SOLID PHASE PEPTIDE SYNTHESIS ON A BEADED CELLULOSE SUPPORT

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DARREN ROSS ENGLEBRETSEN

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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,-tetramethyl-uronium 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

CHAPTER 1

INTRODUCTION

CHAPTER 1 INTRODUCTION

Solid phase peptide synthesis (SPPS) was conceived by R.B. Merrifield in 1959 (see Henahan, 1971). A preliminary report on SPPS was published in 1962, followed by a full paper in 1963 (Merrifield 1962, 1963). Letsinger and Kornet (1963) also reported SPPS, but they used a different chemical pathway to that developed by Merrifield. Present SPPS procedures are almost always based on Merrifield's original strategy. Merrifield was awarded the Nobel Prize for chemistry in 1984 in recognition of the importance of his contribution to peptide synthesis (see Merrifield, 1985).

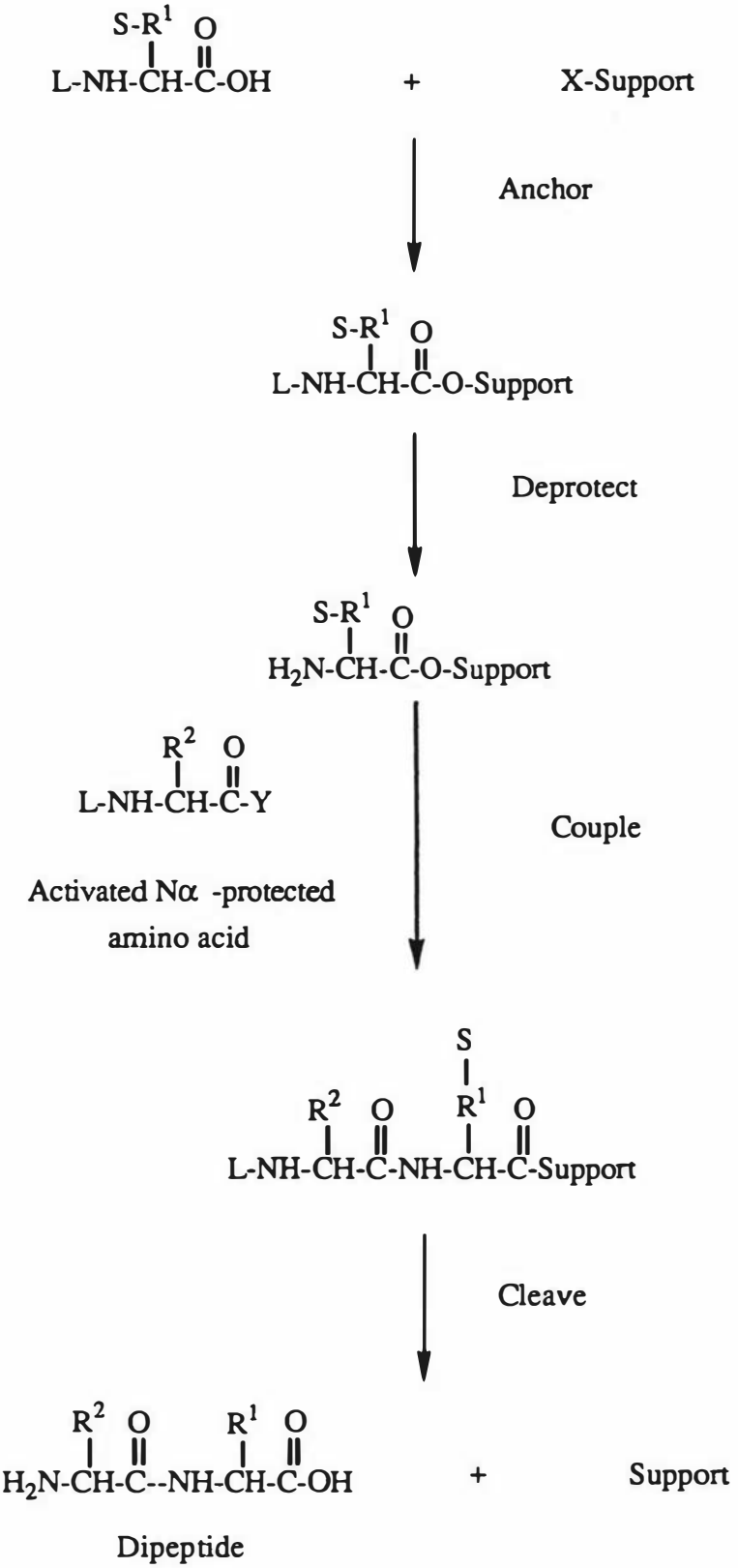
1.1 STRATEGY OF SOLID PHASE PEPTIDE SYNTHESIS

The basic idea of SPPS was to react an Nα-protected amino acid with a suitably functionalised insoluble polymeric ***support*** to ***anchor*** the Nα-protected amino acid via an ester bond. The Nα-protection was then cleaved to expose the amine group (***deprotection*** of the support-bound amino acid). A second Nα-protected amino acid was ***activated*** via its carboxyl group, and ***coupled*** to the support-bound amino acid. An excess of the activated Nα-protected amino acid was used to ensure quantitative reaction. These steps constituted one ***cycle*** of solid phase peptide synthesis. Washing steps were included after the deprotection and coupling steps.

Repetition of the cycle gave stepwise elongation of the support-bound peptide chain. At completion of the synthesis, the peptide was liberated into solution by ***cleavage*** from the support. A diagram of the solid phase peptide synthesis process is given as Figure 1.1.

In the SPPS method the solid support is confined to one reaction vessel, which is fitted with a glass frit or other filter. All of the deprotection, coupling, and associated washing steps are carried out in the same vessel. At the end of each step the solutions are separated from the solid support by filtration. Confinement of the solid support to a single vessel eliminates mechanical losses. One of the features of a repeated stepwise process, such as SPPS, is that it can be readily automated, thus allowing high productivity levels to be attained.

Figure 1.1 Solid Phase Peptide Synthesis (from Stewart and Young, 1984)



L = Labile Nα-amino protecting group

S = Semipermanent side chain protecting group

X = Support-bound reactive group

Y = Carboxyl activating group

The outline given above is a simplified description of a very complex process. Excellent reviews of SPPS include those of Erickson and Merrifield (1976), Barany and Merrifield (1979), Barany et al (1987), and Fields and Noble (1990). In addition, two useful texts concerned with the practical aspects of SPPS are available (Stewart and Young, 1984; Atherton and Sheppard, 1989).

1.2 THE SOLID SUPPORT

To be useful for SPPS a solid support must satisfy the general requirements set out by Erickson and Merrifield (1976):

> *It must contain reactive sites at which the peptide chain can be attached and later removed, and yet it must be stable to the physical and chemical conditions of the synthesis. The support must allow rapid unhindered contact between the growing peptide chain and the reagents. It must be readily separable from the liquid phase at every stage of the synthesis and be physically stable during these operations. In addition, the support must provide enough points of attachment to give a useful yield of peptide per unit volume and must minimize the interactions between bound peptide chains, although the limits of these requirements are not yet clear.*

Both discontinuous batchwise and continuous flow reactors may be employed for SPPS, depending on the physical properties of the support. Cross linked polymers such as polystyrene (Merrifield, 1963) or polyamide resin (Atherton et al, 1975) are usually used in a batchwise peptide synthesiser. The solid support used in continuous flow synthesisers is usually polyamide polymerised within the pores a rigid macroporous support such as kieselguhr (Atherton et al, 1981b). Several other supports have been evaluated for use in SPPS. These include controlled pore glass (Albericio et al, 1989), cotton fabric (Lebl and Eichler, 1989) cellulose paper (Frank and Doring, 1988a), polypropylene membrane (Daniels et al, 1989), polyethylene film (Berg et al, 1991), bovine serum albumin (Hansen et al, 1991), beaded polydextran gel (Vlasov and Bilibin, 1969), and silica gel (Zapevalova et al, 1979). The list given above is only a selection of the many types of supports which have been evaluated.

One of the objectives of the work described in this thesis was to evaluate Perloza beaded cellulose (see Chapter 2 for a description of Perloza) as a support for solid phase peptide synthesis. The ultimate goal was to develop a methodology to enable synthesis of peptide-ligands directly onto Perloza, so that the peptide-resin conjugate could be applied to affinity chromatographic separations. Previous work on the use of carbohydrate matrices as supports for SPPS is summarised in the introductions to Chapters 3 and 5. Although few investigations of beaded carbohydrates as supports for SPPS have been carried out, Erickson and Merrifield (1976) stated that

> *The open structure and hydrophilic nature of the naturally occurring carbohydrates make them attractive supports for the solid-phase synthesis of larger peptides and proteins. Work with these supports is not sufficiently advanced to allow a prediction of their eventual value.*

It was anticipated that this study would go some way in providing information about the value of carbohydrate supports for solid phase peptide synthesis.

1.3 THE ANCHORING BOND

The type of bond anchoring the peptide to the support is of critical importance. Usually the bond is a benzyl ester between the carboxyl group of the peptide and a substituted benzyl alcohol anchored to the support. The type of substitution in the benzene ring can be modified to give different degrees of ester linkage stability to various cleavage reagents (Barany et al, 1987). The bond linking the peptide to the support must be stable to the reagents used to remove the Nα-amino protection, yet be cleavable in high yield at the end of the synthesis. It must be possible to anchor the initial Nα-protected amino acid to the support without racemisation, in high yield, and without side reactions. Any remaining functional groups on the support must then either be chemically stable to the conditions used during the synthesis, or they must be rendered inert by chemical modification.

1.4 PROTECTION OF FUNCTIONAL GROUPS

Most chemical methods of peptide synthesis involve assembly of the peptide from the C- to the N-terminus, which is opposite to the direction of assembly in vivo. The reason for peptide synthesis from the C-terminus is that the methodology allows use of urethane based Nα-amino protecting groups, which protect the activated amino acid from racemisation (Geiger and Konig, 1981). On the other hand, assembly of a peptide from the N- to C-terminus using reagents and methodologies currently available results in serious racemisation of the products (Stewart, 1980).

Several urethane based groups have been employed to provide temporary Nα-amino protection during SPPS (Geiger and Konig, 1981). However, only two Nα-amino protecting groups are commonly used: the acid labile tertiary butyloxycarbonyl (Boc) group (Carpino, 1957a,b; McKay and Albertson, 1957; Anderson and MacGregor, 1957; Merrifield, 1964a,b); and the base labile 9-Fluorenylmethoxycarbonyl (Fmoc) group (Carpino and Han, 1970, 1972; Chang and Meienhofer, 1978; Atherton et al, 1978a,b). The Boc and Fmoc Nα-amino protecting groups, and their associated SPPS chemistries, are discussed in Chapters 3 and 5 respectively.

Some of the amino acids have side chain functional groups which could lead to branching during peptide synthesis, for example the N^{ε}-amino group of lysine. Therefore, side chain functional groups must be protected during a synthesis to prevent unwanted reactions. Side chain protecting groups must be stable to the reagents used for the removal of the temporary Nα-protection, yet be cleaved in quantitative yield at completion of the synthesis. Protecting groups used to block reactive side chains depend on the chemistry employed for the synthesis (usually Boc or Fmoc). The side chain protecting groups used in Boc and Fmoc SPPS chemistry are discussed in Chapters 3 and 5 respectively.

1.5 FORMATION OF THE AMIDE BOND

Many methods are available for formation of the amide bond (Gross and Meienhofer, 1979). Coupling agents used in SPPS must satisfy a number of conditions. The coupling agent or combination of agents used must

not lead to side reactions resulting in racemisation of the amino acids. Although racemisation is possible at any and all stages of a synthesis, the coupling of two amino acids is the step which is most prone to racemisation.

The yield of the coupling reaction must be quantitative, that is, greater than 99.9% under ideal conditions. In SPPS, an excess of coupling reagent and Nα-protected amino acid is used to drive the coupling reaction to completion. Also, double or even triple coupling is often employed in the attempt to achieve 100% coupling yields. In reality, couplings do not always go to completion, regardless of the coupling agent used, the time of reaction, or the number of times the coupling is carried out.

The coupling agent must not give rise to side reactions which lead to a contaminated peptide product. As an example, the amide side chain of Nα-protected asparagine or glutamine may be dehydrated to form a nitrile during activation by dicyclohexylcarbodiimide (DCC). The side chain nitrile derivative is also incorporated into the growing peptide chain, resulting in a peptide contaminated by the nitrile derivative. Peptide cleavage results in two peptides which are similar except for the nitrile side chain of one of them. During purification the nitrile containing peptide must be identified and separated from the target peptide. Separation of the two peptides can sometimes be difficult because of their chemical similarity. Nitrile formation can be eliminated by using the active ester method to couple Nα-protected asparagine and glutamine (Bodanszky, 1979). Many of the side reactions which occur during coupling have been identified, and ways of reducing or eliminating them discovered.

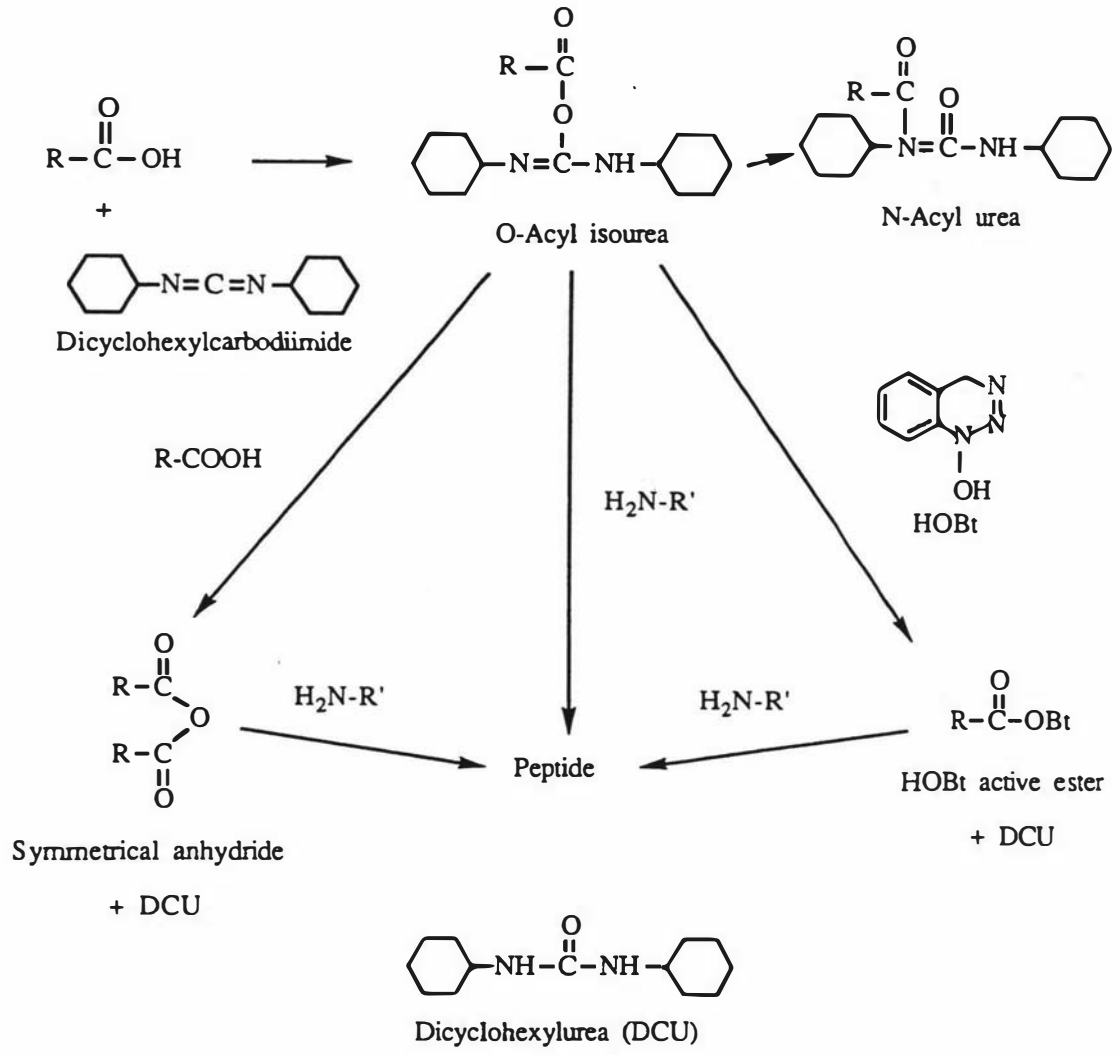
Many methods have been employed for formation of the amide bond in SPPS (Gross and Meienhofer, 1979), but until recently only three methods were commonly encountered:

i) carbodiimide mediated coupling (Merrifield, 1963).
ii) formation of a symmetrical anhydride of the Nα-protected amino acid for reaction with the support-bound peptide (Wieland et al, 1971; Hagenmaier and Frank, 1972).

iii) formation of the HOBt active ester of the Nα-protected amino acid for reaction with the support-bound peptide (Konig and Geiger, 1970a,b,c).

Carbodiimides (dicyclohexylcarbodiimide (DCC) or diisopropylcarbodiimide (DIC)) are usually used as activating agents for all of these reactions. These three methods of activating Nα-protected amino acids are illustrated in Figure 1.2. A number of coupling reagents have more recently started to achieve some popularity, these include BOP (Rivaille et al, 1980), HBTU (Schnolzer et al, 1991), and TBTU (Reid and Simpson, 1992).

Figure 1.2 DCC Mediated Coupling Reactions (from Stewart and Young, 1984)



1.6 MONITORING THE EXTENT OF THE COUPLING REACTION

It is desirable to be able to assess the extent of completion of a coupling reaction. Several tests have been developed to determine the extent of completion of coupling. Two of the tests require removal of a small amount of the supported peptide from the reaction vessel, namely the ninhydrin assay (Kaiser et al, 1970; Sarin et al, 1981) and the trinitrobenzenesulphonic acid (TNBS) test (Hancock and Battersby, 1976). The picric acid test (Gisin, 1972; Hancock et al, 1975; Hodges and Merrifield, 1975; Arad and Houghten, 1990) is carried out on the whole of the support-bound peptide. All of the methods referred to above involve interruption of the reaction sequence for analysis. The Fmoc continuous flow method of peptide synthesis offers the possibility of monitoring deprotection and coupling spectrophotometrically (see Chapter 5), but the method is not sensitive enough to determine the degree of completion of the coupling reaction.

1.7 CLEAVAGE OF THE PEPTIDE FROM THE SUPPORT

The method used to cleave the peptide from the support depends on the chemistry and anchor group employed. Ideally, the cleavage conditions would not result in damage to the peptide chain, formation of side products, or racemisation of any of the amino acids. The most commonly used cleavage conditions are acidolytic. Other conditions, dependent on the anchor group, have been proposed (see Barany et al, 1987). The amino acid side chain protecting groups are often chosen so that they are removed by the same reagent used to cleave the peptide from the support, resulting in a combined cleavage and side chain deprotection step to liberate the peptide from the support.

1.8 PURIFICATION AND ANALYSIS OF SYNTHETIC PEPTIDES

In addition to the target peptide, a crude peptide obtained after cleavage may contain scavengers (such as anisole), salts of the cleavage reagent, organic compounds derived from the cleaved side chain protecting groups, and deletion and termination peptides.

The first step in purification is to separate the peptide from organic contaminants such as scavengers, by-products from side chain protecting group cleavage, and residual cleavage reagent. This can often be accomplished by precipitation of the crude peptide with ether. Unless the peptide is very hydrophobic it will be insoluble in the ether, whereas the organic contaminants will be soluble. The ether-soluble organic contaminants can be removed by filtration of the peptide from the ether. The peptide is then dissolved in 5-10% aqueous acetic acid, although in some cases higher concentrations of acetic acid are required to dissolve the peptide. The peptide solution is filtered from the resin, and lyophilised. As an alternative to ether precipitation, the crude peptide may be dissolved in water or acetic acid solution, and the aqueous solution extracted several times with ether in order to remove scavengers and by-products from side chain protecting group cleavage. Following ether extraction the aqueous solution is lyophilised. The crude peptide may then be subjected to further purification steps.

Gel filtration was commonly used for purification of synthetic peptides prior to the development of high performance liquid chromatography. Currently, gel filtration is usually used for desalting of peptides after synthesis, and as a first step for removal of low molecular weight peptide impurities generated during synthesis and/or cleavage.

Ion exchange chromatography is not commonly used for peptide purification, but may be useful in certain cases. For example, tuftsin was purified by ion exchange chromatography (Nozaki et al, 1977). Details of the ion exchange chromatographic process are given by Scopes (1987).

For purification of hydrophobic peptides the technique of hydrophobic interaction chromatography (HIC) may be useful. Details of the hydrophobic chromatographic process are given by Scopes (1987).

The preparation of synthetic peptides of high purity in a relatively short time would not be possible without the use of modern separation techniques, particularly high performance liquid chromatography (HPLC) (Hancock, 1984, 1990). The crude peptide is dissolved in a buffer solution, typically 0.1% TFA/water. In some cases acetic or formic

acid, or organic solvent such as acetonitrile or isopropanol, may have to be added to solubilise the peptide. The peptide solution is filtered and loaded onto the HPLC column. Peptides are separated by running a solvent gradient from the initial polar aqueous solvent to a less polar organic solvent (typically 0.1% TFA in acetonitrile). HPLC provides a means of both purifying and analysing synthetic peptides.

A new analytical technique which can be applied to the analysis of peptides is capillary electrophoresis (CE) (Karger, 1989). Peptides are separated on the basis of their mobility in an electric field at a given pH. One of the advantages of capillary electrophoresis is that very small volumes of sample are required, typically 1-5 nl.

An important check on the purity of a synthetic peptide is amino acid analysis. A sample of the peptide is hydrolysed in acid under vacuum, the hydrolysate is dried and the residue is dissolved in an appropriate buffer. The hydrolysate solution is then subjected to amino acid analysis (Stewart and Young, 1984). A synthetic peptide may also be sequenced from the N-terminus by Edman degradation as a further check on its composition (Shively, 1986).

Other techniques of peptide characterization include Mass Spectroscopy (MS) and HPLC-MS (McNeal, 1988; Hunt at al, 1990), Nuclear Magnetic Resonance (Markley, 1987), Circular Dichromism and Optical Rotatory Dispersion (Franks, 1988), and X-Ray crystallography (Kent et al, 1991).

1.9 OTHER METHODS OF PEPTIDE SYNTHESIS

Apart from SPPS, a number of other methods for chemically synthesising peptides are employed. In classical solution phase peptide synthesis (Katsoyannis and Schwartz, 1977) an Nα-amino protected amino acid is activated via its carboxyl group and coupled to a carboxyl protected Nα-amino acid . The protected dipeptide is separated from the reaction mixture and purified. If only a dipeptide is required both the carboxyl and Nα-amino protecting group are cleaved and the product purified. If a longer peptide is required the Nα-amino group of the dipeptide is deprotected, the product purified, and the third Nα-amino protected amino acid is coupled. The product is then purified. Repetition of the

cycle results in stepwise elaboration of the peptide chain. Much time is involved in the repeated purifications and reactions required by this method, and losses of product occur at every purification step.

A variation of the solution phase method is the liquid phase method for peptide synthesis (Mutter and Bayer, 1979). A soluble polymer chain is used to provide carboxyl protection and also to solubilise the growing peptide chain. A commonly used solubilising polymer is polyethylene glycol (PEG). In a typical synthesis, an Nα-protected amino acid is coupled to the hydroxyl group of the PEG. The Nα-amino protecting group is cleaved, and the amino acyl-PEG is separated from the reaction liquor either by quantitative precipitation with ether, or by ultra-filtration. The amino acyl-PEG is washed free of excess reagents and redissolved in the usual solvent. The second Nα-protected amino acid is coupled to the amino acyl-PEG. Repetition of the cycle results in stepwise elaboration of the peptide chain. At completion of the synthesis the peptide is cleaved from the PEG and purified. As well as PEG, soluble polystyrene has also been used for soluble polymer supported peptide synthesis (Green & Garson, 1969). The liquid phase method is similar to the solid phase method in that the cleaved peptide will contain deletion peptide impurities if the coupling reactions are not quantitative.

The fragment synthesis technique is used to assemble long peptides from smaller peptide fragments. The fragments are made either by solution or solid-phase methods, but the fragment coupling reactions are generally carried out in solution. All of the reactive groups of two fragments to be coupled are protected, except for the amino terminal of one and the carboxyl terminal of the other. The coupling is preferably done with either a glycine or proline residue as the C-terminal amino acid, to minimize racemisation (Stewart, 1980). The assembled, protected peptide is then either purified, fully deprotected, and repurified, or purified and selectively amino or carboxyl deprotected for attachment to another fragment. In this way a long peptide can be built up from a number of smaller subunits. One of the main problems encountered, when using this strategy, is poor solubility of the growing protected peptide chain in the solvents employed for the synthesis.

Peptides may also be produced using recombinant DNA methods (Wetzell and Goedell, 1983). This is sometimes the most efficient way of producing large quantities of a desired peptide. However, for providing useful quantities of many different biologically active peptides in short times, a chemical means of peptide synthesis is usually employed.

1.10 ADVANTAGES OF CHEMICAL METHODS FOR PEPTIDE SYNTHESIS OVER BIOLOGICAL METHODS

A number of useful alterations can be made to chemically synthesised peptides which cannot be attained in peptides from biological sources. For example, D-amino acids can be incorporated into peptides which do not naturally contain them. Incorporation of D-amino acids into a biologically active peptide can significantly increase the half life of the peptide by reducing the rate of degradation of the peptide by L-amino acid specific enzymes (Davies, 1977). Non-protein or unnatural amino acids can be incorporated into a peptide chain for conformational or activity studies, for example, see Rajashekhar and Kaiser (1986), and Jacobson et al (1989). Synthetic peptides labelled at specific sites, for example with deuterium (Upson and Hruby, 1976), tritium, carbon^{14}, or with chromophores (Scully and Kakkar, 1979), can be made using standard chemical peptide synthesis methodology.

One final advantage of chemical means of peptide synthesis is the time required for a synthesis. Relatively large quantities of peptide can often be chemically synthesised and purified in a shorter time than is required either for establishment of a recombinant DNA source, or for isolation of the peptide from a natural source.

1.11 ADVANTAGES OF SOLID PHASE PEPTIDE SYNTHESIS OVER OTHER CHEMICAL MEANS OF SYNTHESISING PEPTIDES

Solid phase peptide synthesis offers a number of advantages over the other chemical methods of peptide synthesis. SPPS can be automated, thus freeing personnel for other duties involved with SPPS, in particular for peptide purification. Losses of peptide are minimised because the peptide is attached to the insoluble support throughout the synthesis until cleavage. Automated procedures have been standardised

to such an extent that personnel not specifically trained in peptide chemistry can routinely synthesise peptides of acceptable purity. Amino acid coupling cycles typically take two to three hours, which are much faster than for the other methods. Faster coupling cycles, coupled with automated peptide synthesisers, allow synthesis of peptides in a shorter time compared to the other chemical methods of peptide synthesis.

The major disadvantage of SPPS over solution methods is that the peptides are not purified after each coupling cycle, as they are in solution methods. Because purification of intermediates is not possible in SPPS, the solvents and reagents used must be of the highest purity obtainable, in order to minimise product contamination from this source. If coupling reactions in SPPS are not quantitative, or if unexpected side reactions occur during a synthesis, the final peptide product will be contaminated. Separation of similar peptide impurities from the peptide of interest can be time consuming and sometimes impossible. However, despite this disadvantage, solid phase peptide synthesis is the method of choice for most peptide syntheses, on account of its speed, simplicity, and the lack of need for highly trained personnel to carry it out.

1.12 PEPTIDES AS LIGANDS IN AFFINITY CHROMATOGRAPHY

One of the many uses of synthetic peptides is for immobilisation onto hydrophilic solid supports for use as ligands for affinity chromatographic purifications. The affinity chromatographic method makes use of the unique specificity of the protein-ligand interaction as a means of purifying proteins. The principle of affinity chromatography is relatively simple. A ligand (which may range in size from a simple organic molecule to a complex biological macromolecule) is covalently immobilised to a hydrophilic insoluble support, often via a spacer arm. A solution containing the target protein, and usually other contaminating proteins, is introduced to the ligand-support. Both column flow and batchwise processes may be employed (Angal and Dean, 1989). If the experimental conditions (pH, ionic strength, polarity, temperature, length of spacer arm, orientation or conformation of the ligand, etc.) favour binding, the target protein will bind to the immobilised ligand. Contaminating proteins can be removed by thorough washing. The bound

target protein can then be recovered by altering the solution parameters (pH, ionic strength, polarity, addition of chaotropes, addition of denaturants, etc.) to favour dissociation of the protein-ligand complex.

The outline of affinity chromatography given above is a simple description of a complex process, and a large number of variables must be considered in order to develop a successful affinity chromatographic purification method. Lowe and Dean (1974), Turkova (1978), Lowe (1979), Dean et al (1985), Mohr and Pommerening (1985), and Angal and Dean (1989), give comprehensive discussions of the affinity chromatographic technique.

The methods most commonly used to immobilise peptides to affinity chromatographic supports result in anchoring of the peptide via its (or one of its, if the peptide contains lysine residues) amine group. While anchoring via an amine group is generally satisfactory, a number of potential problems or limitations of the methodology exist. If a peptide amino terminus must be presented for recognition and binding of the target protein, immobilisation of the peptide-ligand via the Nα-amino group is obviously inappropriate. In some cases it is possible to protect the Nα-amino group of the peptide, immobilise the peptide via a side chain lysine N^{ε}-amine group, and then cleave the Nα-protecting group to expose the free Nα-amino group (see Robinson et al, 1976; Kuyas et al, 1990; both discussed in Chapter 6). Although such a strategy may be usable in some cases, it may not always be applicable.

An alternative means of achieving directed orientation of a peptide-ligand Nα-amino terminus into the aqueous phase is to protect the Nα-amino group as discussed above, immobilise the peptide via its C-terminal carboxyl group, and then cleave the Nα-amino protecting group to expose the Nα-amino group (see Chaiken, 1979). One limitation of this method is that it may not be useful if the peptide-ligand contains more than one carboxyl group (for example, if it contains Asp or Glu). In this case anchoring of the peptide may occur at one of several sites. Anchoring of the peptide-ligand via a side chain carboxyl rather than the C-terminal carboxyl may alter the conformation of the ligand, leading to non-binding by the target protein.

Another method of achieving directed orientation of an immobilised peptide-ligand N-terminus into the aqueous phase would be to synthesise the peptide directly onto the chromatographic support using current SPPS methodology. It was envisaged that such a strategy would offer a number of potential advantages over the alternative strategies (outlined above) which have been used to give directed orientation of a peptide N-terminus into the aqueous phase. The advantages include:

i) the N-terminus of the peptide would have the required orientation.
ii) high peptide-ligand substitutions could be achieved , if required.
iii) the attachment point of the peptide-ligand would be unambiguous (see discussion above).

However, some disadvantages were also envisaged. Current procedures of peptide-ligand immobilisation allow the use of purified, characterised, peptides; that is, the integrity of the peptide-ligand can be verified before immobilisation. If a peptide was synthesised directly onto a support via a non-cleavable covalent linkage, only indirect methods (amino acid analysis, titration) would be available for assessment of the integrity of the ligand (see Chapter 6 for a more comprehensive discussion). In addition, if the peptide-ligand synthesised onto the support possessed side chain protected amino acids, cleavage of the side chain protecting groups would be necessary to generate the peptide-ligand. In this case, the support would have to be stable to the side chain deprotection conditions employed.

A small number of studies have been published (Smith et al, 1977; Geysen et al, 1984; Frank et al, 1991; all discussed in Chapter 6) in which peptide-ligands were synthesised directly onto insoluble supports for use as ligands for binding biological macromolecules. However, in none of the studies cited were the peptide-supports used for affinity chromatographic purification of the biomolecules.

The studies reported in this thesis were directed towards development of a methodology which would allow synthesis of a protein-specific peptide-ligand directly onto a hydrophilic matrix. Perloza beaded cellulose resin (see Chapter 2) was chosen for this study because it fulfilled many of the conditions, given above, required for a support to be useful

for solid phase peptide synthesis. In addition, Perloza also fulfilled the conditions required for a support to be useful for affinity chromatography (see Chapter 6). Therefore, an investigation was carried out to determine whether peptide-ligands could be synthesised directly onto Perloza. The utility of the peptide-Perloza as a support for affinity isolation of target proteins was assessed.

To be useful for SPPS, Perloza would have to be compatible with the solvents commonly used. The solvent compatibility of Perloza was therefore investigated. In addition, the resin would have to be provided with amine functional groups, preferably separated from the cellulose with a spacer arm, which would itself be linked to the cellulose via a chemically stable bond. Various means of functionalising Perloza were investigated. The results of these and a number of other basic studies are reported in Chapter 2.

A number of studies were required to define SPPS methodologies compatible with Perloza. Both Boc (Chapter 3) and Fmoc (Chapters 4 and 5) methodologies were investigated for use with Perloza. It was anticipated that both the standard Boc and Fmoc solid phase peptide synthesis strategies would have to be modified to give SPPS methodologies suitable for use with Perloza. A number of "standard" peptides were assembled using the new methodologies to validate Perloza as a support for SPPS.

Finally, once Perloza-compatible Boc and Fmoc solid phase peptide synthesis methodologies were established, they were used to synthesise peptide-ligands directly onto amine substituted Perloza. The peptide-resins were then tested for their utility as adsorbents for the isolation of proteins with specific affinity for the immobilised peptides. To a large extent, these aims were met in this study. In addition, a novel means of achieving oriented immobilisation of a peptide-ligand onto Perloza was investigated. The results of these studies are reported in Chapter 6.

CHAPTER 2

FUNCTIONALISATION OF PERLOZA BEADED CELLULOSE

CHAPTER 2 FUNCTIONALISATION OF PERLOZA BEADED CELLULOSE

2.1 INTRODUCTION

Perloza beaded cellulose (Chemopetrol, 1988) was chosen for this study from a number of commercially available hydrophilic matrices, such as Sephadex LH20, polyvinyl alcohol beads (Riedel-de Haen), hydrophilic azlactone beads (3M), and viscose particulate cellulose (Life Technologies (NZ) Ltd.). Perloza is a beaded, non covalently cross-linked regenerated cellulose. Perloza type MT, selected for this study, is supplied swollen in water, and is available in three particle diameter ranges: Fine (80-100 µm), Medium (100-250 µm), and Coarse (250-500 µm). Three porosities, corresponding to molecular weight exclusions of 100, 200, and 500 kDa, are available in each diameter range.

Perloza is manufactured by the Thermal-Sol-Gel-Transition (TSGT) process (Stamberg, 1988; Gemeiner et al, 1989), in which a suspension of cellulose xanthate solution in an immiscible inert solvent is heated to solidify the cellulose. By using appropriate conditions spherical beads result. Following solidification the xanthate is decomposed and the cellulose regenerated by alkaline treatment. The spherical beads consist of interlinked amorphous and crystalline regions with pores containing the aqueous phase. The structure is stabilised entirely by hydrogen bonds, no covalent cross linking of the cellulose chains is employed.

Several types of Perloza are manufactured, for example Perloza MF, MT, SF, ST and KryoPerloza. Perloza type MT was the sole type of Perloza beaded cellulose used in this study. Perloza MT is

> *...intended as material for separation gel chromatographic techniques, as a vehicle for the immobilisation of enzymes and as the initial material for the preparation of a number of modified spherical derivatives of cellulose.* (Chemopetrol, 1988)

Perloza has relatively high mechanical strength for a "soft" matrix, which results in good resistance to deformation. The high mechanical strength gives Perloza good column flow characteristics, that is,

increasing the pressure on a column of Perloza results in an increase of the flow rate rather than pressure induced collapse of the matrix. Perloza gel columns do not collapse at flow rates which collapse other spherical beaded carbohydrate matrices such as dextran gels. The good column flow properties of Perloza are an important consideration if the resin is to be used in a continuous flow peptide synthesiser. In addition, the material is not brittle, which makes it stable to attrition by agitation. Perloza has been tested for its mechanical stability by stirring an aqueous suspension with hard foreign objects, such as glass beads, without deleterious effects (Chemopetrol, 1988). This is important if the resin is to be shaken during batchwise processes, such as in an ABI 430A peptide synthesiser. A resin which suffers attrition during shaking would generate fines which would clog the filter of the ABI 430A reaction vessel.

Perloza is stable to osmotic shocks, so that it withstands variation of ionic strength or pH. This is important if the material is to be used for chromatographic separations, where a drastic change of pH or ionic strength may be required to effect a separation or to clean the resin.

Perloza beaded cellulose has a large number of chemically reactive hydroxyl groups, allowing subsequent chemical modification to be readily accomplished.

A primary amine was desired as a starting point for further chemical functionalisation of Perloza resin. Ideally the amine would be separated from the cellulose backbone by a spacer arm, and the spacer arm would be linked to the cellulose by a stable bond such as an ether. The chemistry chosen should allow control of the level of amine substitution. It was also considered desirable that the chosen methodology should be able to achieve high levels of amine substitution if required, for example up to 2 mmole of amine per g of dry resin.

Following introduction of an amine functional group, it would be possible to further modify the resin by reaction with the anhydrides or active esters of a wide range of carboxylic acid-containing ligands.

A number of methods for substituting carbohydrate matrices with a primary amine group were considered for use with Perloza (see below).

Several of these methods were investigated in this study. Other methods were considered but eliminated for the reasons discussed below.

2.1.1 Cyanogen bromide activation of cellulose

Polysaccharides react with cyanogen bromide in aqueous alkaline media to give a reactive imidocarbonate moiety (Reactifs IBF, 1983). Following formation of the reactive imidocarbonate, an amine containing ligand is introduced. For this study either a diamine or mono-N-protected diamine would be required for reaction with the activated matrix to yield a matrix-bound amine group.

Advantages of the method are:

i) simple.

ii) degree of activation reproducible and controllable.

Disadvantages are:

i) cross-linking could occur if a diamine was used for coupling, although this could probably be minimised by use of a large excess of the diamine, or eliminated by use of a mono-N-protected diamine.

ii) the isourea formed can bear a positive charge (Reactifs IBF, 1983).

iii) cyanogen bromide is a toxic reagent.

iv) in acidic and basic media (pH<5 or >10) partial hydrolysis between the ligand and the support can occur.

One of the major objectives of the studies reported in this thesis was synthesis of peptides directly onto Perloza for affinity chromatographic applications. The possibility of the matrix bearing a positive charge may have provided another potential point for non-specific binding during affinity chromatography, which was not considered desirable. Also, it was anticipated that some affinity chromatographic separations, for example antibody isolation, would require use of elution buffers with a pH below pH 5. Cyanogen bromide activation would not provide a stable link between the peptide ligand and the matrix below this pH. Cyanogen bromide activation was not considered suitable for use with Perloza, in this study, for these reasons.

2.1.2 s-Triazine activation of cellulose

Polysaccharides react with cyanuric chloride at pH 9-11 to yield a dichloro-s-triazinyl complex, which can then react with nucleophiles such as primary amines (Reactifs IBF, 1983). For this study either a diamine or mono-N-protected diamine would be required for reaction with the activated matrix to yield a matrix-bound amine group.

The advantage of the method is that it is simple. However, as for activation with cyanogen bromide, if a diamine was used for reaction, cross-linking of the matrix may occur. Also, the nitrogen link between the triazine and the spacer arm would bear a positive charge (Reactifs IBF, 1983). Finally, this approach has been tried by Eichler at al (1990) for peptide synthesis using cotton fabric as the support. They reacted cotton with cyanuric chloride, then coupled 4-amino-nitrobenzene to the dichloro s-triazine. The nitro group was then reduced to an amine using zinc in ethanolic ammonia, giving a support with an amine substitution of 0.05 mmole/g. However, they found that storage of the product led to an inexplicable decrease of the amino group substitution to 0.003-0.004 mmole/g. The cyanuric chloride approach was not considered suitable for use with Perloza for these reasons.

2.1.3 Reaction of cellulose with ethylenimine

Raw cotton fibres react with ethylenimine vapour at 100-110^{o}C to yield aminoethyl cellulose with a nitrogen substitution of 0.24 mmole/g. Reaction at higher temperatures, 140-170^{o}C, gives nitrogen substitutions in the range of 2.9-17.1 mmole/g (Soffer and Carpenter, 1954). At the higher nitrogen substitutions it was possible that polymerisation of the ethylenimine was occurring. This approach was not tried with the Perloza because the conditions required were not attainable without the use of specialised apparatus.

2.1.4 Reaction of carboxymethylcellulose with a diamine in the presence of a condensing agent

Carboxymethylcellulose reacts with diamines in the presence of coupling agents such as 1-ethyl-3-(dimethylaminopropyl)-carbodiimide hydro-

chloride (EDC) or N-ethoxycarbonyl-2-ethoxy-1,2, dihydroquinoline (EEDQ) to yield an amine substituted resin (Reactifs IBF, 1983). This approach was initially tried with Perloza but was abandoned because:

i) reaction yields were only about 50%, leaving a significant remaining carboxyl substitution.
ii) other approaches were showing better promise of success at the time, so this approach was not pursued. However, further work may prove that the approach is viable.

2.1.5 Epichlorohydrin activation of cellulose followed by reaction with ammonia

In hot concentrated alkaline solution epichlorohydrin reacts with cellulose to form epoxy activated matrices (Reactifs IBF, 1983). The epoxide may then be reacted with ammonia to yield a matrix with a primary amine group.

Disadvantages of the method are:

i) cross linking can occur during epoxidation and also during reaction with ammonia.
ii) the reactive epoxide is slowly lost by alkaline hydrolysis as the coupling reaction proceeds.

Additionally, Elgar and Ayers (1991) report only limited success with the epichlorohydrin method. The procedure may be worth pursuing, however, especially if a method of introducing a primary amine cheaply and simply is desired, for example in a large scale-up situation.

2.1.6 Esterification of a cellulose support with an Nα-protected amino acid, followed by cleavage of the Nα-protecting group to expose the amine

Nα-protected amino acids may be esterified directly to a polysaccharide using the carbonyldiimidazole (CDI), mixed anhydride, acid chloride (Vlasov et al, 1969), or dimethylaminopyridine (DMAP) catalysed DCC/HOBt methods (Eichler et al, 1990, 1991). The Nα-protecting group may then be cleaved and a peptide synthesised directly from the anchored amino

acid. Alternatively, the amine group may be further modified with a linker molecule prior to peptide synthesis. These two approaches have been used for SPPS by Vlasov et al (1969) on Sephadex LH20, and by Eichler et al on paper (1988) and cotton fabric (1990).

If a peptide-support was intended to be used for large scale affinity purifications, sodium hydroxide would probably be used for column washing because it is the cheapest cleaning and sterilising agent available for column regeneration (Hancock, 1990). One disadvantage of attachment of a peptide-ligand to a carbohydrate support via an ester bond is that the ester is labile to aqueous alkaline solutions. Indeed, alkaline hydrolysis is the method usually used to cleave peptides synthesised directly onto a carbohydrate matrix (Vlasov et al, 1973; Orlowska et al, 1975; Eichler et al, 1988). One of the aims of this study was to develop a practical, robust means of synthesising peptide ligands onto a carbohydrate support for affinity chromatographic processes. Attachment of a peptide-ligand to Perloza by esterification was not investigated because of the lability of the peptide-resin link to conditions which could possibly be required for column regeneration.

2.1.7 Carbonyldiimidazole (CDI) activation of cellulose followed by reaction of the carbonylimidazole cellulose with a diamine

Carbonyldiimidazole (CDI) reacts with cellulose in anhydrous dioxane to yield carbonylimidazole substituted cellulose, which will react with excess diamine to yield amino-cellulose with the amino-spacer arm bound to the cellulose via a urethane bond (Bethell et al, 1979). A disadvantage of the method is that cross-linking may occur, although it should be possible to minimise cross linking by using a large excess of diamine. This procedure has the advantage of being simple, and it was therefore applied to Perloza in this study.

2.1.8 Reaction of cellulose with 2-aminoethylsulphuric acid

2-Aminoethylsulphuric acid (2AES) in concentrated alkaline solution reacts with cellulose at 120^{o}C to yield aminoethyl cellulose (Guthrie, 1947; Reeves and Guthrie, 1953). Although the reaction conditions used were probably too harsh, with the risk of drying out the resin, the approach was modified for trial with Perloza.

2.1.9 Reduction of cyanoethyl cellulose by diborane

Cellulose reacts with acrylonitrile in the presence of sodium hydroxide solution to yield cyanoethyl cellulose (Compton, 1963; Bikales, 1971). The cyanoethyl moiety is linked to the cellulose via a stable ether bond. Cyanoethyl cellulose may be reduced by diborane to yield aminopropyl cellulose (Daly and Munir, 1984). This was the method chosen for production of amine substituted Perloza.

Advantages of this approach are:

i) cyanoethyl substitution controllable (Compton, 1963), high nitrogen content should be possible.

ii) cross linking not possible.

iii) a stable ether linkage of the amino-spacer to the support is generated.

One disadvantage of the method is that the reduction may not go to completion.

It was considered that the diborane reduction of cyanoethyl Perloza offered potential advantages over the other methods considered. Experimental trials showed this to be the case, and the method was ultimately chosen for generation of amine substituted Perloza for further experiments.

In summary, methods 2.1.7 (CDI activation), 2.1.8 (2-aminoethylsulphuric acid reaction), and 2.1.9 (diborane reduction of cyanoethyl cellulose) were investigated in this study.

2.2 MATERIALS AND METHODS

2.2.1 Chemicals and Equipment

Perloza MT resins were purchased from Tessek Ltd. Prague, Czechoslovakia. Reagent grade boron trifluoride etherate, diglyme, ethanolamine, phenol, sodium hydroxide, and sulphuric acid were purchased from BDH (NZ) Ltd. Analytical reagent grade hydrochloric acid, dimethylformamide (DMF), and methanol, and HPLC grade tetrahydrofuran (THF) were also purchased from BDH (NZ) Ltd. DMF was distilled under vacuum from calcium hydride. BDH "Convol" sodium hydroxide and hydrochloric acid solutions were used for titrations. Analytical reagent grade dioxane and diethyl ether, and reagent grade pyridine, were purchased from Ajax Chemicals Ltd., Sydney. Drum grade dichloromethane (DCM) was dried over magnesium sulphate and distilled prior to use. Drum grade 95% ethanol was used as supplied. Reagent grade acrylonitrile, 1,6-diaminohexane, diisopropylethylamine (DIEA), sodium borohydride, and triethylamine were from Riedel-de Haen. Calcium hydride was purchased from Aldrich. Reagent grade 1,1'-carbonyldiimidazole (CDI) was from Merck. Ninhydrin was from Koch-Light Laboratories. Water, unless otherwise noted, was from a Millipore MilliQ system fed from a reverse osmosis unit. Analytical reagent grade picric acid was from Merck.

A Radiometer TTT 80 autotitrator was used for all NaOH and HCl titrations. Elemental analyses were performed by the Microanalytical Unit, Chemistry Dept., University of Otago, Dunedin, NZ. Infrared spectra were recorded on a Bio Rad FTS-7R spectrometer. UV-vis absorbances were read using a Phillips Pye Unicam PU 8610 spectrophotometer.

2.2.2 Determination of the ratio of dry to wet weight of resin

A sintered glass funnel was dried at 110^{o}C for 60 minutes, allowed to cool to room temperature in a dessicator, then weighed to 4 decimal places. A sample of wet resin was weighed into the funnel. The resin was washed with ethanol, then diethyl ether, and excess solvent was removed by vacuum filtration. The funnel and contents were dried at 110^{o}C for 30 minutes, allowed to cool to room temperature in a

dessicator, and reweighed. The weight of dry resin was found by difference and the ratio of the dry to wet weight of the resin was calculated and expressed as a percentage.

2.2.3 Swelling of Perloza 100 Medium in different solvents

Perloza resin is supplied preswollen in water. A study of the swelling properties of the resin in other solvents was made.

A sample of water wet Perloza 100 Medium was ***solvent exchanged*** to dioxane by successively washing with 25% dioxane/water, 50% dioxane/water, 75% dioxane/water, then finally with 100% dioxane. From dioxane it was exchanged to a variety of other solvents including dimethylformamide (DMF), dichloromethane (DCM), ethanol, methanol, and diethyl ether. The resin was transferred to a 10 ml measuring cylinder, covered with 5 ml of the test solvent, and allowed to stand for 24 hours. The swollen volume of the resin was noted. The resin was quantitatively transferred to a dried preweighed sintered glass funnel and the dry weight determined. The ratio of the volume the resin occupied in its swollen state to its dry weight was calculated.

2.2.4 Reswelling of lyophilised Perloza 100 Medium

This experiment was to assess whether Perloza resin could be dried and then reswollen to its original volume.

One hundred and ten grams of water wet Perloza Medium resin were lyophilised. Samples of the dried resin, 1.00 g, were suspended in 10 ml of test solvents such as DMF, DCM, dioxane, ethanol, methanol and water. The samples were equilibrated with the solvents for 72 hours. The volume of the swollen resin was measured. The ratio of the swollen volume (in ml) to the resin dry weight was calculated.

2.2.5 Methods for analysis of amine-substituted Perloza resin

A number of standard analytical methods were used to analyse amine-substituted Perloza. In some cases the methods were modified to make them compatible with Perloza.

2.2.5.1 Trinitrobenzenesulphonic acid (TNBS) test for free amine groups

The TNBS test (Hancock and Battersby, 1976; Stewart and Young, 1984) was used as a qualitative assay to determine whether amine groups were present on functionalised Perloza. Development of a bright orange colour on the resin beads indicated the presence of amine groups.

2.2.5.2 Ninhydrin method for determination of amine substitution of functionalised Perloza resin

The ABI modification (Applied Biosystems Inc., 1988) of the ninhydrin assay of Sarin et al (1981), developed for use with amino-polystyrene resins, was assessed for use with amine-substituted Perloza. All reagents were prepared according to the procedures described by ABI. Resin samples were washed with ethanol, then ether, and dried at 110^{o}C for 5-10 minutes. The solutions used for the assay were: Reagent 1: 80% phenol-ethanol, Reagent 2: 0.0002M KCN in pyridine, Reagent 3: 0.28M ninhydrin in ethanol. A sample of dried resin, 1-10 mg, was weighed into a test tube (4 decimal places). Reagent 1 (75 µl), Reagent 2 (100 µl), and Reagent 3 (75 µl) were added to the tube. The sample was incubated at 100^{o}C for 7 minutes. Sixty percent ethanol/water, 4.8 ml, was added to the sample, which was vortexed, then allowed to cool to room temperature. A blank devoid of resin was also run. The absorbance of the solution at 570 nm was determined within 10 minutes. If the absorbance was greater than 1 AU the sample was diluted ten fold and the absorbance read again. The absorption coefficient of the ninhydrin complex, used for calculation of the amount of amine present, was 15000.

The ninhydrin assay was also used as a qualitative test for the presence of amine groups. Generation of a deep blue colour indicated the presence of amine groups.

2.2.5.3 HCl titration of resin-bound amine groups

Amine-substituted Perloza resin was washed with 0.5M sodium hydroxide solution, followed by water until the effluent was neutral to pH paper. A sample of the resin was placed in a polypropylene beaker, and the amine groups were titrated using 0.100M HCl to an end point pH of 4.00.

The resin was transferred quantitatively to a dried, preweighed sintered glass funnel, washed thoroughly with distilled water, and the dry weight determined. The amine substitution (mmole/g dry resin) was calculated.

2.2.5.4 Picrate titration of resin-bound amine groups

Initial experiments to titrate lyophilised amino-Perloza using the Gisin (1972) method were unsuccessful. A modified procedure using 50% aqueous ethanol as solvent for all steps was successful for titrating lyophilised amino-Perloza. This method was subsequently adopted as standard for picrate titration of amine-substituted Perloza.

Amine-substituted Perloza resin was placed into a dried, preweighed sintered glass funnel. A saturated solution of picric acid in 50% aqueous ethanol was added to the resin. After standing for 5 minutes, excess picric acid was washed away with 50% aqueous ethanol. The bound picrate was quantitatively eluted into a 50 ml volumetric flask with either a solution of 5% DIEA or 10% triethylamine in 50% aqueous ethanol. The volume was made up to 50 ml. The eluate was diluted with 50% aqueous ethanol as appropriate so that the final absorbance of the solution was less than 1.0 AU. The absorbance of the solution was read at 358 nm. The amount of picrate present was calculated using an extinction coefficient of 14500. The dry weight of the resin was determined by the standard procedure. The amine substitution of the resin, in units of mmoles of amine per g of dry resin (mmole/g) was calculated.

2.2.5.5 Preparation of resin samples for elemental analysis

Resin samples to be subjected to elemental analysis were washed with ethanol, then ether, and dried at 110°C for at least 10 minutes. Alternatively, the samples were lyophilised or dried under high vacuum before being sent for analysis.

2.2.6 Comparison of different methods for the determination of the amine substitution of amino-Perloza resins

Aminohexyl-Perloza made using the carbonyldiimidazole / 1,6-diamino-hexane (CDI/DAH) reaction (see Section 2.2.8) was analysed by three

methods: HCl titration of the water-wet resin, picrate titration of a reswollen lyophilised sample, and elemental analysis of a dried sample.

In a second comparison aminopropyl Perloza, made by diborane reduction of cyanoethyl Perloza (see Section 2.2.10), was titrated using both HCl and picrate methods.

2.2.7 Reaction of Perloza 100 Medium with 2-aminoethylsulphuric acid

2-Aminoethylsulphuric acid (2AES) was synthesised using the procedure of Rollins and Calderwood (1938). Initial experiments with Perloza which duplicated the reaction conditions used by Guthrie (1947) were unsuccessful.

The following procedure was most successful. Water swollen Perloza 100 Medium, 11.59 g, was placed in a stainless steel bomb reactor. A solution of 2AES (20% w/v) : NaOH (25% w/v), 20 ml, was added. The bomb was sealed, placed in a boiling water bath, and heated for 30 hours. A TNBS test of the resin was positive, as was a qualitative ninhydrin test.

2.2.8 Carbonyldiimidazole (CDI) activation of Perloza 100 Medium

The results of preliminary experiments showed that no benefit was gained by using a quantity of CDI greater than 4 mmoles per gram of dry Perloza 100 resin. The procedure adopted for CDI activation of Perloza is illustrated in the following experiment. Note: care must be taken to exclude water from the reaction, as CDI is very readily decomposed by water.

Water wet Perloza 100 Medium resin 44.3 g (4.4 g dry) was placed in a sintered glass funnel and solvent exchanged to dioxane. The resin was then washed twice more with dioxane, and excess dioxane was removed by filtration. The resin was transferred to a 60 ml screw capped reaction vessel. Dioxane, 40 ml, was added, followed by 2.90 g (17.9 mmole) CDI. The resin was mixed for two hours on the Rototorque. The resin was then washed thoroughly with dioxane, and samples were taken to determine the carbonylimidazole substitution level.

2.2.8.1 Titration to determine carbonylimidazole substitution of Perloza after reaction with CDI

Samples of dioxane-wet carbonylimidazole-Perloza resin were placed in polypropylene beakers. Ten ml of 0.100M sodium hydroxide was added and the samples were hydrolysed overnight at 4°C to cleave the imidazole. The pH was adjusted to 3 using 1M hydrochloric acid, and nitrogen was bubbled into the solution for ten minutes to expel carbon dioxide. The solution was titrated to a pH 5.00 end point using 0.100M NaOH. This was the start point for the titration of the imidazole hydrochloride which resulted from the initial addition of the HCl. The solution was titrated with 0.100M NaOH to an end point pH of 8.50. The volume of 0.100M NaOH consumed was noted. The resin was transferred quantitatively to a dried, pre-weighed sintered glass funnel, washed with water, and the dry weight determined as described above. The carbonylimidazole substitution level (mmoles/g of dry resin) was calculated.

2.2.8.2 Reaction of carbonylimidazole-Perloza 100 Medium with 1,6-diaminohexane

A solution of 8.5 g (70.7 mmoles) 1,6-diaminohexane (DAH) in 50 ml dioxane was added to the CDI activated Perloza from the previous experiment. The resin was mixed for 66 hours, then washed thoroughly with dioxane followed by ethanol, until no 1,6-diaminohexane was detected in the effluent using the TNBS test. Because a TNBS test of the resin gave a positive result, samples of the resin were titrated with 0.100M HCl to determine the amine substitution level.

2.2.9 Cyanoethylation of Perloza 100 Medium

A number of preliminary experiments were carried out to establish a general procedure for making cyanoethyl Perloza.

2.2.9.1 Preliminary cyanoethylation experiments on Perloza 100 Medium

The procedure reported by Compton (1963) was modified for use with Perloza. Initial experiments carried out by mixing acrylonitrile with Perloza suspended in 0.5M sodium hydroxide solution resulted in low

resin nitrogen substitution levels. It was noted that the acrylonitrile was immiscible in the 0.5M NaOH solutions used. Therefore the effect of using water miscible co-solvents, which would also dissolve acrylonitrile, was investigated.

Water-wet Perloza 100 Medium was washed twice with 0.5M sodium hydroxide solution. Excess solution was removed by vacuum filtration to leave the resin moist with the sodium hydroxide solution. The resin was transferred to a conical flask, and co-solvent and acrylonitrile were added. After stirring for 30 minutes to 2 hours the resin was collected by filtration, and washed with water until the pH of the effluent was neutral to pH paper. Samples were taken for elemental analysis.

2.2.9.2 General procedure for cyanoethylation of Perloza

The following procedure, based on the results of the preliminary experiments, was adopted.

Perloza resin was weighed into a sintered glass funnel, and washed twice with 0.5M NaOH solution. After standing for 5 minutes excess NaOH solution was removed by vacuum filtration. The moist resin was transferred to a conical flask, and a volume of dioxane in ml equal to the initial weight of the water-wet resin in grams was added. Acrylonitrile was added and the resin was stirred for one hour. The reaction was mildly exothermic. The resin was collected by filtration and washed thoroughly with water until the pH of the effluent was neutral to pH paper. A sample of the resin was dried for elemental analysis.

2.2.10 Reduction of cyanoethyl Perloza using diborane

A series of preliminary experiments were carried out to establish reaction conditions for reduction of cyanoethyl Perloza to yield aminopropyl Perloza. Initially, reductions were carried out at room temperature with stirring of the resin. Although successful, the products from batch to batch were of variable quality. Sometimes the aminopropyl Perloza contained fines, which blocked filters and gave low flow rates, while at other times a slimy, viscous mass formed. The later reductions at room temperature gave high reduction yields with

products which gave good flow rates (see Section 2.3.8). A modification of a literature procedure, which required reflux of the cyanoethyl Perloza with a diborane solution, was finally adopted for the majority of the reductions of cyanoethyl Perloza.

2.2.10.1 Reduction of cyanoethyl Perloza at room temperature

Reduction of cyanoethyl Perloza at room temperature is illustrated by the following experiment.

Water-wet cyanoethyl Perloza (50 g wet, nitrogen substitution 2.25 mmole/g) was washed thoroughly with THF, followed by washing with THF distilled from sodium wire. The resin was transferred to a 3-necked round-bottomed flask with 50 ml distilled THF. A solution of sodium borohydride, 4.25 g (112 mmoles) in 50 ml diglyme was added to the resin, followed by 23 ml (187 mmole) of boron trifluoride etherate. Gas was evolved on addition of the boron trifluoride etherate. The mixture was stirred, using a magnetic stirrer and follower, at room temperature under oxygen-free-nitrogen for 69 hours. The resin was poured into 500 ml water, whereupon a gas was usually evolved. The pH of the supernatant was about 1. After standing 20 minutes, the resin was collected by filtration, washed with 0.1M NaOH solution, then with water until the pH of the effluent was neutral to pH paper. Samples were taken for picrate titration and elemental analysis.

2.2.10.2 Reduction of cyanoethyl Perloza under reflux

A 3-necked round-bottomed flask was fitted with a nitrogen inlet and a paraffin bubbler. THF distilled from sodium wire was added, followed by sodium borohydride. The mixture was stirred, cooled to $0^{o}C$, and oxygen-free-nitrogen was passed through the flask. The volume of boron trifluoride etherate required to give 100% reaction to form diborane was added slowly to the stirred mixture. The mixture was stirred at $0^{o}C$ for ten minutes. A white precipitate of $NaBF_4$ formed. The solution was stirred at room temperature for a further 15-30 minutes, then vacuum filtered through a sintered glass funnel to another 3-necked round-bottomed flask. The nitrogen inlet was connected to the flask which contained the diborane solution, and a flow of nitrogen was maintained as the diborane solution was cooled to $0^{o}C$.

Cyanoethyl Perloza was weighed into a sintered glass funnel and solvent exchanged to THF. It was then washed three times with THF, followed by three washes with THF distilled from sodium wire. Excess THF was removed by vacuum filtration.

The cyanoethyl Perloza was added slowly to the stirred diborane solution, at $0^{o}C$, while the flow of nitrogen was maintained. Evolution of gas occurred as the resin was added to the diborane solution. If the rate of resin addition was too fast resin was sometimes expelled from the top of the flask. A reflux condenser was fitted to the flask after all of the resin had been added. Nitrogen was exhausted to the paraffin bubbler from the top of the condenser. The suspension was refluxed for three hours under a constant slow flow of nitrogen. The reaction was then allowed to cool to room temperature. Ethanol (95%) was added cautiously to decompose excess diborane, gas was evolved. The resin was collected by filtration on a sintered glass funnel and washed twice with 1M HCl. After standing for 5-10 minutes excess HCl was removed by vacuum filtration and the resin was washed with water until the pH of the effluent was neutral to pH paper. The resin was washed twice with 0.5M NaOH solution. After standing for 5-10 minutes excess NaOH solution was removed by vacuum filtration and the resin was washed with water until the pH of the effluent was neutral to pH paper. Resin samples were taken for picrate titration and elemental analysis. The results of the picrate titration and the elemental analysis of the original cyanoethyl resin were used to calculate the percentage reduction of the starting cyanoethyl resin.

2.3 RESULTS AND DISCUSSION

The experiments described in this chapter were undertaken:

i) to determine whether Perloza was compatible with solvents commonly used for peptide synthesis.
ii) to establish a method for introducing a spacer arm bearing an amine group, in controllable yields, onto Perloza.

Successful outcomes of both studies were necessary before investigations could be undertaken into the feasibility of using Perloza as a support for solid phase peptide synthesis employing the established Boc and Fmoc chemistries.

2.3.1 Swelling of Perloza 100 Medium in different solvents

The results of an investigation into the swelling properties of Perloza in various solvents are given in Table 2.1. Perloza was found to have a limited range of swollen volumes in solvents ranging from water (11.5 ml/g) to diethyl ether (8.3 ml/g). The solvents likely to be used for SPPS were DCM (9.3 ml/g), dioxane (9.4) ml/g, and DMF (10.4 ml/g). From these results the resin shrinkage in changing from DMF to DCM would be 10%, which would be acceptable in a continuous flow or batch system should a change of solvents be required during a peptide synthesis.

Table 2.1 Swelling properties of Perloza in various organic solvents

Solvent	Vol solvent swollen resin/wt dry resin (ml/g)
DMF	10.3
Diethyl ether	8.3
Ethanol	9.9
Dioxane	9.4
DCM	9.3
Water	11.5
THF	9.4
Methanol	10.6

2.3.2 Reswelling of lyophilised Perloza 100 Medium

The results of experiments to determine the extent to which lyophilised Perloza reswelled in various solvents are given in Table 2.2. The results indicated that Perloza could not be dried and then reswollen to its original volume in any of the solvents tested under the conditions used. Therefore, the importance of maintaining the resin in a solvent swollen state at all times is stressed.

Table 2.2 Reswelling of Perloza after lyophilisation

Solvent	Vol solvent swollen resin/wt dry resin (ml/g)
DMF	2.0
Methanol	2.0
Water	3.7
Ethanol	1.4
Dioxane	1.45
DCM	1.3

2.3.3 Methods for analysis of amine-substituted Perloza resin

2.3.3.1 Ninhydrin assay for determination of the amine substitution level of functionalised Perloza resin

It was found during the ninhydrin assay that heating the dried resin samples resulted in reswelling of the resin. The degree of resin reswelling was not investigated.

The results of analyses of amine-substituted Perloza by the ninhydrin assay and by picrate titration were compared by graphical means (Figure 2.1). Comparison of results (see Figure 2.1) of analyses of amine-substituted Perloza by the two methods indicated that the ninhydrin assay sometimes gave nitrogen substitution levels considerably lower than those obtained by the picrate titration. The results of the picrate titration were shown to agree closely with the results of two other methods (HCl titration and elemental analysis, see Section 2.3.4) for determining the amine substitution of functionalised Perloza.

Because the ninhydrin method did not always give the same result as the picrate titration, it was mainly used as a semi-quantitative assay of amine substitution, and the results were treated with some reservation. In some cases, for example if resin samples were dried, the ninhydrin assay was the only practical means of determining the amine substitution level of the sample. The advantages of the ninhydrin assay were that it was considerably faster than the picrate titration method, it could be used with dried resin samples, and only small quantities of resin (less then 5 mg) were required. At low amine substitution levels the ninhydrin assay appeared to be more sensitive than the picrate titration. It was decided not to investigate means of obtaining consistent agreement between the ninhydrin assay and the picrate titration because the picrate titration was found to be adequate for most of the analyses performed.

2.3.3.3 HCl titration of resin-bound amine groups

Determination of the amine substitution of aminohexyl Perloza was straightforward using the HCl titration, but it was time consuming. One possible improvement to the HCl titration method used in this study would have been to wash the resin with dilute NaOH solution to convert the resin bound amine-hydrochloride back to the free amine prior to dry weight determination. The presence of the hydrochloride salt made a significant difference to the final calculated amine substitution level, and had to be allowed for by calculation. Also, care had to be exercised when transferring the resin to the sintered glass funnel for the dry weight determination. Less than quantitative transfer would result in an anomalously high amine substitution level.

2.3.3.4 Picrate titration of resin-bound amine groups

Initial experiments using the original procedure of Gisin (1972) with lyophilised aminohexyl Perloza were unsuccessful because the dichloromethane solvent used did not reswell the Perloza to any extent. However, picrate titration of lyophilised aminohexyl Perloza using 50% aqueous ethanol as solvent did give a result almost identical to that previously found by titration of the water-swollen resin with HCl. If the resin had initially been solvent swollen the Gisin method may have

worked. However, it was found to be more convenient to use 50% aqueous ethanol as the solvent when dealing with Perloza, so the Gisin procedure was modified as described in the experimental section.

The extinction coefficient of DIEA picrate in 50% aqueous ethanol was found to be 14600 ± 3% at a lambda max. of 360 nm, compared to the literature value of 14500 at 358 nm in 95% ethanol (Gisin, 1972). The literature extinction coefficient of 14500 was used in this study. No difference was found between the absorbance of solutions of DIEA picrate and NEt_3 picrate at 358 vs 360 nm. Therefore all measurements were made at the literature wavelength of 358 nm.

The modified Gisin method used to determine the amine substitution of amino-Perloza was originally developed using 5% DIEA in 50% aqueous ethanol for picrate elution. However, a temporary shortage of DIEA necessitated use of triethylamine solution for elution of picrate. The triethylamine solution was made to 10% v/v with 50% aqueous ethanol. No difference was found between the extinction coefficient of DIEA picrate and NEt_3 picrate in 50% aqueous ethanol. Accordingly, a 10% solution of triethylamine in 50% aqueous ethanol was used for the majority of picrate titration results reported in this study.

One problem encountered was entrapment of picric acid in the sinter of the funnel. Thorough washing of the sinter was required to ensure all of the unbound picric acid was removed. Failure to ensure trapped picric acid was washed away resulted in anomalously high amine substitution levels. When due care was taken the picrate titration was more convenient and less time consuming than the HCl titration. In addition, all of the manipulations were carried out in the same sintered glass funnel, thus eliminating resin transfer errors.

2.3.4 Comparison of different methods for the determination of the amine substitution of amine functionalised Perloza resins

A comparison of three methods of determining the amine substitution of aminohexyl Perloza was made. The amine substitution of aminohexyl Perloza was determined by picrate and HCl titrations. In addition elemental analysis of the resin gave a figure for the total nitrogen substitution. The three results are given in Table 2.3.

Table 2.3 Comparison of picric acid, HCl and elemental analysis results for determination of amine substitution of aminohexyl Perloza

Analysis method	Amine substitution
HCl titration	1.01 mmole/g
Picrate titration	0.98 mmole/g
Elemental analysis	0.93 mmole/g

The theoretical amine substitution was derived from the elemental analysis result by dividing the total nitrogen substitution by 2. This result would be expected to be the same as the titration result if there was no cross linking between resin-bound carbonylimidazole groups by 1,6-diaminohexane (see Section 2.2.8). Any cross linking would reduce the number of amine groups available for titration. Therefore, if cross linking had occurred, the titration result would be expected to be lower than the theoretical substitution calculated from the elemental analysis result.

A comparison of the results given in Table 2.3 showed good agreement between the HCl result of 1.01 mmole/g vs the picric acid result of 0.98 mmole/g. Both results were higher than the theoretical amine substitution of 0.93 mmole/g derived from the elemental analysis. Although the elemental analysis result was lower, the result of the comparison was encouraging because it indicated very little cross linking had occurred during reaction of 1,6-diaminohexane with the carbonylimidazole activated Perloza resin.

In a second experiment, aminopropyl Perloza was titrated using the HCl and picrate methods. The HCl titration result of 1.95 mmole/g was again 3% greater than the picrate titration result of 1.89 mmole/g. A possible explanation for the difference in the results of the two titration methods was that the end point pH of 4.00 used in the HCl titration may have been slightly lower than the true end point pH.

The picrate titration was ultimately chosen for routine use for determination of amine substitution of functionalised Perloza because:

i) it was the more convenient of the two titration methods.
ii) resin transfer errors were eliminated.

2.3.5 Reaction of Perloza 100 Medium with 2-aminoethylsulphuric acid

Aminoethyl cotton fabric has been made with amine substitution levels up to 0.81 mmole/g using the 2-aminoethylsulphuric acid procedure of Guthrie (1947) and Reeves and Guthrie (1953). In their procedure cotton fabric was heated at 120°C, while moist with a concentrated solution of NaOH and 2-aminoethylsulphuric acid, to introduce aminoethyl groups. These conditions were tried with Perloza, but the resin dried out. Since it was previously shown that it was impossible to reswell dried Perloza resin to its original volume (Section 2.3.2), the procedure was modified to avoid drying of the resin. After reaction in a bomb at 100°C for 31 hours, the resulting aminoethyl Perloza had an amine substitution level of 0.052 mmole/g as determined by the ninhydrin assay. It did not seem likely that amine substitution levels much higher than 0.052 mmole/g could be obtained using this method. As it was considered desirable to be able to obtain amine substitution levels of up to 2 mmole/g, if required, this method was abandoned.

2.3.6 Carbonyldiimidazole / 1,6-diaminohexane (CDI/DAH) functionalisation of Perloza

Carbonylimidazole Perloza was made using a procedure based on that of Bethell et al (1979). Preliminary experiments showed no advantage in using more than 4 mmole CDI per gram of dry resin. The intermediate carbonylimidazole substituted Perloza, carbonylimidazole substitution 2.21 mmole/g, was reacted with 1,6-diaminohexane to yield aminohexyl Perloza. Titration of the aminohexyl Perloza with 0.100M HCl to a pH 4.00 end point gave an amine substitution of 0.98 mmole/g, which represented a coupling yield of 49%, assuming that cross linking had not taken place. Cross linking was to be avoided if possible because it alters the pore size distribution of the matrix, which is undesirable for separation chromatographic applications.

The 0.98 mmole/g amine substitution level achieved using the CDI/DAH reaction was useful, however this method was not pursued once successful

reduction of cyanoethyl Perloza had been demonstrated. One possible drawback of the aminohexyl urethane Perloza is the questionable stability of the urethane link at extremes of pH. No investigations were undertaken to determine the pH-dependent stability of the urethane-Perloza linkage.

Although the CDI/DAH method was not pursued in this study it did provide a very simple means of introducing a primary amine onto Perloza.

2.3.7 Cyanoethylation of Perloza

The results of preliminary cyanoethylation experiments are given in Table 2.4. The standard cyanoethylation procedure was developed from the final experiment reported in Table 2.4.

Table 2.4 Initial Perloza cyanoethylation experiments

Weight wet resin	Volume of co-solvent	Volume of acrylonitrile	Reaction time	Nitrogen substitution (mmole/g)
31 g	15 ml ethanol	25 ml	60 min	1.10
10 g	20 ml dioxane	8 ml	60 min	4.21
30 g	60 ml dioxane	25 ml	120 min	n.d.*
30 g	60 ml dioxane	25 ml	60 min	4.85
50 g	50 ml dioxane	25 ml	60 min	3.66

n.d.* not determined, the resin formed a gel

The results of a number of standard cyanoethylations of Perloza are given in Tables 2.5 and 2.6. The standard reaction scheme (Section 2.2.9.2) gave cyanoethyl Perloza with controllable nitrogen substitution levels of up to 3.66 mmole/g.

Table 2.5 Cyanoethylation of Perloza 100 Medium

N substitution (mmole/g)	K_{acr}*
3.66	0.200
2.94	0.156
2.25	0.107
1.35	0.074
1.28	0.091
1.12	0.072
1.09	0.065
0.88	0.059
0.83	0.065
0.80	0.059
0.66	0.059
0.48	0.073
0.42	0.034
0.38	0.032

$$* K_{acr} = \frac{\text{vol acrylonitrile (ml)}}{\text{vol acrylonitrile (ml) + vol dioxane (ml) + wt water-wet resin (g)}}$$

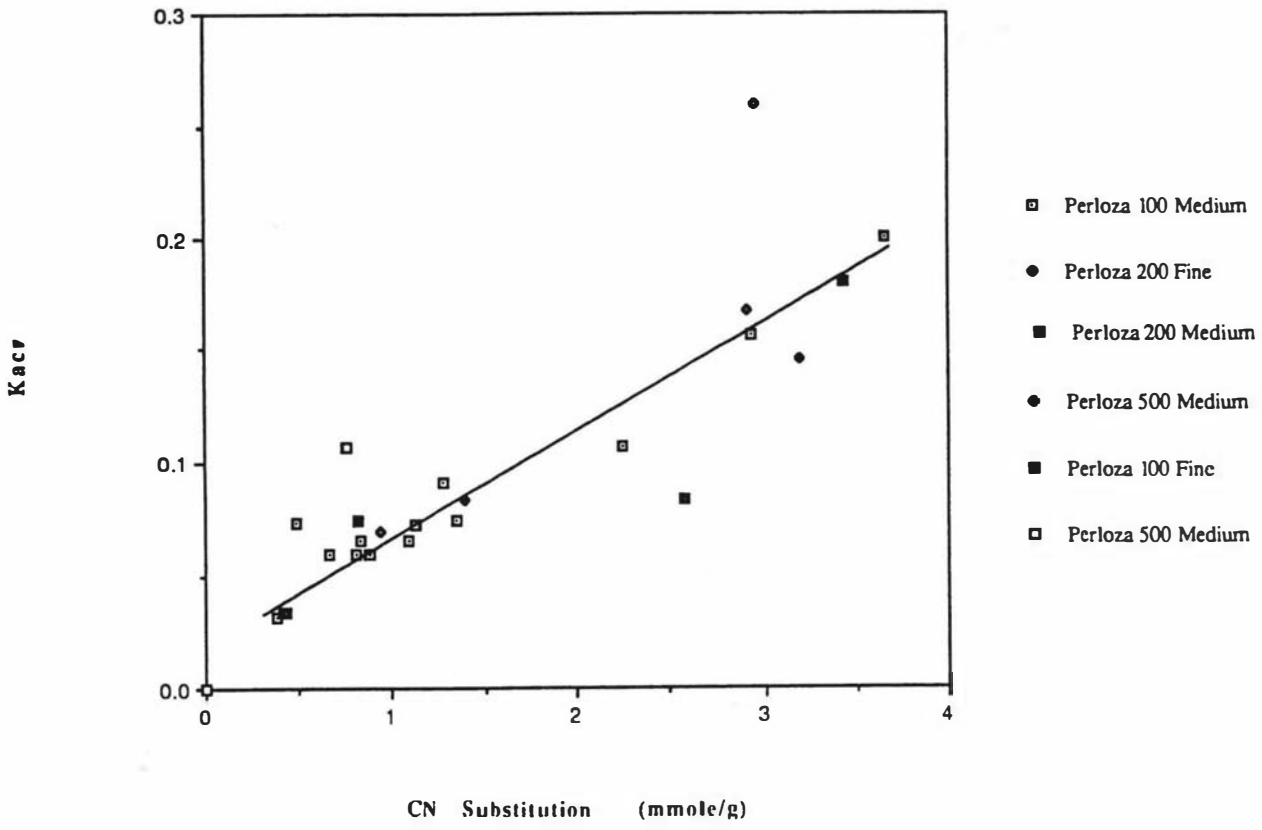
Table 2.6 Cyanoethylation of grades of Perloza other than 100 Medium

N substitution (mmole/g) (grade of Perloza)	K_{acr}
0.82 (100 Fine)	0.074
3.19 (200 Fine)	0.146
1.40 (200 Fine)	0.083
3.43 (200 Medium)	0.180
2.58 (200 Medium)	0.083
0.43 (200 Medium)	0.034
0.42 (200 Medium)	0.034
2.96 (500 Fine)	0.259
2.91 (500 Fine)	0.167
0.94 (500 Fine)	0.069
0.76 (500 Medium)	0.107

Higher substitution levels using the standard scheme were not attempted, however during the initial experiments a nitrogen substitution level of 4.85 mmole/g was achieved without destruction of resin integrity. The resin at 4.85 mmole/g nitrogen substitution was found to be highly swollen in NaOH solution and consequently difficult to filter. Neutralisation of the NaOH by acetic acid made the resin easy to filter. Attempts to achieve higher nitrogen substitution levels resulted in formation of a sticky mass and loss of the resin's free flowing, beaded character.

All of the results from the standard cyanoethylation procedure were graphed by plotting the nitrogen substitution vs K_{acr} (Figure 2.2). Most of the points for Perloza 100 Medium fell on a straight line. The main use of this graph was for estimating the volume of acrylonitrile required to achieve a desired cyanoethylation substitution, given a predetermined amount of wet resin to be cyanoethylated. Points were added as data became available, but only three or four initial data points were required for a useful calibration graph to be obtained.

Figure 2.2 Graph of nitrogen substitution level of cyanoethyl Perloza resins vs K_{acr}



The nitrogen substitution results of cyanoethylation of other grades of Perloza were also plotted vs K_{acr} in Figure 2.2. The points for other grades of Perloza often fell close to the line plotted for Perloza 100 Medium. The graph was therefore of some use for predicting the volume of acrylonitrile required for cyanoethylation of other grades of Perloza. However, it would be likely that separate calibration graphs would be required for the different grades of Perloza.

The results of the cyanoethylation experiments showed that it was possible to achieve controllable, low to high levels of cyanoethyl substituted Perloza. The next step was to investigate reduction of cyanoethyl Perloza with diborane.

2.3.8 Reduction of cyanoethyl Perloza with diborane

Diborane is a powerful reducing agent (Brown and Korytnyk, 1960) generated by reaction of sodium borohydride with boron trifluoride in diglyme solvent (Brown and Tierney, 1958). THF may also be used as solvent. Although sodium borohydride is only sparingly soluble in THF it dissolves as the reaction proceeds. The order of addition can be important. Diborane is generated more smoothly if a diglyme solution of sodium borohydride is added to a THF or diglyme solution of boron trifluoride. A slight excess of boron trifluoride is desirable. Diborane solutions in THF may be exposed to air without spontaneous ignition, however it is best to keep air exposure to a minimum as diborane is readily hydrolysed by atmospheric moisture (Brown et al, 1970).

A nitrile reacts with diborane to form an N,N,N-trialkylborazole (Emeleus and Wade, 1960). Subsequent hydrolysis of the N,N,N,-trialkylborazole yields a primary amine. Nitriles are reduced in high yields at room temperature by diborane solutions. Two nitrile groups are reduced for each diborane molecule (Brown and Subba Rao, 1960).

Only one reference to reduction of cyanoethyl cellulose was found. Daly and Munir (1984) claimed to have achieved quantitative reduction of cyanoethylated bleached wood pulp using refluxing borane-dimethyl

sulphide (BH_3.DMS) or borane-THF (BH_3.THF). In their procedure, they took 2.0 g of cyanoethyl cellulose (nitrogen substitution 5.5 mmole/g, 11 mmole nitrogen), suspended it in 30 ml THF, added 5 ml 2M borane-DMS or 10 ml 1M borane-THF (10 mmoles borane) and refluxed for three to five hours. After work up of the reaction they found no nitrile band in the infrared spectrum.

On a stoichiometric basis alone the yield would have been expected to be 91%, because one borane molecule reduces one nitrile group, and the ratio of nitrile to borane was 11:10. In addition, diborane reacts with alcohols in the order primary>secondary>tertiary (Brown et al, 1970), and the hydroxyls of the cyanoethyl cellulose would have been expected to react with the diborane, resulting in destruction of diborane. As noted in the Section 2.2.10.2 of this study, evolution of gas accompanied addition of cyanoethyl Perloza to diborane solutions. Because the cyanoethyl Perloza was in very dry THF, the only explanation for the evolution of gas was decomposition of diborane by resin-bound hydroxyl groups. Daly and Munir also noted that the final nitrogen substitution was the same as that found before reduction, however, examination of their elemental analysis data revealed that the final nitrogen substitution was 5.1 mmole/g, compared to the initial substitution of 5.5 mmole/g. Although they claimed quantitative reduction, Daly and Munir noted that

> *The observed ion-exchange capacities are approximately 50% of the theoretical values estimated from the initial cyanoethyl substitution, although no loss of functionality was evident in the elemental analysis data.*

Daly and Munir explained the lower than expected ion-exchange capacities by stating that

> *The derivative must exist in the free amine form, but the charge development on high DS derivatives must limit the extent of protonation in aqueous acid.* (DS = Degree of Substitution)

The most likely explanation now would appear to be that they achieved less than quantitative reduction of the cyanoethyl cellulose. From

their statements it appears the real value was about 50%. One observation which may have led Daly and Munir to assume they had achieved quantitative reduction was the absence of a nitrile stretching band at about 2250 cm^{-1} in the infrared spectrum. In this study it was sometimes found that a nitrile band was absent after a reduction of cyanoethyl Perloza even though the picrate titration and elemental analysis data suggested unreacted nitrile was still present. For example, cyanoethyl Perloza (initial nitrogen substitution 1.50 mmole/g) after reduction had a nitrogen substitution of 1.53 mmole/g, and an amine substitution of 1.26 mmole/g by picrate titration (84% reduction yield). However, examination of the infrared spectrum did not reveal the expected nitrile band between 2200 and 2300 cm^{-1}. The absence of the expected nitrile band could not be explained, unless it was too weak to be seen under the conditions used.

The results of investigations into the reduction of cyanoethyl Perloza using diborane at room temperature are given in Table 2.7.

Table 2.7 Reduction of cyanoethyl Perloza 100 Medium at room temperature

Initial CN substitution (mmole/g)	Excess of diborane and reaction time	Final NH_2 substitution (mmole/g)	% Reduction
3.66	11X, 65 hr	2.77	75
2.94	9X, 24 hr	1.90	65
2.94	10X, 102 hr	1.80	61
2.94	10X, 67 hr	2.55	75
2.25	13X, 69 hr	1.82	81
1.35	22X, 67 hr	1.07	79
1.28	23X, 17 hr	1.09	85
1.50	19X, 22 hr	1.26	84

Daly and Munir (1984) were unable to reduce cyanoethyl cellulose at room temperature using diborane. However, it was found in this study that it was possible to reduce cyanoethyl Perloza at room temperature in high yield using diborane. A large excess of diborane, typically 9-23X, was

used. Reduction times varied from 17-102 hours. The highest yields were obtained in the last four experiments, 79-84% (Table 2.7).

Considerable care was taken in the last four experiments to ensure solvents were dry. The yields of the last four experiments compared favourably with those obtained by reduction under reflux (see Tables 2.8 and 2.9). The flow properties of aminopropyl Perloza made by room temperature reduction of cyanoethyl Perloza varied due to generation of fines. During the room temperature reductions the suspension was magnetically stirred, and it was likely that the grinding action of the magnetic flea was responsible for most of the fines generated. The resins reduced for 17-22 hours exhibited good flow properties because they contained little or no fines. A better method of mixing the room temperature reductions, for example by swirling, might have resulted in fewer fines.

The results of investigations into the reduction of cyanoethyl Perloza using diborane under reflux (temperature 67^{o}C) are given in Tables 2.8 and 2.9.

Table 2.8 Reduction of cyanoethyl Perloza 100 (Medium unless noted)

Initial CN substitution (mmole/g)	Excess of diborane and reaction time	Final NH_2 substitution (mmole/g)	% Reduction
1.12	17.1X	0.76	68
1.09	17.5X	0.87	80
0.97	19.8X	0.85	88
0.88	21.8X	0.81	94
0.83	23.0X	0.83	100
0.80	23.8X	0.69	86
0.66	28.9X	0.64	97
0.48	66.0X	0.44	92
0.42	19.4X	0.28	67
0.38	21.8X	0.37	97
0.82 (P100 Fine)	39.9X	0.70	85

Table 2.9 Reduction of cyanoethyl Perloza 200 and 500

Initial CN substitution (mmole/g)	Excess of diborane and reaction time	Final NH_2 substitution (mmole/g)	% Reduction
P200CN Medium			
0.42	31.7X	0.30	71
0.43	28.6X	0.43	100
P500CN Fine			
2.91	14.4X	2.63	90
1.03	16.3X	0.85	83
0.65	34.0X	0.34	52
0.94	20.6X	0.69	73
P500CN Medium			
0.76	26.3X	0.65	86
0.69	15.3X	0.56	81
0.38	21.1X	0.31	82

Reduction of cyanoethyl Perloza by an excess of diborane in refluxing THF solution resulted in products containing no fines. Reduction yields varied from 52-100%. No common factor was found to explain the variation in reduction yields. An explanation for the less than quantitative reduction yields could be that the diborane was consumed by reaction with the cellulose hydroxyls, except that gas was always generated on adding ethanol in the work up, which indicated that diborane was still present at the end of the reaction. Possibly the time allowed, three hours, was insufficient to give 100% reduction, however in some cases 100% reduction was achieved in three hours.

The post-reduction nitrogen substitution was sometimes seen to be less than the initial substitution. Loss of nitrogen could be explained by hydrolysis of the nitrile to carboxylic acid, or loss of acrylonitrile from the reversible reaction given in Scheme 2.1. However, many of the reductions showed a post reduction nitrogen substitution level similar or identical to the initial level. Obviously a number of factors were responsible for the variation in the reduction yields obtained in this study.

Scheme 2.1

$$\text{Cellulose-O-CH}_2\text{-CH}_2\text{-CN} \xleftrightarrow{\text{NaOH}} \text{Cellulose-OH} + \text{CH}_2\text{=CH-CN}$$

Regardless of some unanswered questions regarding reduction yields, aminopropyl Perloza made by reflux reduction exhibited good flow properties and did not contain fines. The readily controllable range of nitrile and hence amine substitutions, and the availability of low to high amine substitutions as required, made the procedure attractive for this study. The resin-bound amine groups were found to be reactive and readily accessible for further reaction. In addition, the ether bond linking the aminopropyl group to the resin was likely to have greater chemical stability than the urethane of the aminohexyl urethane-Perloza made by the CDI/DAH procedure.

The cyanoethyl reduction method also gives a spacer arm which is shorter than the hexyl spacer of the CDI/DAH resin. This would possibly result in less hydrophobic interaction of the spacer arm with solutes in aqueous media (O'Carra, 1978) should the aminopropyl derivative be used for chromatographic purposes.

The results of these studies provided a basis for further investigations into the use of Perloza as a support for solid phase peptide synthesis using existing Boc and Fmoc protocols. Perloza was shown to be compatible with solvents, such as DMF, which are commonly used for peptide synthesis by both Boc and Fmoc methodology. It was expected that polar solvents such as DMF would result in favourable solvation of the carbohydrate matrix of Perloza.

The lower than quantitative reduction yields would result in the presence of resin-bound cyanoethyl groups. The presence of cyanoethyl groups resulting from incomplete reduction was not envisaged as being detrimental if the resin was to be used as a support for peptide synthesis or affinity chromatography, as they would be expected to be relatively inert. It should be borne in mind, however, that at extremes of pH in aqueous solvents, some hydrolysis of nitrile groups to carboxylic acid may be expected to occur.

2.4 CONCLUSIONS

1) Perloza appeared to be suitable for use with a variety of solvents. Although the external volume of the gel in different solvents was similar, the effect of the different solvents on the internal structure of Perloza was not known.

2) Perloza should be kept damp with solvent, it should not be allowed to dry out.

3) CDI/DAH activation may be used to generate aminohexyl Perloza with a useful primary amine substitution level and minimal cross linking.

4) Cyanoethylation of Perloza can give controllable nitrile substitutions over the range of 0-4.5 mmole/g. Subsequent diborane reduction of cyanoethyl Perloza is an efficient method for synthesising aminopropyl substituted Perloza.

5) The ninhydrin assay was not used extensively in this study. More work would be required before the assay would be useful for routine use with Perloza. Modifications of the currently used ninhydrin assay would probably be required to adapt the procedure for use with Perloza. In this study, a similar situation was experienced with the Gisin picrate titration.

CHAPTER 3

SOLID PHASE PEPTIDE SYNTHESIS ON PERLOZA:

BOC CHEMISTRY

CHAPTER 3 SOLID PHASE PEPTIDE SYNTHESIS ON PERLOZA: BOC CHEMISTRY

3.1 INTRODUCTION

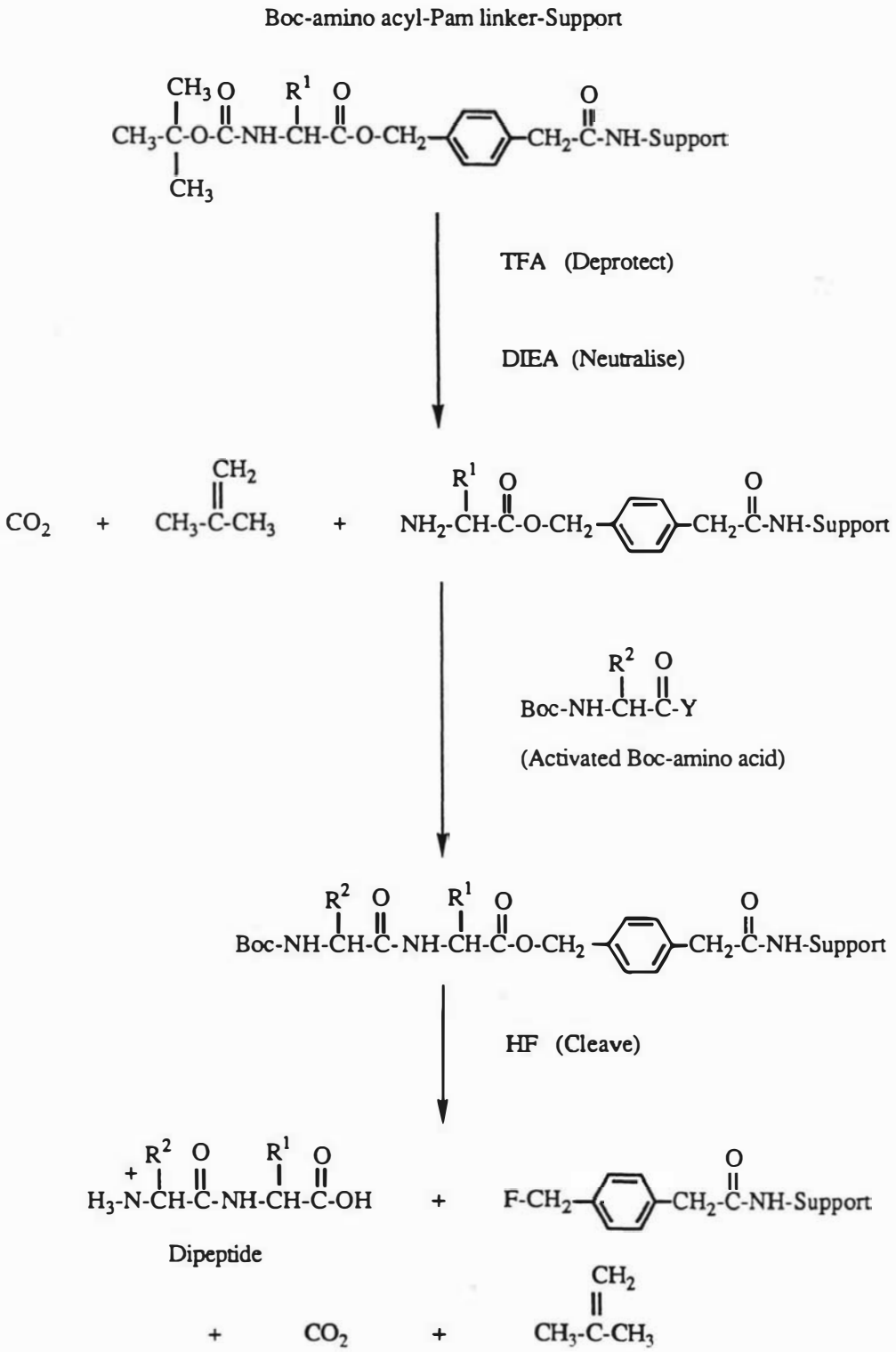
The tertiary butyloxycarbonyl (Boc) group was developed as an acid labile amine protecting group (Carpino 1957a, 1957b) and applied to solution peptide synthesis by McKay and Albertson (1957) and Anderson and MacGregor (1957). The Boc group was first used for solid phase peptide synthesis by Merrifield (1964a,b), and has come to be the most commonly used acid labile Nα-amino protecting group (Erickson and Merrifield, 1976). The Boc group is cleaved by 25% TFA in DCM, 4M HCL in dioxane (Stewart and Young, 1984), 10% sulphuric acid in dioxane (Houghten et al, 1986), and boron trifluoride in acetic acid (Hiskey et al, 1971; Schnabel et al, 1971). A diagram of the Boc method of SPPS is given as Figure 3.1.

The solid support usually used for SPPS by the Boc method is 1% divinylbenzene cross linked polystyrene (Erickson and Merrifield, 1976), although many other supports have been investigated (Barany et al, 1987). The polystyrene support was first functionalised for SPPS by chloromethylation of pendant benzene rings by chloromethyl methyl ether in the presence of a Lewis acid catalyst such as $SnCl_4$ (Merrifield 1963). The chloride was then displaced by a Boc-amino acid, as its triethylammonium salt, to anchor the Boc amino acid to the support via a benzyl ester. However, the benzyl ester linkage was not found to be completely stable to the acid solutions used for Boc cleavage. For example, Gutte and Merrifield (1971) noted an average 1.4% cleavage of the anchoring benzyl ester bond during each cycle in a synthesis of the 124 amino acid residue polypeptide Ribonuclease A. In that synthesis 50% TFA/DCM was used for Boc cleavage.

The phenylacetamidomethyl (PAM) linker was developed in response to the reported cleavage of peptide from the traditional benzyl ester during acidic Boc cleavage (Mitchell et al, 1976b). The amino acyl-PAM ester linkage was found to be 100 times more stable to refluxing TFA than the benzyl ester previously used, and is now one of the most commonly used linkers for solid phase peptide synthesis by the Boc methodology. A PAM linked Boc-amino acid may be introduced to aminomethyl polystyrene

(Mitchell et al, 1976a) by DCC mediated coupling of Boc-amino acyl-4-oxymethyl phenylacetic acid (Mitchell et al, 1976b, 1978). Alternatively, 4-bromomethyl phenylacetic acid may be coupled to aminomethyl polystyrene and a Boc-amino acid subsequently coupled either as its triethylammonium salt (Mitchell et al, 1976b), or it may be coupled in the presence of potassium fluoride (Toth and Penke, 1991).

Figure 3.1 Boc SPPS methodology illustrated by the synthesis of a dipeptide



Reactive side chains of Nα-Boc amino acids must be protected during the course of peptide synthesis. For example, the side chain hydroxyls of serine and threonine are protected as their benzyl ethers, and the side chain carboxyls of aspartic and glutamic acids are protected as their benzyl esters. The N^{ε}-amino of lysine is protected by the p-chloro-carbobenzyloxy (N^{ε}-pCl-CBZ) group, the N^{G} of Arg is protected by the tosyl (Tos) group, and the N^{im} of His is protected by the tosyl group (if HOBt is not to be used for coupling of any later Boc-amino acids) or by the 2,4-dinitrophenyl (Dnp) group if HOBt is to be used for following couplings. The side chain thiol of cysteine is protected by the p-methoxybenzyl (S-pMeO-Bzl) group, and finally the N^{in} of Trp is protected with the formyl (N^{in}-CHO) group. All of the side chain protecting groups given are cleaved by strong acid, for example liquid HF, except the 2,4-dinitrophenyl group on the N^{im} of His and the formyl group on the N^{in} of Trp. The His N^{im}-Dnp group is generally removed, prior to peptide cleavage, by thiolysis of the peptide-resin, while the Trp N^{in}-formyl group is cleaved, after peptide cleavage, by aqueous piperidine.

A currently popular method for coupling most Boc amino acids is via their symmetrical anhydrides, preformed in solution using DCC (Wieland et al, 1971; Hagenmaier and Frank, 1972). Boc-Asn and Boc-Gln cannot be coupled as their symmetrical anhydrides because of side chain dehydration to form the nitrile. They are instead coupled as their HOBt esters (Konig and Geiger, 1970a,b,c). Boc-Arg(N^{G}-Tos) is also coupled as its HOBt ester instead of the anhydride to prevent intramolecular lactam formation. Other methods of activating Boc-amino acids for coupling include TBTU (Reid and Simpson, 1992), HBTU (Schnolzer et al, 1991), and BOP (Coste et al, 1989). The progress or extent of completion of coupling is usually monitored by the quantitative ninhydrin assay (Sarin et al, 1981).

Cleavage of the peptide from the resin support is usually accomplished by strong anhydrous acid. For example, liquid hydrogen fluoride, at $0^{o}C$, containing anisole as scavenger, is commonly used for peptide cleavage (Sakaibara et al, 1967, 1971). The scavenger is included to trap carbocations formed from cleavage of side chain protecting groups. Peptide cleavage using liquid HF must be carried out in a special HF

resistant all-teflon apparatus. Trifluoromethanesulphonic acid (TFMSA) (Bergot et al, 1986), with scavengers, may also be used for peptide cleavage. One of the advantages of using TFMSA rather than liquid HF is that the cleavage can be carried out in glass apparatus. This is not possible with HF because HF reacts with glass.

Carbohydrate supports that have been used for SPPS by Boc methodology include Sephadex LH20 (Vlasov and Bilibin, 1969; Orlowska et al, 1975; Erickson and Merrifield, 1976), cellulose paper (Eichler et al, 1989), and cellulose cotton (Eichler et al, 1991).

Merrifield initially investigated Sephadex LH20 as a support for solid phase peptide synthesis in 1959, but really satisfactory conditions were not found. The work was resumed in 1965, and the tetrapeptide LAGV was synthesised. The method was still not found to be entirely satisfactory, as inexplicable side chain termination was observed during several separate syntheses of the tetrapeptide LAGV (Erickson and Merrifield, 1976).

Vlasov and Bilibin (1969) coupled Boc-glycine directly onto Sephadex LH20 using CDI, a mixed anhydride, or Boc-glycine chloride. Boc cleavage with TFA, followed by coupling of Boc-amino acids as their active esters, and cleavage by hydrolysis, furnished a pentapeptide. The nonapeptide bradykinin was synthesised on a similar support using DCC for coupling and 1M p-toluenesulphonic acid in acetic acid for Boc cleavage (Vlasov et al, 1973).

Orlowska et al (1975) synthesised a pentapeptide fragment of Substance P on LH20 using Boc methodology. Boc-glycine was anchored to the support hydroxyl groups in the presence of CDI to give an initial amino acid substitution of 0.25 mmole/g. Boc cleavage was by 1M p-toluenesulphonic acid in acetic acid. Difficulties were encountered in cleaving the peptide from the support. Methanolic NaOH, 0.1M, was found to be the most effective cleavage reagent.

Eichler et al (1989) anchored Fmoc-alanine to Whatman 540 cellulose paper by reaction of the paper with Fmoc-alanine chloride, to anchor the amino acid to the paper via an ester bond. The Fmoc group was cleaved

with piperidine in DMF, and three hexapeptides (YVPKXA, YEETXA, YKQIXA; X= 6-aminohexanoic acid) were then synthesised by a combination of Boc and Fmoc methodology. Fmoc-amino acids used were Fmoc-Lys(Boc), Fmoc-Pro and Fmoc-Val. The benzyl side chain protecting groups of Thr and Glu were cleaved using boron tris trifluoroacetate in TFA (Pless and Bauer, 1973). The peptides were then cleaved from the cellulose by alkaline hydrolysis.

Eichler et al (1991) used cellulose cotton as a support for the SPPS of a hexapeptide and eight heptapeptides by the Boc/Bzl and Fmoc/tBu methods. The C-terminal amino acids were anchored directly to the cotton by N-methylimidazole (NMI) catalysed carbodiimide/HOBt activation. Comparison by HPLC of the products produced by the Boc and Fmoc methods showed that the peptides made using the Boc/Bzl methodology were considerably more heterogeneous than those made using the Fmoc methodology. This was explained by Eichler et al in terms of inferiority of the method used to cleave the peptides made using the Boc methodology. Incomplete cleavage of peptide side chain protecting groups by boron tris trifluoroacetate in TFA, as well as subsequent side reactions during alkaline cleavage (for example aspartimide formation during alkaline treatment of Asp(OBzl) containing peptides), were seen as major problems with the methodology employed in their study.

The published results showed that solid phase peptide synthesis could be carried out on known carbohydrate supports using the Boc methodology. However, as discussed above, these supports each presented their own problems when used for SPPS with the Boc methodology. Therefore, it was decided to investigate the feasibility of using the less well known Perloza beaded cellulose for SPPS by Boc methodology. An initial attempt was made to synthesise the tetrapeptide LAGV directly onto aminopropyl Perloza. As the synthesis proved successful the work was extended by anchoring a C-terminal Boc-amino acid to the support via a cleavable linker, followed by syntheses of short test peptides. The base-labile glycolamide linker (Baleux et al, 1984) was chosen as the anchoring group because of its low cost, straightforward chemistry, and availability. The approach taken to anchor Boc-amino acids to Perloza was to react either α-chloroacetic or α-bromoacetic acid anhydride with aminopropyl Perloza, and to then displace the halide with the cesium

salt of a Boc-amino acid. This furnished a glycolamide ester link between the Boc-amino acid and the Perloza. Peptide synthesis was carried out by coupling Boc-amino acids as their HOBt esters. The peptides were cleaved by alkaline hydrolysis.

3.2 MATERIALS AND METHODS

3.2.1 Chemicals and Equipment

Aminopropyl Perloza beaded cellulose was made as described in Chapter 2. Nα-Boc-L-amino acids (Ala, Gly, Leu, Phe, Tyr(Bzl), Val) were supplied by Bachem, Torrance, California. Authentic leucine-enkephalin was purchased from Vega.

Electronic grade acetic acid was from Rhone-Poulenc, NZ. Analytical reagent grade acetic anhydride was obtained from Ajax Chemicals, Sydney. Trifluoroacetic acid was from Halocarbon, N.J., and was distilled before use. Reagent grade α-bromoacetic acid and N-methylmorpholine (NMM) were purchased from BDH, NZ. N-methylmorpholine (NMM) was distilled from barium oxide. The α-chloroacetic acid was from J.T. Baker Ltd. Dimethylaminopyridine (DMAP) was from Riedel-de Haen. Reagent grade dicyclohexylcarbodiimide (DCC), diisopropylcarbodiimide (DIC), and 1-N-hydroxybenzotriazole monohydrate (HOBt.H_2O) were purchased from Aldrich. Methanesulphonic acid (MSA) was from May and Baker. Dimethylacetamide (DMA) was from BDH, NZ. All other reagents were from sources listed in the previous Chapter.

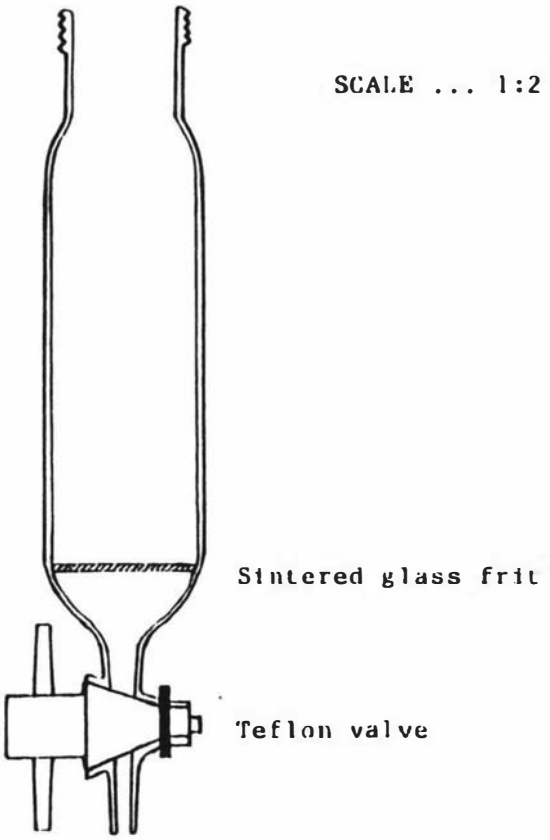
Peptide syntheses were carried out manually using a Rototorque wheel (Cole Parmer, Chicago) for end over end mixing, or semimanually using an LKB Biochrom Ltd. Biolynx 4175 continuous flow peptide synthesiser. A screw capped reaction vessel, fitted with a glass frit and teflon valve, was made for use with the Rototorque (Figure 3.2).

Amino acid analyses were performed using a Pharmacia LKB Alpha Plus apparatus equipped with a Spectra Physics Chromjet integrator. Peptide-resins and amino acyl-resins were washed with ether and dried at 110°C prior to hydrolysis. Hydrolyses of peptides and peptide-resins were performed in 6M HCl plus 0.1% phenol, 0.3-0.5 ml, under nitrogen, at

110°C for 18-24 hours. The hydrolysates were dried in vacuo and made up in pH 2.2 0.2M citrate buffer to give a final equivalent peptide concentration of 2.5 x 10^{-4}M. The substitution levels reported in this thesis (in units of mmole/g) were based on the number of mmoles of amino acid or peptide per gram of dry amino-acyl-resin or peptide-resin.

HPLC analyses and purifications were carried out using a Waters system comprised of 2 model 510 pumps, a Waters model 410 absorbance detector, WISP sample injector (analytical HPLC only), and Pharmacia chart recorder. Acetonitrile (HiPerSolv grade) was purchased from BDH. The mobile phase solvent systems for HPLC consisted of buffer A (MilliQ water containing 2% acetonitrile, 0.1% TFA) and buffer B (0.1% TFA in acetonitrile). Analytical HPLC was carried out using a Vydac 4.6 x 250 mm C_4 column, with a linear gradient run from 0% B to 60% B over 60 minutes. A 10 x 250 mm Synchroprep C_{18} column was used for the preparative HPLC purification runs. Generally 50 to 90 mg of crude peptide were purified in each prep HPLC run.

Figure 3.2 Reaction vessel used for SPPS of LAGV onto aminopropyl Perloza (made by Mr. G. Platt, glassblower, Massey University)



3.2.2 Synthesis of Leu-Ala-Gly-Val directly onto aminopropyl Perloza

An attempt was made to synthesise the Merrifield test peptide Leu-Ala-Gly-Val (Merrifield, 1963) directly onto aminopropyl Perloza using 10% sulphuric acid in dioxane (Houghten et al, 1986) for cleavage of the Boc group.

DMF-wet aminopropyl Perloza (amine substitution 1.89 mmole/g, 1.3 mmole amine) was transferred to a screw capped reaction vessel (Figure 3.2). Boc-amino acids were activated by dissolving 2 mmole Boc-amino acid, 2 mmole HOBt.H_2O, and 2 mmole DCC in 3 ml DCM plus 1 ml DMF. After stirring for 30 minutes a precipitate of dicyclohexylurea (DCU) formed. The DCU precipitate was removed by filtration, and washed with 20 ml DMF. The filtrates were pooled and added to the resin. The resin and Boc-amino acyl-OBt ester were mixed overnight (Rototorque, 16-18 hours). The solution was filtered from the resin, which was then washed with DMF, and fresh Boc-amino acyl-OBt ester was recoupled, usually with 0.05 g DMAP, for three hours.

The aminopropyl resin was double coupled with 2 mmoles Boc-valine-OBt ester. The residual amine substitution was 0.10 mmole/g as determined by the ninhydrin assay. The expected Boc-valine substitution was 1.30 mmole/g (1.28 mmole/g found by amino acid analysis). The resin was washed 3X dioxane and the Boc group cleaved by treatment with 10% sulphuric acid in dioxane (Houghten et al, 1986). Samples were taken at intervals for picrate titration to determine the progress of Boc cleavage by the sulphuric acid solution. The results of the titrations suggested that cleavage of valine from the Perloza was occurring. After 60 minutes treatment with 10% sulphuric/dioxane the amino acyl Perloza was washed 2X dioxane, 2X 20% triethylamine/dioxane, 3X dioxane, and 3X DMF. The next amino acid, Boc-glycine, was coupled to the valine-Perloza using the double coupling protocol described above. The synthesis was then paused while an investigation was carried out to determine whether 10% sulphuric acid/dioxane would cleave aminopropyl groups from aminopropyl Perloza.

3.2.2.1 Treatment of aminopropyl Perloza with 10% sulphuric acid in dioxane

Aminopropyl Perloza, initial amine substitution 1.89 mmole/g, was solvent exchanged from water to dioxane. A solution of sulphuric acid in dioxane, 10%, was added and the resin mixed. Samples of resin were withdrawn at intervals for picrate titration. The results of this study indicated that cleavage of resin-bound aminopropyl groups by 10% sulphuric acid in dioxane had occurred.

3.2.2.2 Treatment of aminopropyl Perloza with a number of candidate Boc cleavage reagents

Aminopropyl Perloza was treated with a number of Boc cleavage reagents to determine which gave the least cleavage of amine groups from the support. The reagents tested were 1M HCl in dioxane, 1.5M boron trifluoride etherate in dioxane (BF_3/dioxane), 1M boron trifluoride etherate in THF, and 2.5M methanesulphonic acid (MSA) in dioxane. The amine substitution was determined by picrate titration after exposure to the reagent.

The results of this study suggested that boron trifluoride etherate in dioxane would possibly be suitable as a Boc cleavage reagent. Boron trifluoride in benzyl alcohol had already been shown to be suitable for Boc cleavage (Epton et al, 1980). A 1M solution (12.3% v/v) of boron trifluoride etherate in dioxane (BF_3/dioxane) was tested for its efficiency in cleaving the Boc group. Picrate titration of Boc-Gly-Val-Perloza after a 2 hour treatment with the 1M BF_3/dioxane solution confirmed that the Boc group had been cleaved.

The synthesis of Leu-Ala-Gly-Val directly onto aminopropyl Perloza was continued, but with 1M BF_3/dioxane being used to cleave the Boc group. The coupling protocol used for addition of each amino acid was as follows:

i) DeBoc: 1M BF_3/dioxane, 2 hours
ii) Wash 2X dioxane
iii) Wash 2X 20% triethylamine/dioxane
iv) Wash 2X dioxane
v) Wash 3X DMF
vi) Couple 2 mmole Boc-aa-OBt 16-18 hours
vii) Wash 3X DMF
viii) Recouple 2 mmole Boc-aa-OBt 3 hours (plus 0.4 mmole DMAP)
ix) Wash 3X DMF
x) Wash 3X dioxane

At completion of the synthesis a sample of the peptide-resin was taken for amino acid analysis. The result of the synthesis of LAGV on aminopropyl Perloza using Boc chemistry was encouraging enough to warrant further investigation.

3.2.3 Preparation of Perloza with a cleavable glycolamide linkage between the peptide and the resin

3.2.3.1 Coupling α-chloro or α-bromo acetic anhydride to aminopropyl Perloza

The results of initial experiments indicated that the most efficient method of coupling α-haloacetic acid to aminopropyl Perloza was to react two equivalents of the symmetrical anhydride with the resin for 1-2 hours. The reaction did not require a basic catalyst such as DMAP. If DMAP was added, elemental analysis for halogen indicated that the haloacetyl group was also bound to the carbohydrate backbone.

The most efficient method, found in this study, for coupling the anhydride of α-bromoacetic acid to aminopropyl Perloza is given below:

Water-wet aminopropyl Perloza 500 Medium (0.56 mmole/g, 1 mmole amine) was washed 2X dioxane and 3X DMF. The anhydride of α-bromoacetic acid (2 mmole, 2 eq) was made by reacting 4 mmole α-bromoacetic acid with 2.1 mmoles of DIC in 6 ml DCM. After 10 minutes 10 ml DMF was added. The anhydride solution was added to the aminopropyl Perloza. After 1 hour the resin was filtered and washed 3X DMF, 4X 95% ethanol, and 2X

dioxane. Samples of the α-bromoacetamido Perloza were dried for elemental analysis and ninhydrin assay. The amine substitution after one hour reaction was 1.3 μmole/g, indicating 99.8% reaction. Elemental analysis indicated that the α-bromoacetic anhydride had reacted solely with the resin-bound amine groups.

3.2.3.2 Preparation of Boc-amino acid cesium salts

Cesium salts of Boc-amino acids were prepared by dissolving or suspending a weighed amount of the amino acid in 50% aqueous ethanol, and adding a volume of 2.140M cesium bicarbonate calculated to give 100% neutralisation. On adding the cesium bicarbonate any remaining solid dissolved and effervescence was noted. The solution was diluted with water and lyophilised. Often the Boc-amino acid cesium salt was an oil. In some cases the oil was used directly, in others it was treated twice by adding benzene, and then removing the benzene in vacuo to remove any remaining water azeotropically. The resulting white solid was dried in vacuo.

3.2.3.3 Coupling Boc-amino acid cesium salt to α-chloroacetamido Perloza

One eq of dioxane-wet α-chloroacetamido Perloza was washed thoroughly with DMF. The Boc-amino acid cesium salt, 2 eq, was dissolved in DMF, and added to the resin. After reaction for 21-22.5 hours at 55°C, the resin was washed thoroughly with DMF and then with dioxane. Samples were taken for elemental analysis. Another sample was treated with 1M boron trifluoride etherate in dioxane for one hour to cleave the Boc group, washed with dioxane, 20% triethylamine in dioxane, then dioxane, and subjected to picrate titration.

3.2.3.4 Coupling Boc-amino acid cesium salt to α-bromoacetamido Perloza

One equivalent of DMF-wet α-bromoacetamido Perloza was washed with DMF or DMA. The cesium salt of the Boc-amino acid, 2 eq, was dissolved in DMF or DMA and added to the α-bromoacetamido resin. The resin was mixed at room temperature (Rototorque) for up to 48 hours, drained,

washed with DMF, 50% DMF/water, then 3X DMF. Samples of the Boc-amino acyl-glycolamido-Perloza resin were taken for elemental and amino acid analyses. Samples were also taken for picrate titration. The Boc group was cleaved with 1M BF_3/dioxane, 1 hour, before picrate titration (see Section 3.2.3.3).

3.2.4 Treatment of Boc-leucine-glycolamido-Perloza with two potential Boc cleavage reagents

Samples of Boc-leucine-glycolamido-Perloza (0.66 mmole/g by amino acid analysis, calculated for Boc removed) were treated with 1M HCl in dioxane, or 1M BF_3/dioxane. The amine substitution was determined by picrate titration after 60 minutes exposure to the reagent.

The two Boc cleavage reagents were further investigated by treating Boc-leucine-glycolamido-Perloza with each and withdrawing samples at intervals for picrate titration.

3.2.5 Studies of BF_3/dioxane as a cleavage reagent using the glycolamide linker

The concentration and time required for complete Boc removal using BF_3/dioxane were investigated. Boc-valine-glycolamido-Perloza resin samples (Boc-valine substitution 0.67 mmole/g, 0.72 mmole/g after Boc removal, see equation 3.1 below) were treated with 1M and 2M BF_3 in dioxane. Samples of resin were removed after 15 and 60 minute intervals, washed immediately with dioxane, and the amine substitution level was determined by picrate titration. In addition to the batchwise treatment, a resin sample was also exposed to a flow of 1M BF_3/dioxane at 3ml/min for 10 minutes, followed by washing and picrate titration. This experiment served as a comparison of the batch and flow methods of Boc removal using BF_3/dioxane.

Equation 3.1 Calculation of amine substitution of amino acyl-Perloza after Boc cleavage

Boc-valine-CO-O-CH_2-CO-NH-Perloza valine substitution by amino acid analysis: 0.67 mmole/g

↓ i) 1M BF_3/dioxane, ii) 20% NEt_3/dioxane

H_2N-valine-CO-O-CH_2-CO-NH-Perloza

If the initial weight of Boc-valine-glycolamido-Perloza is 1.000g, there will be 0.67 mmole of valine present. If the Boc group is cleaved, the final weight of the valine-glycolamido-Perloza will be:

$$1.000 - (0.67 \times 10^{-3} \times 100 \text{ [MW of Boc + 1 x H]}) \text{ g}$$
$$= 1.000 - 0.067 \text{ g}$$
$$= 0.933 \text{ g}$$

However, there is still 0.67 mmole of valine present. Therefore, the valine substitution as determined by picrate titration of the resin after Boc cleavage will be:

$$\frac{0.67 \text{ mmole}}{0.933 \text{ g}}$$
$$= 0.72 \text{ mmole/g}$$

3.2.6 Reaction of activated Boc-glycine with unmodified Perloza

3.2.6.1 Reaction of Perloza 100 Medium with Boc-Gly-OBt

Perloza 100 Medium (5.0 g water wet, 0.50 g dry) was solvent exchanged via dioxane to DMF and transferred to a Biolynx reaction column. Boc-glycine (1 mmole) was activated for 50 minutes in 4 ml DMF using HOBt.H_2O (1 mmole) and DIC (1 mmole). The Boc-Gly-OBt solution was recirculated through the resin for one hour at 5 ml/min. Samples were taken for amino acid analysis, and recirculation was continued for a total of 17 hours. The resin was washed (all washes at 3 ml/min) with DMF (5 min), dioxane (5 min), 1M BF_3/dioxane (10 min), dioxane (5 min),

5% NMM/DMF (5 min), DMF (5 min). The amine substitution of the resin, after Boc removal, was determined using the ninhydrin assay.

3.2.6.2 Reaction of Perloza 100 Medium with Boc-Gly anhydride

Perloza 100 Medium (4.0 g water wet, 0.40 g dry) was solvent exchanged via dioxane to DMF, and transferred to a Biolynx reaction column. Boc-glycine symmetrical anhydride, 0.5 mmole, was preformed in DMF by reaction, for 45 minutes, of 1.0 mmole Boc-amino acid with 0.5 mmole of DIC. The Boc-glycine anhydride solution, plus 100 µl NMM, was recirculated through the Perloza for one hour at 3 ml/min. The resin was then washed with DMF and the Boc group was cleaved using the cycle given in section 3.2.6.1. The amine substitution of the resin was determined using the ninhydrin assay.

3.2.7 Semimanual peptide synthesis: Boc chemistry

3.2.7.1 Boc-amino acid activation

HOBt active esters preformed in DMF using DIC were used to couple all Boc-amino acids. A three times excess of activated Boc-amino acid over resin-bound amino acid was used. Boc-amino acid (1 mmole) was dissolved in 4 ml 0.5M HOBt/DMF. DIC (1 mmole) was added and the solution was stirred for forty minutes prior to coupling.

3.2.7.2 Peptide synthesis cycle using the LKB Biolynx 4175 continuous flow peptide synthesiser

The following single coupling cycle (0.33 mmole scale) was used for the Boc methodology with the LKB Biolynx 4175 peptide synthesiser:

Biolynx continuous flow peptide synthesis cycle: Boc methodology

Function	Time	Flow rate
Wash dioxane	5 min	3 ml/min
DeBoc, 1M BF_3/dioxane	15 min	3 ml/min
Wash dioxane	5 min	3 ml/min
Wash DMF	5 min	3 ml/min
Load activated Boc-amino acid*		2 ml/min
Recycle		3 ml/min
Clean loader		
Recycle stop	1 hour	
Wash DMF	5 min	3 ml/min

* 100 µl NMM was also loaded to neutralise BF_3-amine complex remaining after Boc cleavage.

3.2.8 Leu-Ala-Gly-Val

Boc-valine-glycolamido-Perloza resin (0.31 mmole valine, Boc-valine substitution 0.67 mmole/g) was transferred to a Biolynx reaction column. The peptide was assembled using one hour coupling times for the Boc-amino acids. After addition of Boc-leucine the Boc group was cleaved, the resin was washed with dioxane, 20% NMM/dioxane, dioxane, and water. The peptide was cleaved from the support using 2 eq of NaOH in 12 ml water for three hours. After cleavage the resin was filtered off and washed with 20 ml 5% acetic acid. The filtrates were pooled and lyophilised. The crude peptide was purified by HPLC. A ninhydrin assay of the cleaved resin indicated a peptide cleavage yield of 96%.

3.2.9 Leucine-enkephalin

Tyr-Gly-Gly-Phe-Leu

Leucine-enkephalin was synthesised using the same general procedure given for Leu-Ala-Gly-Val. The peptide was cleaved from the support using 3 eq of NaOH in 11 ml water for three hours. Ninhydrin analysis of the cleaved resin indicated a peptide cleavage yield of 95%. The

tyrosine of the crude Leu-enkephalin was protected by the benzyl ether. The crude side chain protected Leu-enkephalin was purified by HPLC. The benzyl ether protecting group of the peptide tyrosine hydroxyl was cleaved by catalytic hydrogenation (Anwer and Spatola, 1980), and the fully deprotected peptide was purified by HPLC.

3.2.10 Attempts to synthesise ACP 65-74

Val-Gln-Ala-Ala-Ile-Asp-Tyr-Ile-Asn-Gly

Several attempts were made to synthesise the acyl carrier protein 65-74 sequence (Hancock et al, 1973) using the glycolamide linker, however none of these syntheses were successful.

3.3 RESULTS AND DISCUSSION

3.3.1 Treatment of aminopropyl Perloza with Boc cleavage reagents

Sulphuric acid in dioxane was initially used as a Boc cleavage reagent for the synthesis of Leu-Ala-Gly-Val directly onto aminopropyl Perloza. Boc-valine-Perloza was treated with 10% sulphuric acid in dioxane, and samples were taken at intervals for picrate titration to determine the progress of the cleavage. The picrate titration results, given in Table 3.1, indicated a time dependent decrease of the amine substitution of the amino acyl-Perloza. The results therefore suggested that use of 10% sulphuric acid in dioxane for Boc cleavage from Boc-valyl-Perloza resulted in loss of valine from the support.

Table 3.1 Cleavage of Boc from Boc-valine-Perloza with 10% sulphuric acid/dioxane

Time	15 min	30 min	60 min
Amine substitution	1.16 mmole/g	1.11 mmole/g	0.96 mmole/g

The expected amine substitution after quantitative Boc removal was 1.47 mmole/g (initial Boc-valine substitution: 1.28 mmole/g by amino acid analysis, see Equation 3.1).

Aminopropyl Perloza was treated with 10% sulphuric acid in dioxane to investigate the chemical stability of this proposed SPPS support to this reagent. Samples of the resin were taken at intervals for picrate titration to determine the amine substitution. The results of this study are given in Table 3.2.

Table 3.2 Treatment of aminopropyl Perloza with 10% sulphuric acid in dioxane

Time of exposure / hr	0	3	17.5	23	73
% decrease in amine substitution	0	61	75	78	85

The results given in Table 3.2 showed that treatment of aminopropyl Perloza with 10% sulphuric acid/dioxane resulted in significant loss of

amine groups from the support. The lowering of the substitution level with time can be explained if aminopropyl Perloza consists of ordered crystalline regions linked by poorly ordered amorphous regions (Chemopetrol, 1988; Stamberg, 1988; Gemeiner et al, 1989). The amorphous regions would be expected to contain most of the aminopropyl groups because they would have been more accessible to the reagents used in their generation. The amorphous regions of cellulose, which are most susceptible to acid hydrolysis (Bertoniere and Zeronian, 1987), would be preferentially cleaved by the 10% sulphuric acid/dioxane solution. As these regions would also be expected to contain the majority of the aminopropyl groups, the amine substitution would be expected to decrease as the amorphous regions were hydrolysed. Although 10% sulphuric acid in dioxane gave significant cleavage of amine groups from aminopropyl Perloza, it did not appear to degrade the flow properties of the matrix.

A search for an alternative Boc cleavage reagent was undertaken. The criterion used to determine which reagent was chosen for further studies was that it would give the least cleavage of amine groups from aminopropyl Perloza. A number of reagents were tested. The results of this investigation are given in Table 3.3.

Table 3.3. Treatment of aminopropyl Perloza with candidate Boc cleavage reagents

Reagent	Time of exposure	% decrease in amine substitution
1M HCl/dioxane	24 hr	19%
1.5M BF_3/dioxane	69 hr	7%
1M BF_3/THF	16 hr	Gel formed
2.5M MSA/dioxane	21 hr	18%

The results of this experiment showed that, of the reagents tested, 1.5M BF_3/dioxane gave the lowest decrease in amine substitution over time. Boron trifluoride in benzyl alcohol had already been shown to be suitable for Boc cleavage (Epton et al, 1980). A 1M BF_3/dioxane solution was tested and found to be suitable for cleavage of the Boc group. Picrate titration (results not shown) indicated that after a two

hour treatment of Boc-Gly-Val-Perloza with 1M BF_3/dioxane, quantitative cleavage of the Boc group had been achieved. The reagent was used for subsequent Boc cleavage in the synthesis of LAGV directly onto aminopropyl Perloza.

3.3.2 Synthesis of LAGV directly onto aminopropyl Perloza

BF_3/dioxane (1M) was used as the Boc cleavage reagent to complete the synthesis of LAGV directly onto aminopropyl Perloza (see Section 3.3.1). At completion of the synthesis amino acid analysis of the peptide-resin gave the expected amino acid ratios: Leu 1.05 [1], Ala 1.01 [1], Gly 1.09 [1], Val 1.00 [1]. The amino acid analysis results suggested that it was possible to use Perloza as a support for solid phase peptide synthesis using the Boc methodology. The encouraging result of this study prompted investigation of SPPS on Perloza using a cleavable glycolamide linker to anchor the peptide to the support.

3.3.3 Coupling of Boc-amino acid cesium salts to α-haloacetamido Perloza

The glycolamide linker was introduced by Baleux et al (1984) for use with a polyacrylic support. In their study 3 eq of α-bromoacetic anhydride were reacted with support-bound amine groups to give an α-bromoacetamido resin. Displacement of the bromine by Boc-amino acid cesium salts in DMF (5 eq, 48 hours) afforded Boc-amino acyl-glycolamido resin in quantitative yield. The amino acyl-glycolamide linkage was stable to cleavage by anhydrous acids such as liquid HF, trifluoromethanesulphonic acid, and hot TFA, but was cleaved quantitatively by basic reagents such as dilute NaOH, ammonia, and alkoxides (Baleux et al, 1986). The lithium salt of 2-mercaptoethanol in THF was also shown to cleave the peptide-glycolamide linkage (Shekhani et al, 1990).

Both α-chloroacetic and α-bromoacetic anhydride were coupled in high yield to aminopropyl Perloza. Only 2 eq of the anhydride were required to achieve > 98% coupling. DMAP was not required as catalyst. Addition of DMAP resulted in coupling of the anhydride to the resin hydroxyls as determined by elemental analysis. It was also found that α-chloroacetic anhydride appeared to be more reactive than α-bromoacetic

anhydride, as it would sometimes couple to the resin hydroxyls even in the absence of DMAP (up to 10% of the total chlorine present).

The results of the coupling reactions of Boc-amino acid cesium salts with α-chloroacetamido and α-bromoacetamido Perloza are given in Tables 3.4 and 3.5 respectively.

Table 3.4 Coupling of Boc-amino acid cesium salts to α-chloroacetamido Perloza

Amino acid	Time at 55°C (hours)	Initial Cl (mmole/g)	Final Cl (mmole/g)	Amino acid substitution* (mmole/g)
Val	22.5	1.01	0.12	0.63
Leu	21	1.32	0.42	0.72

* Picrate titration result

Table 3.5 Coupling of Boc-amino acid cesium salts to α-bromoacetamido Perloza

Amino acid (Solvent)	Rxn time (hours)	Residual Br (mmole/g)	Amine substitution* (mmole/g)	Amino acid substitution# (mmole/g)	Yield**
Val (DMA)	26	0.06	0.75	0.72	87%
Leu (DMA)	24	0.05	0.68	0.66	80%
Gly (DMF)	42	Nil	0.72	n.d.	81%
Gly (DMF)	48	Nil	0.28	n.d.	80%

*Picrate titration result

#Amino acid analysis result

**Yield based on quantitative substitution of halide by Boc-amino acid, see Equation 3.2 below

Equation 3.2 Reaction of Boc-Gly cesium salt with α-bromoacetamido Perloza

$$Br\text{-}CH_2\text{-}CO\text{-}NH\text{-}Perloza + Boc\text{-}NH\text{-}CH_2\text{-}COO^- Cs^+ \downarrow$$

$$Boc\text{-}NH\text{-}CH_2\text{-}CO\text{-}O\text{-}CH_2\text{-}CO\text{-}NH\text{-}Perloza + CsBr$$

For 1.000 g α-bromoacetamido Perloza, initial bromine substitution 1.00 mmole/g, the expected substitution of glycine (assuming quantitative reaction) is:

$$\frac{1.00 \text{ mmole}}{1.000 + (1 \times 10^{-3} \times [176 - 79.9])\text{ g}} = 0.91 \text{ mmole/g}$$

If, for example, the final Boc-Gly substitution found by amino acid analysis was 0.80 mmole/g, then the yield would be calculated as:

$$\frac{0.80}{0.91} \times 100\% = 88\%$$

Most of the Boc-amino acid cesium salt coupling experiments were carried out using α-bromoacetamido Perloza. It was found (Table 3.4) that coupling Boc-amino acid cesium salts to α-chloroacetamido Perloza required elevated temperature (55°C), and the reaction had not gone to completion after 22.5 hours. Elemental analysis revealed that significant amounts of chlorine remained on the resin. It was considered desirable that a support to be used for SPPS not contain residual reactive halides.

It was thought that bromine would be more readily displaced by Boc-amino acid cesium salts than chlorine, and this proved to be the case, as

comparison of the results given in Tables 3.4 and 3.5 shows. The reaction of Boc-amino acid cesium salts with α-bromoacetamido Perloza proceeded in high yield at room temperature after 24 hours, compared to the elevated temperature required for reaction with α-chloroacetamido Perloza. Both DMA and DMF were used as solvents for the reaction of Boc-amino acids with α-bromoacetamido Perloza. Although some bromine remained after 24 hours, as determined by elemental analysis, extension of the reaction time to 48 hours resulted in loss of all bromine. In the last two results given in Table 3.5 loss of bromine with less than quantitative coupling of amino acid was noted. This indicated that hydrolysis of bromine had occurred, probably due to water present in the Boc-amino acid cesium salts. Although Baleux et al (1984) obtained quantitative yields in coupling Boc-amino acids to α-bromoactamido resin, other workers have encountered difficulties using the cesium salt method with bromine substituted supports. For example, Nicolas et al (1991) encountered problems in coupling Fmoc-amino acid cesium salts to α-(4-bromomethyl-3-nitrobenzamido) benzyl polystyrene resin. They could not obtain reproducible results, and yields were only 65-85%. They postulated that water from the hygroscopic Fmoc-amino acid cesium salts, and impurities in the DMF, such as formic acid and dimethylamine, could also couple with the reactive bromomethyl group. In this study it was possible that, even after lyophilisation, water was still present in the oily Boc-amino acid cesium salts, which resulted in hydrolysis of bromine.

Although the coupling yields using the α-bromoacetamido Perloza were not quantitative, useful amino acid substitution levels were obtained. In addition, little or no bromine remained on the resin, which was desirable. It should be noted that in Table 3.5 the results of the picrate titrations and amino acid analyses (where carried out) agreed quite closely, showing that the usually acid-stable amino acyl-glycolamido-Perloza linkage was cleaved under the acid hydrolysis conditions used to prepare the resin samples for amino acid analysis.

3.3.4 Use of BF_3/dioxane as Boc cleavage reagent with the glycolamide linker

The next step in this study was to determine whether BF_3/dioxane was suitable for use as a Boc cleavage reagent with the glycolamide linker.

A second cleavage reagent, 1M HCl/dioxane, was also examined in case the boron trifluoride solution was unsuitable. Boc-leucine-glycolamido Perloza (Leu substitution 0.66 mmole/g by amino acid analysis, calculated for Boc removed) was treated with a 1M solution of each cleavage reagent for one hour. Comparison of the picrate titration results given in Table 3.6 indicated that the 1M solution of boron trifluoride etherate in dioxane had fully cleaved the Boc group after one hour.

Table 3.6 Treatment of Boc-leucine-glycolamido-Perloza for one hour with 1M BF_3/dioxane or 1M HCl/dioxane

Reagent	Leucine substitution (by picrate titration)
1M HCl/dioxane	0.61 mmole/g
1M BF_3/dioxane	0.66 mmole/g

Initial leucine substitution by amino acid analysis: 0.66 mmole/g

The performance of the two Boc cleavage reagents was further compared by treating Boc-leucine-glycolamido Perloza with each and withdrawing samples at intervals for picrate titration. The results, given in Table 3.7, indicated that treatment with 1M HCl/dioxane started to result in loss of amino acid after one hour. There were probably two competing reactions taking place. Up to between 30 minutes and one hour, Boc cleavage predominated, but after one hour cleavage of the amino acid from the support was occurring. The results also showed that 1M HCl/dioxane was going to take between 30 minutes and one hour to give near quantitative Boc cleavage. On the other hand, 1M BF_3/dioxane gave quantitative Boc cleavage after 15 minutes. It did appear that treatment with 1M BF_3/dioxane resulted in loss of a small amount of amino acid after 24 hours, however this result was only just outside the estimated experimental error of the picrate titration (± 0.01 mmole/g). These results indicated that 1M BF_3/dioxane was suitable for Boc cleavage with the glycolamide linkage and Perloza resin.

Table 3.7 Treatment of Boc-leucine-glycolamido-Perloza with Boc cleavage reagents

	Time	Amine substitution*
1M HCl/dioxane	5 min	0.31 mmole/g
	15 min	0.51 mmole/g
	30 min	0.61 mmole/g
	60 min	0.61 mmole/g
	2 hr	0.45 mmole/g
	5 hr	0.49 mmole/g
1M BF_3/dioxane	15 min	0.68 mmole/g
	30 min	0.68 mmole/g
	60 min	0.66 mmole/g
	2 hr	0.67 mmole/g
	24 hr	0.65 mmole/g

Initial leucine substitution by amino acid analysis: 0.66 mmole/g

A final study was undertaken to compare the effect of different concentrations of boron trifluoride on the Boc cleavage rate. Different systems for effecting Boc cleavage, batchwise vs flow, were also investigated. The results, given in Table 3.8, showed that after 15 minutes 2M BF_3/dioxane was slightly more effective than 1M BF_3/dioxane, but after 60 minutes there was no difference between the results of the two concentrations of the reagent.

Comparison of the batchwise results with the flow system result suggested that the 10 minute flow treatment with 1M BF_3/dioxane was as efficient as a one hour batch treatment with either 1M or 2M BF_3/dioxane. The results given in Table 3.8 indicated that 1M BF_3/dioxane was suitable for Boc removal in a batch system using 60 minutes exposure, or in a continuous flow system at 3 ml/min for 10 minutes.

Table 3.8 Results of treatment of Boc-valine-glycolamido-Perloza with BF_3/dioxane

BF_3 concentration and deBoc method	Reaction time (minutes)	Amine substitution* (mmole/g)
1 M (Batch)	15	0.67
	60	0.71
2 M (Batch)	15	0.71
	60	0.72
1 M (Flow, 3 ml/min)	10	0.74

*Picrate titration result

Resin valine substitution by amino acid analysis: 0.72 mmole/g

3.3.5 Picrate titration of Boc-amino acyl-glycolamido Perloza

BF_3/dioxane (1M, 1 hour), was used to cleave the Boc group from all Boc-amino acyl resins prior to picrate titration. Amino acid analysis of resins after picrate titration indicated that the 10% triethylamine solution used to elute the picrate in the titrations also cleaved the amino acid from the base-labile glycolamide linker. The difference between the found and true result depended on the amino acid substitution level, and the formula weight of the amino acid involved. For example, valine-glycolamido-Perloza, amino acid substitution 0.72 mmole/g by amino acid analysis, would have an uncorrected amino acid substitution by picrate titration of 0.78 mmole/g (see Equation 3.3). Because the difference fell outside the estimated error of the picrate titration (± 0.01 mmole/g), a simple calculation was applied to the results of all picrate titrations of amino acyl-glycolamido Perloza resins to determine the true amino acid substitution.

Equation 3.3 Calculation of expected result of picrate titration of amino acyl-glycolamido Perloza from an amino acid analysis result

$$Picrate^{-}H_3N^{+}\text{-valine-CO-O-}CH_2\text{-CO-NH-Perloza}$$

$$\downarrow \quad 10\%\ NEt_3 /\ 50\%\ \text{aqueous ethanol}$$

$$Picrate^{-}NEt_3^{+} + \text{valine} + HO\text{-}CH_2\text{-CO-NH-Perloza}$$

For 1.000 g of valyl-glycolamido- Perloza resin, if the initial valine substitution was 0.72 mmole/g by amino acid analysis, the apparent amine substitution by picrate titration would be:

$$\frac{\text{initial amount of valine (mmole)}}{\text{final weight of resin after treatment with } NEt_3 \text{ solution (g)}}$$

$$= \frac{0.72 \text{ mmole}}{1.000 - (0.72 \times 10^{-3} \times [117 - 17])\ \text{g}}$$

$$= \frac{0.72}{0.928}$$

$$= 0.78 \text{ mmole/g}$$

This calculation was reversed to get the true amino acid substitution result from a picrate titration result.

3.3.6 Coupling of activated Boc-amino acids to Perloza

When using carbohydrate matrices as supports for SPPS the possibility always exists that activated Nα-protected amino acids will couple directly to the support hydroxyls and serve as initiation points for synthesis of deletion peptides. If deletion peptides were formed on the carbohydrate hydroxyls, they would be cleaved using the same reagents used for peptide cleavage from the glycolamide linker. That is, support-bound deletion peptides would also be cleaved by alkaline hydrolysis. The presence of significant amounts of deletion peptides,

similar in sequence to the target peptide, would complicate peptide purification. The longer deletion peptides would be similar in structure to the target peptide, and may therefore be difficult to separate from the target peptide.

Before proceeding to the synthesis of a peptide on functionalised Perloza, a study was carried out to evaluate the extent of reaction of activated Boc-glycine with unmodified Perloza. Perloza 100 was reacted with Boc-glycine symmetrical anhydride and Boc-glycine-OBt active ester to determine the amount of amino acid that would couple to the matrix during a typical SPPS coupling cycle. Boc-glycine was used because it is the least sterically hindered of the Boc-amino acids, and may therefore be expected to couple to the matrix in the highest yield of all the Boc-amino acids. After coupling for one hour, the Boc-amino acyl-Perloza was treated with BF_3/dioxane to cleave the Boc group. The amino-acyl-Perloza was analysed to determine the amino acid substitution. Reaction of Perloza with Boc-glycine-OBt active ester for one hour gave an amino acid substitution of 14 µmole/g by amino acid analysis. After 17 hours reaction time the amino acid substitution was 50 µmole/g by the ninhydrin assay. Reaction of Perloza for one hour with Boc-glycine anhydride gave an amino acid substitution of 9 µmole/g by the ninhydrin assay.

The carbohydrate-bound amino acid substitution levels found in this study were relatively low. Also, it would be expected that many of the reactive hydroxyls of functionalised Perloza would have been substituted during the initial reaction with with acrylonitrile. Therefore, it was proposed that peptide quality would not be significantly compromised by formation of peptides coupled directly to the carbohydrate backbone when using Perloza as a support for SPPS.

3.3.7 Peptides synthesised on Perloza using Boc chemistry

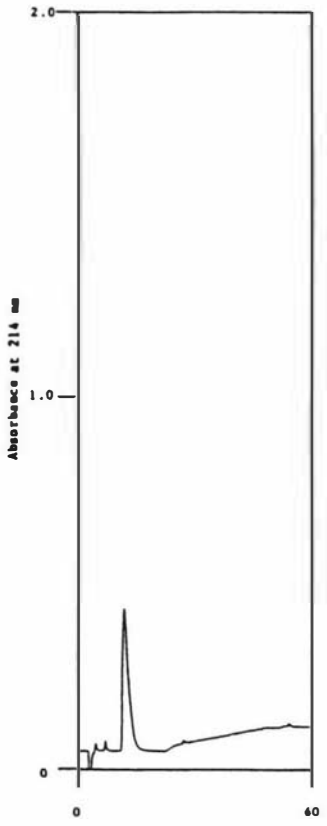
Peptides assembled on Boc-amino acid-glycolamido-Perloza resin were Leu-Ala-Gly-Val, and Leu-enkephalin. The amino acids were coupled in a three fold excess as the preformed HOBt active esters. Coupling times were for 1 hour using a semi-manual flow apparatus. The peptides were cleaved with NaOH (2-3 equivalents) for 3 hours. The excess of NaOH was

kept low to minimise the possibility of peptide amide bond hydrolysis. Ninhydrin assays of the cleaved resins indicated approximately 95% of the support-bound peptides had been cleaved. The crude peptides were purified by HPLC.

3.3.7.1 Leu-Ala-Gly-Val

The purified Merrifield test peptide, Leu-Ala-Gly-Val (Merrifield, 1963) was obtained in 66% yield based on the initial amino acid substitution. The purified peptide gave a single peak on analysis by HPLC (Figure 3.3), and gave the expected ratios on amino acid analysis: Gly 1.00 [1], Ala 1.02 [1], Val 1.02 [1], Leu 1.00 [1].

Figure 3.3 HPLC trace of purified Leu-Ala-Gly-Val

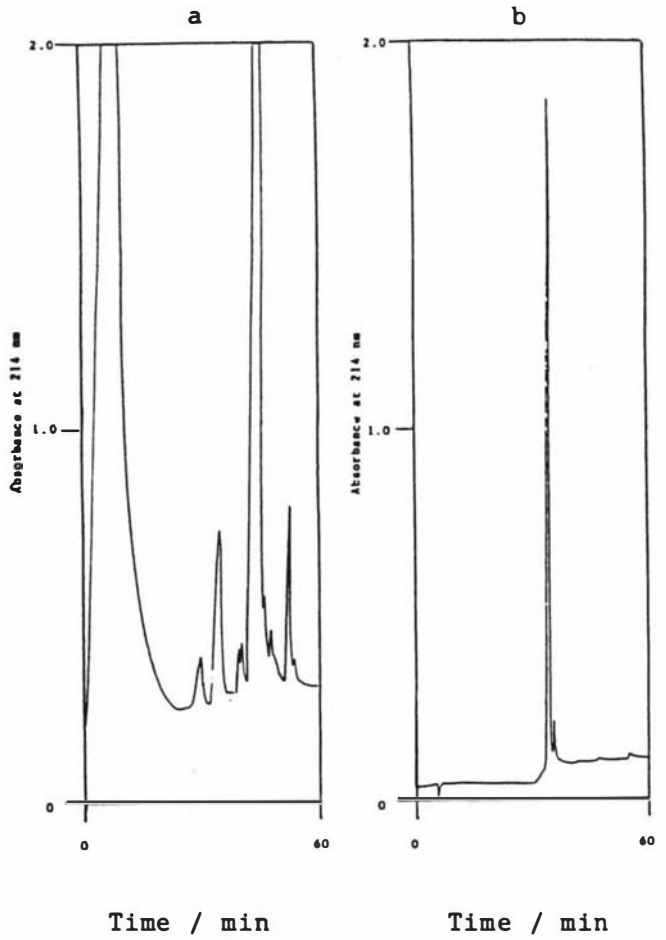


3.3.7.2 Leucine-enkephalin

Tyr-Gly-Gly-Phe-Leu

Side chain protected Leu-enkephalin was cleaved from the glycolamide linker. The tyrosine benzyl ether protecting group was cleaved by catalytic hydrogenation. The Leu-enkephalin was purified by HPLC to give a 49% yield of peptide based on the initial amino acid substitution. The synthetic Leu-enkephalin gave a single peak on HPLC (Figure 3.4), coeluted on HPLC with authentic Leu-enkephalin, and gave the expected ratios on amino acid analysis: Gly 2.00 [2], Leu 0.97 [1], Tyr 0.99 [1], Phe 1.00 [1].

Figure 3.4 HPLC traces of a) crude, b) purified, Leu-enkephalin



The cleavage of side chain protected Leu-enkephalin provided preliminary evidence that the glycolamide linker/Perloza combination could be used to provide protected peptides, for example for fragment syntheses. Neither of the peptides synthesised in this study contained Asp or Glu with benzyl ester carboxyl side chain protection. Asp side chain benzyl esters would be subject to aspartimide formation under the basic conditions employed for peptide cleavage in this study (Tam et al, 1988). However, Shekhani et al (1990) successfully used lithium β-mercaptoethanol (LiβME) in THF to cleave Boc-Arg(Mtr)-Lys(Z)-Asp(OBzl)-Val-Tyr(Bzl) attached to a polystyrene support via a glycolamide linkage. The side chain protecting groups were cleaved by catalytic hydrogenation, and the purified peptide was characterised by direct comparison with an authentic sample. Shekhani et al did not report any side reactions involving the Asp(OBzl) when using LiβME for peptide cleavage. Therefore, LiβME may be a useful reagent for cleavage of protected peptide fragments from peptide-glycolamido-Perloza. Experiments involving the use of LiβME/THF for cleavage of peptides from peptide-glycolamido-Perloza are described in Chapter 4.

Several attempts to synthesise the decapeptide ACP 65-74 using Boc chemistry and the glycolamide linker were unsuccessful. This peptide has been reported to be difficult to assemble using Boc chemistry on polystyrene resin (Hancock et al, 1973), and from the limited results of this study to date it also appeared to be difficult to synthesise on Perloza using Boc chemistry and the glycolamide linker. An approach different to that used in this study may be needed to successfully synthesise ACP 65-74 on Perloza using the Boc methodology. For example, one possible strategy would be to use the PAM linker to anchor the peptide to the Perloza, and to cleave the peptide using a strong acid such as liquid HF.

3.4 CONCLUSIONS

1) The tetrapeptide LAGV was synthesised directly onto aminopropyl Perloza, which demonstrated the potential of Perloza as a support for SPPS.

2) Protic Boc cleavage reagents significantly degraded aminopropyl functionalised Perloza. However, a dioxane solution of the aprotic Lewis acid boron trifluoride gave minimal degradation of aminopropyl Perloza. BF_3/dioxane was found to be suitable for Boc cleavage during the synthesis of LAGV directly onto aminopropyl Perloza.

3) Boc-amino acids could be coupled to α-bromoacetamido Perloza in high yield to give a glycolamide linkage between the Boc-amino acid and the support.

4) BF_3/dioxane was suitable for Boc cleavage using the glycolamide linker.

5) Two peptides, LAGV and Leu-enkephalin, were successfully synthesised on glycolamido-Perloza, cleaved, and purified. Perloza was found to be suitable as a support for synthesis of these small peptides using Boc methodology. These peptides did not contain Asp or Glu with the side chain carboxyl groups protected as their benzyl esters, which are subject to side reactions under basic conditions. At this stage peptide synthesis on glycolamido-Perloza is probably limited to short peptides which do not contain benzyl esters, unless LiβME is found to be usable for cleavage of peptides containing Asp or Glu with carboxyl side chains protected as their benzyl esters.

CHAPTER 4

SOLID PHASE PEPTIDE SYNTHESIS ON PERLOZA: FMOC CHEMISTRY WITH THE GLYCOLAMIDE LINKER

CHAPTER 4 SOLID PHASE PEPTIDE SYNTHESIS ON PERLOZA: FMOC CHEMISTRY WITH THE GLYCOLAMIDE LINKER

4.1 INTRODUCTION

The results of the investigations reported in the previous chapter showed that Perloza could be used as a support for solid phase peptide synthesis using Boc chemistry. In order to extend that study, an attempt was made to synthesise Leu-enkephalin using Fmoc chemistry with the glycolamide linker (Baleux et al, 1984). Fmoc SPPS chemistry is discussed in the introduction to Chapter 5.

Some doubt existed as to the stability of the base-labile glycolamide linker to the piperidine solutions used for Fmoc cleavage. However, Baleux et al (1986) successfully used the glycolamide linker in a synthesis of TP5, the 32-36 sequence of thymopoietin (RKDVY), using Fmoc chemistry and a polyacrylic resin support. The N-terminal arginine was coupled with the Nα-amine protected by the Boc group, and the guanidino side chain protected by protonation. Baleux et al (1986) found that it was possible to take advantage of the acid stability of the glycolamide linker in order to acidolytically cleave side chain protecting groups before aqueous alkaline peptide cleavage. The ability to cleave side chain protection while leaving the peptide bound to the resin led Baleux et al (1986) to propose that

> *...the use of a FMOC/tBu strategy with a glycolamide ester anchorage and a water compatible polyacrylic carrier affords a good method for obtaining specifically immobilised peptides. The highly loaded peptide resin adduct may be used to interact with biological macromolecules (enzymology with an immobilised substrate, purification by affinity chromatography, immunochemistry, etc.) in aqueous media up to pH 9.*

However, Baleux et al do not appear to have reported any studies demonstrating the successful application of this idea. In theory such an approach could also be applied to peptides synthesised using the glycolamide linker with Perloza. In addition, peptide-ligands could be synthesised directly onto aminopropyl Perloza for use as matrices for

affinity purification. However, it would be necessary to be able to cleave amino acid side chain protecting groups without degrading the Perloza.

The study reported in this Chapter addressed the following questions:

i) could a peptide be made on Perloza using the glycolamide linker and Fmoc chemistry?
ii) could amino acid side chain protecting groups be cleaved from peptide-glycolamido-Perloza without degrading the matrix?

4.2 MATERIALS AND METHODS

4.2.1 Chemicals and Equipment

Boc-leucine-glycolamido-Perloza was synthesised as described in Chapter 3. Nα-Fmoc-L-amino acids (Phe, Gly, and Tyr(tBu)) were purchased from Bachem, Torrance, California. Reagent grade piperidine was purchased from BDH (NZ), and was distilled from calcium hydride before use. Purum grade 2-mercaptoethanol (βME) was from Fluka AG, and n-butyl lithium in THF was obtained from Aldrich. All other reagents were from the sources given in previous chapters.

Peptide synthesis was performed using an ABI 430A automated batchwise peptide synthesiser. Slightly modified ABI Fmoc coupling cycles were employed. Fmoc-amino acids were activated using the standard ABI DIC/HOBt activation protocols (Applied Biosystems Inc., 1988).

4.2.2 Leucine-enkephalin synthesised using acid resistant/base labile glycolamide linker with Fmoc chemistry

Boc-leucine-glycolamido-Perloza (leucine substitution 0.72 mmole/g) was treated with 1.35M BF_3/dioxane for two hours to cleave the Boc group. The resin was washed with dioxane, 20% triethylamine in dioxane, then 3X dioxane. The peptide was synthesised using an ABI 430A peptide synthesiser. The scale of the synthesis was 0.32 mmole. A single coupling of a 3X excess of Fmoc-amino acid-OBt ester was employed for amino acid addition.

At the end of the synthesis, the Fmoc protecting group was cleaved using 20% piperidine/DMF for 10 minutes and then 30 minutes, followed by washing 5X DMF. The ratio of dry to wet weight of the DMF-wet peptide-resin was determined using the procedure given in Chapter 2. The final peptide substitution calculated from the starting amino acid substitution was 0.53 mmole/g.

4.2.3 Cleavage of Leu-enkephalin from glycolamido-Perloza

4.2.3.1 Treatment of peptide-resin with 95% TFA followed by LiβME peptide cleavage

A sample of the DMF-wet peptide-resin (0.132g dry, peptide substitution 0.53 mmole/g) was solvent exchanged to water and washed thoroughly. The weight of water-wet peptide-resin was 0.580 g, giving a weight of water of 0.448 g. TFA was added to bring its concentration up to 95%, and the peptide-resin was stirred in this solution for one and a half hours. During the TFA treatment the peptide-resin became highly swollen, and aggregated into lumps. The lumps of aggregated peptide-resin were broken up and washed with dioxane, 20% triethylamine in dioxane, dioxane, and then 3X THF. Even after this washing sequence, the peptide-resin did not filter as quickly as it did before it was treated with TFA. The THF-moist peptide-resin was treated with 18 eq of the lithium salt of 2-mercaptoethanol (LiβME) (Shekhani et al, 1990) in 5 ml THF for three hours. A ninhydrin assay of the dried cleaved resin showed a cleavage yield of approximately 32%. The crude peptide was analysed, and then purified, by HPLC.

4.2.3.2 Treatment of the peptide-resin with 22.5% TFA / 2.5% water in dioxane followed by LiβME peptide cleavage

Dioxane-wet peptide-resin (0.159 g dry) was treated with a solution of 22.5% TFA / 2.5% water in dioxane for one hour. The resin was filtered, washed 2X dioxane, 2X 20% triethylamine/dioxane, 3X dioxane, then 3X THF. It was noted that in this experiment the resin flow properties did not appear to be impaired after treatment with the TFA / dioxane solution. The peptide-resin was treated with 25 eq of LiβME in 6 ml THF for 3 hours. The THF was removed in vacuo, and the peptide was precipi-

tated by addition of 20 ml of ether. The precipitate was washed 2X 10 ml ether, then the peptide was dissolved in 5% acetic acid and separated from the resin by filtration. The solution of crude peptide was lyophilised. A ninhydrin assay of the dried cleaved resin indicated a cleavage yield of approximately 83%. The lyophilised crude peptide was analysed, and then purified, by HPLC.

4.2.3.3 Cleavage of side-chain protected Leu-enkephalin

An attempt was made to cleave Leu-enkephalin from the resin whilst leaving the tyrosine hydroxyl protected as its t-butyl ether. A sample of DMF-wet peptide-resin (0.157 g dry) was transferred to a sintered glass funnel, and washed with THF, then with ether. Excess ether was removed by vacuum using a water aspirator. The peptide-resin was transferred to a round-bottomed flask and treated with 38 eq of LiβME in 7 ml THF. After three hours stirring the THF was removed in vacuo. The peptide was precipitated with 20 ml ether. The peptide and cleaved resin were collected by filtration and washed 2X 10 ml ether. The peptide was dissolved in 50% acetic acid and filtered from the resin. The resin was washed with 5% acetic acid and the combined acetic acid solutions were diluted to a final acetic acid concentration of 10% and lyophilised. A ninhydrin assay of the dried cleaved resin showed a cleavage yield of approximately 81%. The crude peptide was analysed by HPLC.

The lyophilised peptide, with the tyrosine hydroxyl group protected as its t-butyl ether, was treated with 95% TFA / 5% water for one hour to cleave the t-butyl ether. The TFA was removed in vacuo, and the peptide was purified by HPLC.

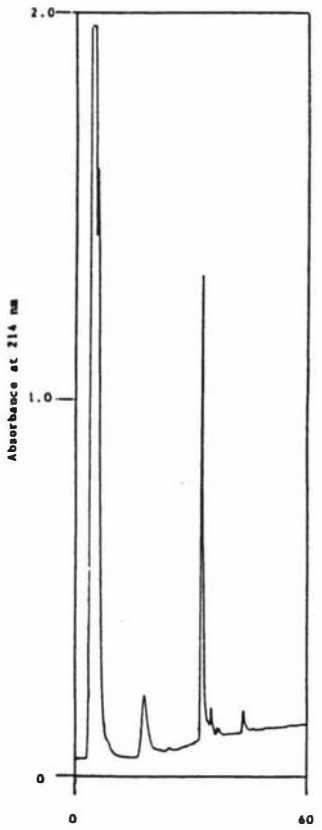
4.3 RESULTS AND DISCUSSION

On completion of the synthesis, the peptide-resin was subjected to picrate titration to determine the peptide substitution level. The peptide substitution was 0.45 mmole/g, which may be compared to the result expected from the initial amino acid substitution of 0.53 mmole/g. The difference may be explained as either an error in the picrate titration, or that some cleavage of the glycolamide linker by piperidine took place during the synthesis. Cleavage of the glycolamide linker by piperidine was not investigated.

4.3.1 Treatment of peptide-resin with 95% TFA followed by LiβME peptide cleavage

The peptide-resin was treated with 95% TFA for one and a half hours to cleave the t-butyl ether of the tyrosine. The peptide was cleaved from the resin using LiβME (Shekhani et al, 1990). The crude peptide was analysed by HPLC (Figure 4.1).

Figure 4.1 HPLC trace of Leu-enkephalin after treatment of peptide-resin with 95% TFA, followed by LiβME peptide cleavage



HPLC analysis of the crude peptide (see Figure 4.1) showed a major peak at 33.2 minutes (Leu-enkephalin) with only a minor peak at 43.9 minutes (Leu-enkephalin with the tyrosine hydroxyl protected as its tBu ether). A ninhydrin assay of the cleaved resin indicated a peptide cleavage yield of approximately 32%. The crude Leu-enkephalin was purified by HPLC. Leu-enkephalin was obtained in 21% yield, based on the initial amino acid substitution. The purified peptide coeluted on HPLC with authentic Leu-enkephalin and had the correct amino acid analysis: Gly 2.00 [2], Leu 0.98 [1], Tyr 1.06 [1], Phe 1.04 [1].

The results of this experiment showed that it was possible to side chain deprotect a peptide (in this case cleave a tyrosine tBu ether) while leaving the majority of the peptide anchored to the support. Subsequently, it was possible to cleave the peptide from the support, albeit in low yield of 32%. In the other experiments described in this Chapter (see Sections 4.2.3.2 and 4.2.3.3) peptide cleavage yields of greater than 80% were obtained using similar cleavage conditions to those employed here. The reason for the low yield in this experiment may have been that the TFA treatment degraded the resin properties in such a way that made it difficult or impossible for the cleavage reagent (LiβME) to gain access to some of the peptide-resin glycolamide linkages. It was noted that treatment of the peptide-resin with 95% TFA resulted in considerable swelling of the resin, accompanied by aggregation of the individual beads into lumps. In this state, the resin was difficult to filter. Even after the lumps had been broken up, it took longer to filter the resin than before TFA treatment.

The significance of the low cleavage yield, and the difficulty in filtering the resin after TFA treatment, relates to use of a peptide-resin as an affinity matrix. Considering that TFA treatment may have made it difficult for the relatively sterically unhindered LiβME to gain access to the resin-bound peptide, it may be expected that larger molecules (for example enzymes), would be even less able to gain access to resin-bound peptide-ligands after TFA treatment. Using Fmoc chemistry, 95% TFA would usually be used for cleavage of the side chain protecting groups of support-bound peptides. It was shown in this experiment that treatment of peptide-Perloza with 95% TFA also resulted in impairment of the flow properties of Perloza after one and a half

hours exposure. One possible alternative method of cleaving side chain protecting groups without degrading the flow properties of the matrix might be to use lower times of exposure of the peptide-resin to TFA, except that some side chain protecting groups used in Fmoc chemistry, notably Arg N^G (Mtr), require cleavage times of 4-6 hours (Atherton et al, 1983b; see also Chapter 5).

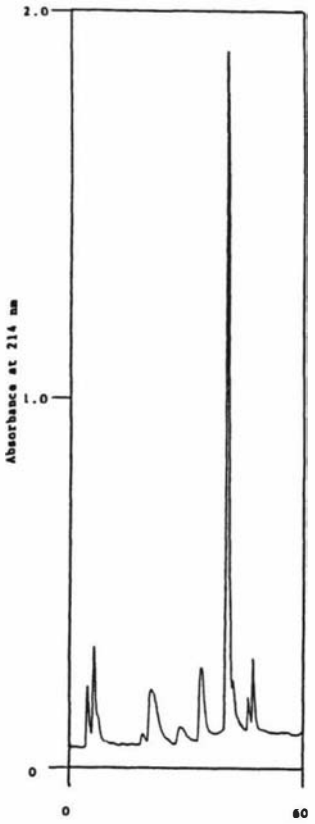
4.3.2 Treatment of the peptide-resin with 22.5% TFA / 2.5% water in dioxane followed by LiβME peptide cleavage

It was found in the previous experiment that concentrated TFA degraded the flow properties of Perloza after one and a half hours exposure (see also Chapter 5). If Fmoc chemistry was to be used for synthesis of Perloza-bound peptides for affinity chromatography, a milder, less matrix-destructive side chain deprotection reagent would be required. Therefore a solution of 22.5% TFA in dioxane was tested for its ability to cleave the t-butyl ether of tyrosine while leaving the peptide bound to the support. Water (2.5%) was also added to this solution to act as a scavenger of t-butyl cations. The TFA / dioxane solution was tested for its ability to cleave the tyrosine t-butyl ether of support-bound Leu-enkephalin whilst leaving the peptide bound to the resin. After a one hour exposure to the cleavage reagent the peptide-resin was washed, and the peptide was cleaved using LiβME. It was noted that the flow properties of the resin were not impaired by this treatment. HPLC analysis of the product (Figure 4.2) gave a major peak with a retention time of 41 minutes, which was close to that expected for Leu-enkephalin containing a tyrosine protected by the t-butyl ether (43.9 minutes, Section 4.3.3). It was concluded that 22.5% TFA / 2.5% water in dioxane was ineffective for cleavage of the tyrosine tBu ether after one hour.

The Fmoc SPPS method offers the greatest potential for use in synthesising peptide-resin affinity matrices, because the conditions used to cleave the amino acid side chain protecting groups employed are much milder than those required for the Boc method. However, in the case of Perloza, the results of the preliminary experiments reported above suggested that concentrated TFA was too destructive to the matrix for use as a reagent for side chain deprotection, although it was effective in cleaving the tyrosine t-butyl ether of support-bound Leu-

enkephalin. The dioxane solution of TFA did not degrade the flow properties of the matrix, but neither was it effective in cleaving the tyrosine t-butyl ether after one hours treatment. Further work on side chain deprotection reagents was required to make synthesis of peptide-ligands directly onto Perloza a practical proposition.

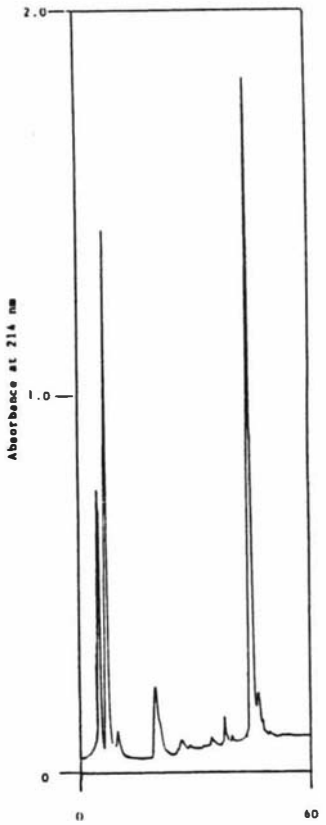
Figure 4.2 HPLC trace of Leu-enkephalin after treatment of the peptide-resin with 22.5% TFA / 2.5% water in dioxane, followed by LiβME peptide cleavage



4.3.3 Cleavage of side-chain protected Leu-enkephalin

Leu-enkephalin, with the tyrosine hydroxyl protected as its t-butyl ether, was cleaved from the resin using LiβME. A ninhydrin assay of the cleaved resin indicated a peptide cleavage yield of approximately 81%. Analytical HPLC of the crude peptide showed a major peak eluting at 43.0 minutes, compared to the 33.2 minute elution time of the fully deprotected peptide (Figure 4.3).

Figure 4.3 Side chain protected Leu-enkephalin after LiβME peptide cleavage



The crude side chain protected Leu-enkephalin was treated with TFA to cleave the tyrosine t-butyl ether. The Leu-enkephalin was then purified by HPLC. Leu-enkephalin was obtained in 59% yield, based on the initial amino acid substitution. The purified peptide coeluted on HPLC with authentic Leu-enkephalin and had the correct amino acid analysis: Gly 2.00 [2], Leu 1.01 [1], Tyr 1.01 [1], Phe 1.03 [1].

This experiment showed that it was possible to cleave side chain protected Leu-enkephalin without damage to the peptide. Further studies must be carried out to determine whether LiβME can be used to cleave peptides containing t-butyl esters of Asp or Glu side chains without incurring side reactions. For example, peptides containing Asp or Glu t-butyl esters are known to undergo intramolecular cyclisation in the presence of nucleophiles (Roeske, 1981).

4.4 CONCLUSIONS

1) Fmoc chemistry and the glycolamide linker may be used with Perloza for synthesising short peptides.

2) It was possible to cleave the t-butyl ether side chain protection from the tyrosine of support-bound Leu-enkephalin, using 95% TFA, while leaving the peptide bound to the resin. Leu-enkephalin could then be cleaved from the support, although the peptide cleavage yield was relatively low. However, the treatment with TFA also impaired the flow properties of the resin. It may be possible to increase the stability of Perloza to TFA by introducing cross linking.

3) Concentrated TFA, while effective in cleaving the t-butyl ether of tyrosine, also appeared to seriously degrade the flow properties of peptide-substituted Perloza. A solution of 22.5% TFA / 2.5% water in dioxane was ineffective in cleaving the tyrosine t-butyl ether of support-bound Leu-enkephalin, but neither did it appear to degrade the flow properties of the support.

4) Side chain protected Leu-enkephalin was cleaved from glycolamido-Perloza using LiβME. One of the applications of side chain protected peptides is in fragment condensations to form longer peptides. Another application of side chain protected peptides could be for immobilisation to chromatographic supports for affinity purifications (see Chapter 6). Further investigations must be carried out to determine if cleavage of side chain protected peptides from the glycolamide linker using LiβME is generally applicable for synthesis of protected peptide fragments.

CHAPTER 5

SOLID PHASE PEPTIDE SYNTHESIS ON PERLOZA:

FMOC METHODOLOGY

CHAPTER 5 SOLID PHASE PEPTIDE SYNTHESIS ON PERLOZA: FMOC METHODOLOGY

5.1 INTRODUCTION

The 9-fluorenylmethoxycarbonyl (Fmoc) amine protecting group, introduced by Carpino and Han (1970, 1972), is cleaved (Figure 5.1) by base promoted β-elimination. Piperidine in DMF is most frequently used for cleavage of the Fmoc group (Fields and Noble, 1990).

Use of the Fmoc group for temporary Nα-amino protection in solid phase peptide synthesis allowed an orthogonal synthesis methodology to be established. An orthogonal system has been defined as

> *...a set of completely independent classes of protecting groups, such that each class of groups can be removed in any order and in the presence of all other classes* (Kneib-Cordonier et al, 1990).

The orthogonal nature of the Fmoc methodology derives from the difference between the cleavage mechanism of the Fmoc Nα-amino protection (base catalysed β elimination), and the acidolytic cleavage of the side chain protecting groups and peptide-resin link. With use of the Fmoc group for Nα-amino protection, it was possible to use side chain protecting groups and peptide-resin linkages more acid-labile than those required for the Boc methodology. It was found, for example, that TFA-labile peptide-resin linkages of the p-alkoxybenzyl ester type (Wang, 1973) could be used in conjunction with tertiary butyl (tBu) based side chain protection for SPPS when using Fmoc for Nα-amino protection. A diagram of the Fmoc SPPS methodology is given as Figure 5.2

The Fmoc/tBu strategy, as it is sometimes known, allowed synthesis of peptides using much milder conditions than those used for the Boc/Bzl strategy. Whereas the Boc methodology required repetitive acidolytic cleavage of the Boc group using TFA, followed by peptide cleavage with strong acid (for example liquid HF), the Fmoc method used a much gentler piperidine treatment for cleavage of the Fmoc temporary Nα-amino protection, followed by a single TFA treatment for cleavage of the peptide from the support.

Figure 5.1 Mechanism of cleavage of the Fmoc Nα-amino protecting group

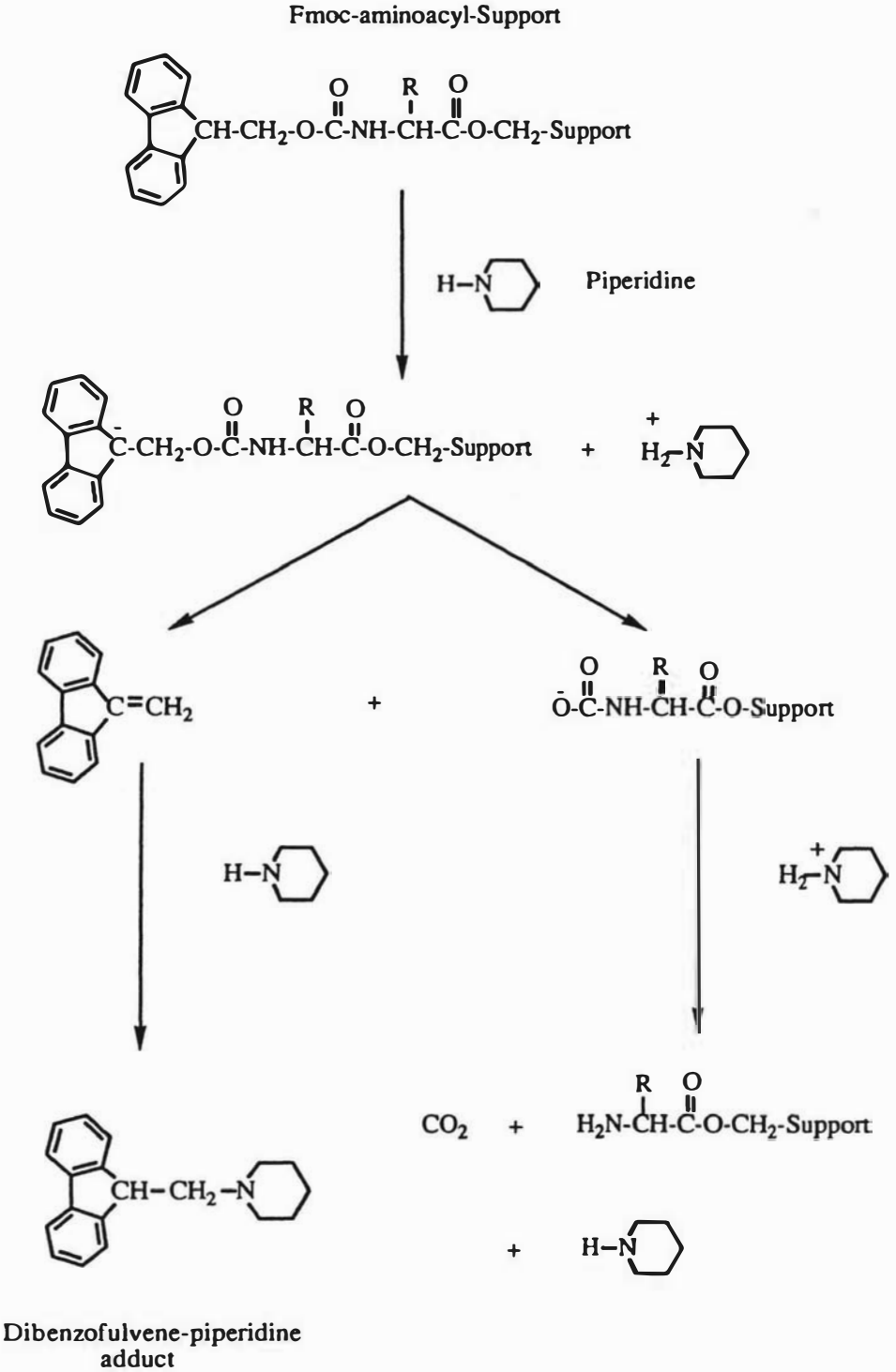
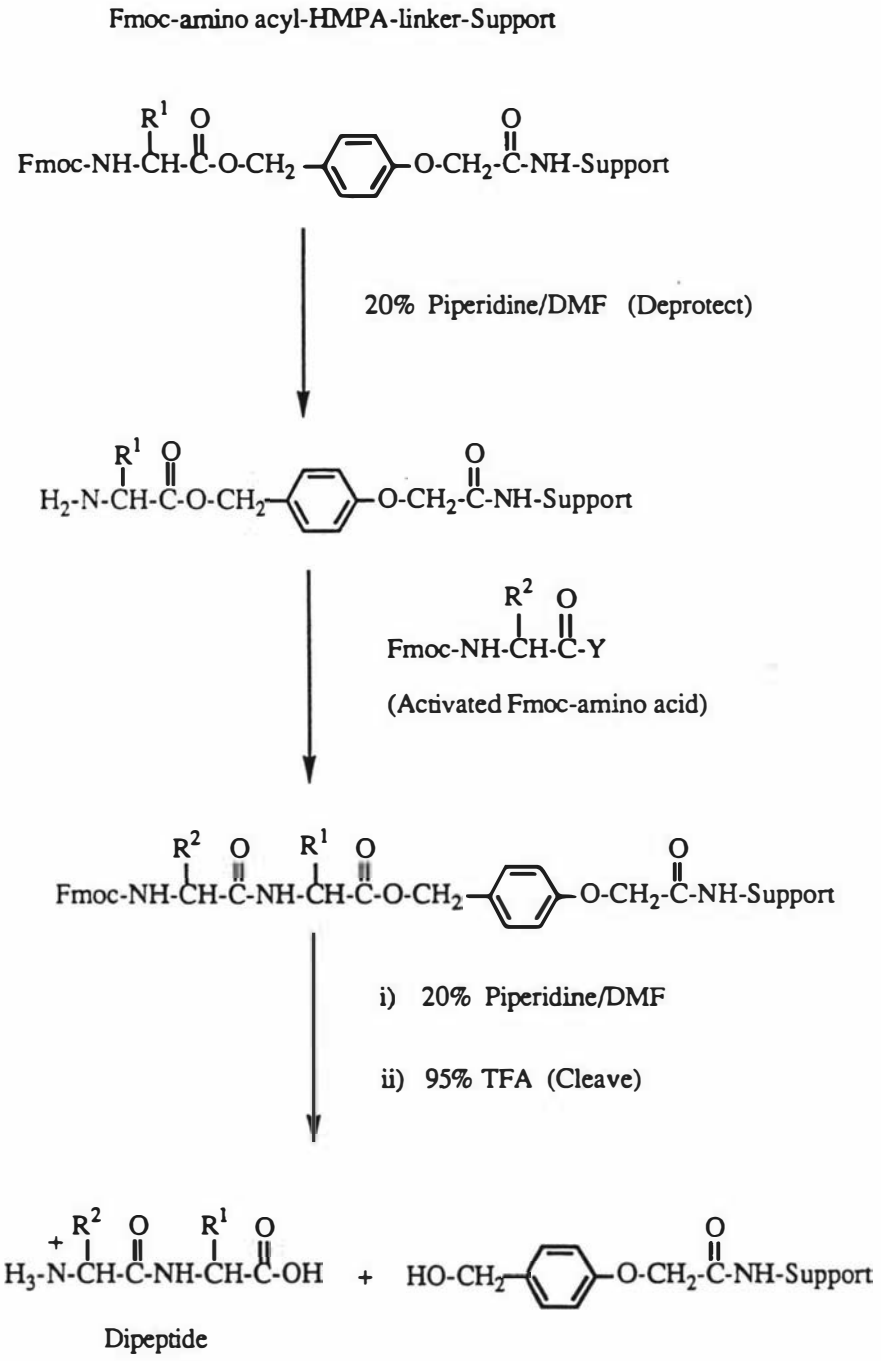


Figure 5.2 Fmoc SPPS methodology illustrated by the synthesis of a dipeptide



The first solid phase peptide syntheses using the Fmoc group for Nα-amino protection were reported in 1978. Chang and Meienhofer (1978) used 1% divinylbenzene cross linked polystyrene as a support for SPPS of the 14 amino acid residue peptide dihydrosomatostatin. Atherton et al (1978a,b) synthesised the decapeptide 65-74 sequence of acyl carrier protein (ACP 65-74), followed by the 31-residue peptide β-endorphin, using a beaded polyamide support. From these beginnings, the Fmoc method has been developed and refined to the point where it is widely used for SPPS. Excellent sources of information on the Fmoc SPPS method include Atherton and Sheppard (1989), and Fields and Noble (1990).

As well as the polystyrene and polyamide matrices previously mentioned, other supports which have been demonstrated as adequate for the Fmoc method include controlled pore glass (Albericio et al, 1987, 1989), polyamide-kieselguhr composite (Atherton et al, 1981b), cellulose paper (Frank and Doring 1988a,b; Eichler et al, 1989), cellulose cotton (Lebl and Eichler, 1989; Eichler et al, 1990, 1991), and Polyhipe^{TM} poly(styrene-co-divinylbenzene) (Small and Sherrington, 1989).

Both discontinuous batchwise and continuous flow automated peptide synthesisers may be used for syntheses by the Fmoc method. The type of synthesiser used depends on the solid support. For example, a batchwise synthesiser such as the ABI 430A is used for beaded polyamide and beaded 1% divinyl-benzene crosslinked polystyrene supports, because if they are used in flow systems the matrix packs down and excessively high back pressures are generated (Atherton et al, 1981b). A polyamide-kieselguhr composite was developed for use in continuous flow synthesisers such as the LKB Biolynx 4175. The inorganic kieselguhr serves to support the polyamide in the column, without generation of high back pressures. However, the polyamide-kieselguhr support cannot be used in a shaken batchwise synthesiser because the fragile kieselguhr suffers attrition and generates fines which clog the filters of the reaction vessel.

A continuous flow system for SPPS was seen as desirable (Sheppard, 1983) because it offered significant advantages over a batchwise system.

> *The removal of excess reactants by a continuous solvent flow through a column bed is inherently more efficient, economical, and*

rapid than batchwise washing. This is particularly true for gelatinous solids with a very high liquid retention. (Sheppard, 1983).

Polyamide-kieselguhr and beaded polyamide supports, used in a flow and batch system respectively, were used for comparative syntheses of an octadecapeptide (Atherton et al, 1983a). Little difference, if any, was found in the efficiency of synthesis on the two polyamide supports using the two different synthesisers.

One advantage of the continuous flow system was that it offered the possibility of real-time monitoring of deprotection and coupling reactions by inserting a UV flow cell into the recirculation circuit, and recording the solution absorbance with time (Sheppard, 1988; Atherton and Sheppard, 1989)

The solid support is usually functionalised with amine or hydroxyl groups. The p-alkoxy benzyl ester TFA cleavable linkage between the support and the C-terminal Fmoc-amino acid may be introduced in one of three general ways.

1) The hydroxymethyl linker molecule may be coupled to an amine functionalised support directly, or the linker may already be present from a different route (Wang, 1973). The C-terminal Fmoc-amino acid may then be esterified to the hydroxyl group. For example, Chang and Meienhofer (1978) used DCC in the presence of DMAP to esterify Fmoc-Cys(S-tBu) to a p-benzyloxybenzyl alcohol resin. Preformed symmetrical anhydrides of Fmoc-amino acids have also been used for loading to hydroxyl supports using DMAP catalyst. However, it was reported that significant racemisation resulted when DMAP was used as catalyst (Atherton et al, 1981a). Procedures to minimise racemisation during loading of the Fmoc-amino acid to hydroxyl supports involving use of HOBt (a racemisation suppressant) have been described (van Nipsen et al, 1985). Another problem with DMAP catalysed esterification of Fmoc-amino acids to hydroxyl functionalised supports is premature Fmoc removal by DMAP, resulting in dipeptide formation (Pedroso et al, 1983). Dipeptide formation may also be reduced by adding HOBt during the loading reaction (Van Nipsen et al, 1985). A full discussion of the problems encountered

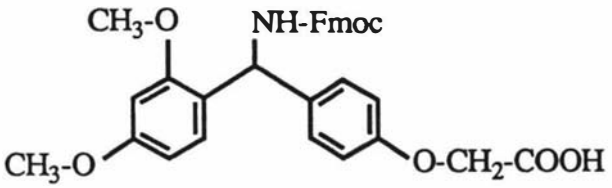
in loading Fmoc-amino acids to hydroxyl functionalised supports is given by Fields and Noble (1990).

2) The Fmoc-amino acid may be coupled to the linker molecule prior to attachment of the linker to the resin. The preformed Fmoc-amino acyl-linker compound is then coupled to an amine-functionalised support. For example, Bernatowicz et al (1989a) synthesised Fmoc-aminoacyl-4-oxy-methylphenoxyacetic acid 2,4-dichlorophenyl esters for coupling to amine-functionalised supports. The 2,4-dichlorophenyl ester served to activate the linker carboxyl for coupling to the amine groups of the support. Pyridine was used to catalyse the reaction. Daniels et al (1991) used a similar procedure except a combination of N-methyl morpholine and HOBt were used to catalyse the coupling reaction.

3) A halomethyl derivative of the linker molecule may be coupled to an amine-functionalised support, followed by displacement of the halogen by the cesium salt of the Fmoc-amino acid. For example, Colombo et al (1983) coupled 4-chloromethylphenoxyacetic acid to aminomethyl polystyrene or norleucyl-poly(dimethylacrylamide) resin to yield a 4-chloromethylphenoxyacetyl functionalised support. The chlorine was then displaced by the cesium salt of the Fmoc-amino acid to result in formation of an ester bond between the Fmoc-amino acid and the linker. One major advantage of this method is that it results in negligible racemisation of the amino acids (Mergler et al, 1989).

A number of linkers, usually of the acid labile p-alkoxybenzyl alcohol type (Wang, 1973), have been used for Fmoc syntheses. The 4-hydroxy-methyl phenoxyacetyl (HMPA) linker (Atherton et al, 1978a) is commonly used for synthesis of peptide acids. Peptide amides may be synthesised using linkers which furnish the amide on cleavage with TFA, a number of these are discussed by Bernatowicz et al (1989b). The p-[(R,S)-α-(9H-fluoren-9-yl)-methoxyformamido}-2,4-dimethoxybenzyl]-phenoxyacetyl linker (Figure 5.3), purchased from Novabiochem, was used in this study for the synthesis of peptide amides.

Figure 5.3 The p-[(R,S)-α-(9H-fluoren-9-yl)-methoxyformamido - 2,4-dimethoxybenzyl]-phenoxyacetyl linker for synthesis of peptide amides



The Fmoc method allowed use of TFA-labile amino acid side chain protecting groups. The protecting groups commonly used are: tBu ethers for the hydroxyls of serine, threonine and tyrosine; tBu esters for the carboxyl side chains of aspartic and glutamic acids, Boc for the N^{ε} group of lysine; triphenylmethyl (Trt) for the N^{im} of histidine; Trt for the sulfhydryl of cysteine; and N^{G}-4-methoxy-2,3,6-trimethyl-benzene-sulphonyl (Mtr) or N^{G}-2,2,5,7,8-pentamethylchroman-6-sulphonyl (Pmc) for the N^{G} of arginine. In addition, the amide side chains of asparagine and glutamine may be protected, for example by the Trt group.

Fmoc-amino acids may be activated for coupling in SPPS by methods including HOBt/DIC (Fields et al, 1989), symmetrical anhydride (Atherton et al, 1978a), pentafluorophenyl active ester (Kisfaludy and Schon, 1983), 2-(1H-benzotriazol-1-yl)-1,1,3,3,-tetramethyluronium hexafluoro-phosphate (HBTU) (Fields et al, 1991), 2-(1H-benzotriazol-1-yl)-1,1,3,3,-tetramethyluronium tetrafluoroborate (TBTU) (Beck-Sickinger et al, 1991) and benzotriazo-1-yl-oxy-tris-(dimethylamino) phosphonium hexafluorophosphate (BOP) (Hudson, 1988).

The extent of completion of coupling may be monitored by withdrawing samples for the qualitative TNBS (Hancock and Battersby, 1976) or quantitative ninhydrin tests (Sarin et al, 1981). Both of these methods require removal of resin samples. Real time monitoring may be accomplished by adding a small amount of bromophenol blue to the solution of activated Fmoc-amino acid. The blue colour fades as the resin bound amine groups are acylated (Krchnak et al, 1988). The

continuous flow Fmoc method also allowed monitoring of the deprotection and coupling steps by insertion of a UV flow cell into the circuit. However, the method is not sensitive enough to determine whether quantitative coupling of the Fmoc-amino acid has taken place.

Peptides made by the Fmoc method are usually cleaved from the support with TFA. Scavengers are included to trap reactive carbocations generated during side chain cleavage. The scavengers used depend on the type of side chain protection employed and the amino acids in the peptide (Applied Biosystems, Inc., 1990a). Typical cleavage mixtures are given in Section 5.2.8. of this Chapter (see also Van Wandelen et al, 1989).

Two groups have reported the use of carbohydrate supports for SPPS using the Fmoc methodology. Frank and Doring (1988a) used Whatman 3MM cellulose paper, in a continuous flow synthesiser, as a support for peptide synthesis using the Fmoc methodology. The paper was functionalised by esterification with 4-methoxytrityl-6[4'(oxymethyl) phenoxy] hexanoic acid, followed by acetylation of remaining reactive hydroxyl groups and cleavage of the 4-methoxytrityl group to expose the benzyl hydroxyl group. The first Fmoc-amino acid of the peptide sequence to be synthesised was coupled to the benzyl hydroxyl via its symmetrical anhydride in the presence of DMAP. The support was acetylated again to block any remaining reactive hydroxyls. Frank and Doring achieved 100% reaction with the benzyl hydroxyls, but Fmoc-amino acid also reacted with hydroxyl groups of the carbohydrate support to become anchored via ester bonds. The paper-bound Fmoc-amino acids were not cleaved by TFA, but were susceptible to hydrolysis by aqueous triethylamine. Because TFA was used for peptide cleavage, Frank and Doring postulated that peptides bound directly to the carbohydrate were unlikely to lead to contamination of the product. A number of heptapeptides were synthesised using paper as the SPPS support.

Eichler et al (1989) used Whatman 540 cellulose paper as a support for SPPS by the Fmoc method. Whatman 540 paper was reacted with Fmoc-glycine chloride to esterify the amino acid to the support. The Fmoc group was cleaved and a preformed Fmoc-amino acyl-4-oxymethyl phenoxy-propanoic acid 2,4,5-trichlorophenyl ester was coupled to the free amine

group (Albericio and Barany, 1985). Five 11-residue peptides were synthesised on the paper using Fmoc chemistry and a manual batchwise synthesis protocol. Peptides were cleaved with 75% TFA, 2% anisole in DCM.

Lebl and Eichler (1989) investigated the use of cotton fabric as a support for SPPS. An approach similar to that described in the previous paragraph was used to synthesise the tetrapeptide Des-Gly^2-Met-enkephalin and the pentapeptide Met-enkephalin on cellulose cotton fabric. The synthesis protocol used by Lebl and Eichler was chosen to demonstrate the practicality of using cotton as a support for continuous peptide synthesis. The peptides were cleaved with 50% TFA, 5% DMS in DCM. In an alternative synthesis strategy, Met-enkephalin-Gly was synthesised directly onto Fmoc-glycine modified cotton fabric. The side chain protecting groups were cleaved by acidolysis, and the peptide was cleaved from the carbohydrate support using 0.1M NaOH. It was found that the yield of Met-enkephalin-Gly synthesised directly onto the carbohydrate backbone of cotton was much lower than the yields of the other two peptides synthesised using the acid labile handle.

Eichler et al (1990, 1991) also described a procedure where glycine-cotton was reacted with the 2,4,5-trichlorophenyl ester of 4-hydroxy-methyl propanoic acid. C-terminal Fmoc-amino acids were esterified to the benzyl hydroxyls by a DCC/HOBt/DMAP procedure. It was found that up to 20% of the total protected amino acid had coupled directly to the support. Eichler et al did not consider this dangerous as acid cleavage would only liberate peptide bound to the linker, peptides bound directly to the carbohydrate support would only be cleaved by alkaline hydrolysis. Peptides up to 8 amino acids in length were made in this study. Eichler et al (1990) described an attempt to use triazine chemistry (discussed in Section 2.1.2 of Chapter 2) with cotton for anchoring a linker molecule to cotton. However, they did not pursue the triazine method because they found an inexplicable decrease in the amine substitution level with time.

It was demonstrated in the studies cited above that it was feasible to synthesise peptides on cellulose supports using the Fmoc methodology. The ultimate goal of this project was to develop a methodology for

synthesis of peptide-ligands directly onto Perloza for use as affinity chromatographic matrices. The Fmoc/tBu strategy of SPPS appeared to offer more potential in this respect than the Boc/Bzl strategy, because the amino acid side chain protecting groups used in the Fmoc method are cleavable using TFA. Although it was shown in Chapter 4 that treatment of a peptide-Perloza conjugate with concentrated TFA impaired the flow properties of the matrix, the Fmoc methodology still offered the greater potential as a route for the synthesis of support-bound peptide-ligands where side chain protected amino acids were present. An investigation was therefore carried out to evaluate Perloza as a support for SPPS using Fmoc methodology. One of the main goals of the studies reported in this Chapter was to determine whether peptides of high quality could be synthesised on Perloza using the Fmoc methodology. One of the requirements of a peptide-ligand synthesised directly onto Perloza would be that the synthesis go in high yield to generate mostly the desired peptide on the support. If Perloza was shown in this study to be a viable support for SPPS by the Fmoc methodology the protocols developed would then be applied to the synthesis of peptide-ligands directly onto aminopropyl Perloza.

The first step was to establish a reliable protocol for anchoring Fmoc-amino acids to aminopropyl Perloza. In this study, Fmoc-amino acids were anchored to aminopropyl Perloza via their preformed HMPA linker-2,4-dichlorophenyl esters. An alternative method of anchoring Fmoc-amino acids to Perloza would have been to couple the hydroxyl–linker (in this case 4-hydroxymethyl phenoxyacetic acid, HMPA) to aminopropyl Perloza, and to then use the DCC/HOBt/DMAP procedure to anchor the Fmoc-amino acids. Two advantages were seen in using the preformed Fmoc-amino acyl-HMPA linker-2,4-dichlorophenyl ester method. Synthesis of the preformed Fmoc-amino acyl-HMPA linker-2,4-dichlorophenyl ester would largely avoid racemisation of the C-terminal amino acid, because the Fmoc-amino acid would be coupled to the linker by nucleophilic displacement of a bromine atom (Mergler et al, 1989; Bernatowicz, 1989a). A second advantage was that esterification of amino acid to the support, as found by Frank and Doring (1988a) and Eichler et al (1990) in their studies, would be avoided. Although support-bound amino acids or peptides were unlikely to interfere in the synthesis of a peptide, they could consume reagents that would otherwise react with the acid

cleavable HMPA-linked amino acid or peptide. Once a means of anchoring Fmoc-amino acids to Perloza had been found, peptide synthesis was investigated using both ABI 430A (batchwise) and LKB Biolynx (continuous flow) peptide synthesisers.

5.2 MATERIALS AND METHODS

5.2.1 Chemicals and equipment

Nα-Fmoc-L-amino acids (Ala, Arg(Mtr), Asn, Asp(OtBu), Cys(tBu), Gln, Glu(OtBu), Gly, His(Trt), Ile, Leu, Lys(Boc), Met, Phe, Pro, Ser(tBu), Trp, Tyr(tBu), Val) were supplied by Bachem, Torrance, California. The peptides luteinising hormone-releasing hormone (LHRH), angiotensins I and II, and adrenocorticotropin sequence 4-11 (ACTH 4-11) were also purchased from Bachem. The peptides were to be used as "standards" for HPLC comparison with the same peptides synthesised using Perloza. L-Pyroglutamic acid was supplied by Sigma. The peptide amide linker p-[(R,S)-α-(9H-fluoren-9-yl)-methoxyformamido}-2,4-dimethoxy-benzyl]-phenoxyacetic acid was purchased from Novabiochem Ltd., (UK). Reagent grade benzene, mercuric acetate, and piperidine were purchased from BDH (NZ) Ltd. Piperidine was distilled from calcium hydride. Analytical reagent grade ethyl acetate was also from BDH (NZ). Reagent grade 1,2-ethanedithiol, and thioanisole, were from Aldrich (USA). Reagent grade p-cresol was from May and Baker (UK). Reagent grade diisopropyl-ethylamine (DIEA) and 2,4-dichlorophenol were from Riedel-de Haen. Reagent grade bromine was from Hopkin and Williams (UK). Celite filter aid (type 545) was from Serva. Drum grade dichloromethane was dried over magnesium sulphate and then distilled. Trifluoroacetic acid was from Halocarbon, New Jersey, and was distilled prior to use. All other reagents were from the sources listed in previous chapters.

Silica gel thin layer chromatography plates (Kieselgel 60 F_{254}) were supplied by Merck.

Peptide syntheses were performed semi-manually using an LKB Biolynx 4175 continuous flow peptide synthesiser. Automated peptide syntheses were performed using an ABI 430A batchwise synthesiser.

5.2.2 Anchoring Fmoc-amino acids to aminopropyl Perloza

Fmoc-amino acids were anchored to aminopropyl Perloza via the 4-hydroxymethyl phenoxyacetyl (HMPA) linker. Fmoc-amino acyl-4-oxymethylphenoxyacetic acid 2,4-dichlorophenyl esters were synthesised. These compounds were reacted with aminopropyl Perloza in the presence of either pyridine or NMM/HOBt as catalysts.

5.2.2.1 Synthesis of Nα-Fmoc-amino acyl-4-oxymethylphenoxyacetic acid 2,4-dichlorophenyl esters

5.2.2.1.1 Synthesis of 4-methylphenoxyacetic acid

p-Cresol, 30 g (0.278 mole), was added to 200 ml of 4.87M NaOH solution. α-Chloroacetic acid, 39.4 g (0.417 mole) was added and the solution refluxed for one hour. A white precipitate of sodium 4-methylphenoxyacetate formed on cooling to room temperature. The solid was collected by filtration, dissolved in 500 ml hot distilled water, and 200 ml 2M HCl solution was added to precipitate the free acid. The solution was allowed to cool to room temperature. A white precipitate formed. The precipitate was collected by filtration, washed with water, and dried under vacuum, yielding 27.96 g (61%) of product. The melting point was 142-142.5°C (lit. 136°C; CRC, 1971). Elemental analysis: expected for $C_9H_{10}O_3$: C, 65.1%; H, 6.0%. Found: C, 64.8%; H, 6.0%.

5.2.2.1.2 Synthesis of 4-bromomethylphenoxyacetic acid

4-Methylphenoxyacetic acid, 15.0 g (0.090 mole), was dissolved in 200 ml benzene in a round-bottomed flask. The solution was refluxed, and a solution of 4.8 ml bromine (0.095 mole) in 30 ml benzene was added over 20 minutes while the flask was irradiated with a 200 watt tungsten lamp. The solution was refluxed for a further 30 minutes, then transferred to a conical flask and allowed to cool to room temperature. On cooling, a white precipitate formed. The precipitate was collected by filtration on a sintered glass funnel, washed twice with benzene, and dried under vacuum. A second crop was obtained on concentration of the filtrate in vacuo, this solid was also collected, washed with benzene and dried

under vacuum. The two crops were combined (13.03 g) and recrystallised from benzene. The product was collected, washed with benzene and dried under vacuum (10.17 g, 46%). Elemental analysis: expected for $C_9H_9BrO_3$: C, 44.1%; H, 3.7%; Br, 32.6%. Found: C, 44.6%; H, 3.9%; Br, 32.5%. Melting point: 130-136°C (uncorr.) (lit 121-123°C; Bernatowicz et al, 1989a).

5.2.2.1.3 Synthesis of 2,4-dichlorophenyl 4-bromomethylphenoxyacetate

4-Bromomethylphenoxyacetic acid, 2.45 g (10 mmole), and 2,4-dichlorophenol, 1.79 g (11 mmole), were dissolved in 30 ml ethyl acetate. The solution was cooled to 0°C, stirred, and DCC, 2.27 g (11 mmole), was added. A precipitate of DCU formed. The solution was stirred at 0°C for one hour, and at room temperature for a further hour. After two hours tlc (ethyl acetate:acetic acid, 9:1) indicated that reaction was complete. The DCU was filtered off, washed twice with ethyl acetate, and the washings combined with the initial filtrate. The ethyl acetate was removed in vacuo, leaving an oil which solidified on cooling. The residue was dissolved in 30 ml ethyl acetate and triturated with 100 ml of n-hexane. After one minute needles started to precipitate. The solution was cooled to -20°C for one hour. The precipitate was collected by filtration, washed with a little n-hexane, and dried under vacuum. The weight of product was 2.73 g (73%). Melting point: 115-118°C (uncorr.) (lit. 114-116°C; Bernatowicz et al, 1989a).

5.2.2.1.4 Reaction of the DIEA salt of Fmoc-phenylalanine with 2,4-dichlorophenyl 4-bromomethylphenoxyacetate

The general procedure, used in this study, for these reactions is illustrated below:

Fmoc-phenylalanine, 2.131 g (5.5 mmole), was dissolved in 10 ml DMF. DIEA, 0.711 g (0.96 ml, 5.5 mmole), was added. The solution was stirred and 1.950 g (5 mmole) 2,4-dichlorophenyl 4-bromomethylphenoxyacetate was added. The solution was stirred for 25 hours. Tlc (ethyl acetate:n-hexane, 1:1) indicated the reaction had gone to completion. Dioxane, 60 ml, was added and the solution was lyophilised to yield an oil which contained a semicrystalline solid. Ether, 20 ml, was added, and the

precipitate of diisopropylethylamine hydrobromide was filtered off and washed with 10 ml ether. The ether filtrates were pooled, and the ether was removed in vacuo to leave an oil. After 2-3 hours the oil had solidified. The solid was recrystallised from ethyl acetate (20 ml): n-hexane (40 ml), with cooling to 4°C. The product was collected by filtration, washed with a little ethyl acetate:n-hexane 1:3, then dried in vacuo. After standing for 17 hours at room temperature a second crop formed, which was also collected, washed and dried. The two crops were combined, weight 1.24 g (71%). Tlc (ethyl acetate:n-hexane, 1:1) showed a single spot, R_f 0.74. Elemental analysis: expected for $C_{39}H_{31}Cl_2NO_7$: C, 67.2%; H, 4.5%; N, 2.0%; Cl, 10.2%. Found: C, 67.1%; H, 4.7%; N, 2.2%; Cl, 10.1%.

In some cases a precipitate of the product did not readily form, even after several attempts to induce crystallisation. In those cases the oily Nα-Fmoc amino acyl-4-oxymethylphenoxyacetic acid 2,4-dichlorophenyl ester was used for reaction with aminopropyl Perloza.

5.2.2.1.5 Reaction of aminopropyl Perloza with Nα-Fmoc-amino acyl -4-oxymethylphenoxyacetic acid 2,4-dichlorophenyl ester

A typical reaction that employed pyridine as the catalyst, and a three fold excess of Nα-Fmoc-amino acyl-4-oxymethylphenoxyacetic acid 2,4-dichlorophenyl ester, is described below:

Water-wet aminopropyl Perloza 100 medium (amine substitution 1.14 mmole/g, 0.63 mmole amine) was solvent exchanged to dioxane and then to DMF, followed by a thorough wash with DMF. Excess DMF was drained and the resin was transferred to a screw capped reaction vessel (Figure 3.2, Chapter 3). Fmoc-leucyl-4-oxymethyl phenoxyacetic acid 2,4-dichlorophenyl ester (2.0 mmole, an oil) was dissolved in 5 ml of DMF and added to the resin, followed by 0.16 ml (2.0 mmole) of pyridine to catalyse the reaction. After mixing (Rototorque) for 19.5 hours, the resin was washed with DMF. Unreacted amine groups were acetylated for 2 hours using 0.25 ml acetic anhydride and 0.06 g DMAP in 3 ml DMF. The resin was washed with DMF, and the residual amine substitution was determined by the ninhydrin assay. The resin was then washed with dioxane, and stored at 4°C.

A sample of the Fmoc-leucyl-HMPA-Perloza was treated with 20% piperidine in DMF for 15 minutes to cleave the Fmoc group. Following Fmoc cleavage the resin was washed with DMF, then with dioxane. The resin was divided into two equal portions. One sample was dried and subjected to amino acid analysis, while the other was used for determination of the amine substitution by picrate titration.

When using a combination of NMM and HOBt as catalysts, a similar procedure was used, except that three equivalents each of NMM and HOBt.H_2O were added to the reaction mixture instead of pyridine.

5.2.3 Determination of the extinction coefficient of fulvene-piperidine adduct

Fmoc-glycine, 297.6 mg (1.00 mmoles) was treated with 100 ml 20% piperidine in DMF, which gave a cloudy solution. The cloudiness was due to formation of a precipitate of glycine. After 15 minutes 0.20 ml of the solution was made up to 25 ml in a volumetric flask using DMF. The absorbance of the diluted solution was 0.456 at 301 nm, which gave an extinction coefficient of 5700 for the fulvene-piperidine adduct. The experiment was repeated, but the cloudy solution was filtered prior to dilution. The extinction coefficient was again found to be 5700.

5.2.3.1 Spectrophotometric determination of Fmoc-amino acid substitution levels of Fmoc-amino acyl-HMPA-Perloza

A sample of DMF-wet Fmoc-amino acyl-HMPA-Perloza was transferred to a sintered glass funnel. The resin was treated with 25 ml 20% piperidine in DMF (approx. 3 X 8 ml). The effluent was collected in a 100 ml volumetric flask. The solution was made up to 100 ml using DMF, then diluted to give a final absorbance of between 0.1-0.8 at 301 nm. The resin was subjected to picrate titration, and dried as described in Chapter 2. The amount of fulvene-piperidine adduct was calculated using the A_{301} absorbance with an extinction coefficient of 5700. The Fmoc substitution (and therefore amino acid substitution) was calculated once the dry weight of the resin had been obtained. The amino acid substitution calculated this way could be compared to that found using the picrate titration. The one experiment therefore combined two

independent ways of determining the amino acid substitution of an Fmoc-amino acyl-HMPA-Perloza resin.

5.2.4 Coupling p-[(R,S)-α-(9H-fluoren-9-yl)-methoxyformamido-2,4-dimethoxybenzyl]-phenoxyacetic acid to aminopropyl Perloza for the synthesis of peptide amides

The amide-linker compound p-[(R,S)-α-(9H-fluoren-9-yl)-methoxy-formamido-2,4-dimethoxybenzyl]-phenoxyacetic acid (Figure 5.3) was purchased from Novabiochem Ltd. Aminopropyl Perloza (amine substitution 0.44 mmole/g, 0.5 mmole amine) was washed with dioxane and then with DMF. To the DMF- moist resin was added 0.75 mmole of the linker compound (1.5 eq.), plus 1 mmole HOBt.H_2O (2 eq). DMF (5 ml) was added and the slurry was mixed thoroughly to dissolve the reagents. DIC, 0.83 mmole (1.66 eq), was added and mixing was continued for 9 hours. The residual amine substitution was 0.035 mmole/g, which indicated 92% reaction. Unreacted amine groups were acetylated for one hour using 0.5 ml acetic anhydride, 0.5 ml pyridine in 5 ml DMF. The resin was washed thoroughly with DMF. A sample of the resin was taken, and the Fmoc group was removed as described in Section 5.2.2.1.5. The amine substitution was determined by picrate titration.

To prepare the resin for peptide synthesis, the Fmoc group was cleaved, the resin was washed with DMF, and the C-terminal Fmoc-amino acid of the peptide to be synthesised was coupled via the DIC/HOBt method described in Section 5.2.5.

5.2.5 Reaction of Perloza 100 Medium with Fmoc-Gly-OBt

Perloza 100 Medium, 3.46 g, was washed with ethanol, and then with DMF. The resin was transferred to a Biolynx reaction column, which was fitted to the Biolynx peptide synthesiser. Fmoc-Gly, 0.297 g (1.00 mmole), and HOBt.H_2O, 0.168 g (1.10 mmoles), were dissolved in 3 ml DMF. DIC, 164 µl (1.05 mmole), was added to the DMF solution, and the solution was stirred for 10 minutes. After 10 minutes Fmoc-Gly-OBt solution was recycled through the Perloza using the Biolynx. After 1 hours recycle at 3 ml/min the Perloza was washed with DMF (3 ml/min, 10 minutes), 20% piperidine in DMF (3 ml/min, 10 minutes), and DMF (3 ml/min, 10 minutes). A sample of the resin was taken for ninhydrin assay.

5.2.6 Stability of aminopropyl Perloza to 20% piperidine in DMF

A sample of aminopropyl Perloza 100 Medium, amine substitution 1.89 mmole/g, was solvent exchanged to dioxane and then washed with DMF. After washing with DMF, 1.59 g of the damp resin was placed in a vial and 10 ml of 20% piperidine in DMF was added. The resin was mixed using the Rototorque. Samples were taken after 15 and 69 hours for determination of the amine substitution by picrate titration. After 15 hours exposure the amine substitution was almost unchanged at 1.87 mmole/g, but after 69 hours it had decreased to 1.77 mmole/g, or a loss of 5.3%. A 15 hour exposure to the reagent would be equivalent to 90X 10 minute treatments using the standard Biolynx SPPS protocol. The result indicated a loss of 0.01% of the initial amine per cycle, which was not envisaged as leading to any problems during SPPS on Perloza.

5.2.7 Peptides synthesised by the Fmoc methodology using Perloza as the solid support

5.2.7.1 Biolynx peptide synthesiser protocols

Nα-Fmoc-amino acyl-4-oxymethylphenoxyacetamido Perloza resin was transferred to a Biolynx reaction column. The weight of resin used was calculated to provide the amount of C-terminal amino acid required for the predetermined scale of the synthesis. The resin was washed for 10 minutes with DMF at 3 ml/min, followed by 20% piperidine/DMF for 10 min at 3 ml/min, followed by DMF at 3 ml/min for 10 min.

Fmoc-amino acyl-OBt active esters, preformed using the method given in section 5.2.5, were used to couple all Fmoc-amino acids except Fmoc-Asn and Fmoc-Gln, which were coupled as their 4-nitrophenyl or pentafluorophenyl active esters in the presence of HOBt. Single-coupling cycles were employed for addition of all amino acids, including Fmoc-Arg(Mtr) (see Section 5.2.7.2).

The standard LKB Biolynx 4175 peptide synthesis cycle was employed (LKB, 1987). The synthesis cycle used to couple each amino acid is given below. On completion of the synthesis, the Fmoc group was cleaved from the N-terminal amino acid. The peptide-resin was washed sequentially with DMF, DCM, and ether, and dried in vacuo.

Biolynx continuous flow peptide synthesis cycle: Fmoc methodology

Function	Time	Flow rate
Load activated Fmoc-amino acid		2 ml/min
Recycle		3 or 5 ml/min
Clean loader		
Recycle stop	After 1-2 hours	
Wash DMF	10 min	3 ml/min
Take sample for ninhydrin (optional)		
20% piperidine/DMF	10 min	3 ml/min
Wash DMF	5 min	3 ml/min
Load 5 ml DMF		2 ml/min
Wash DMF	5 min	3 ml/min

5.2.7.2 Automated syntheses, Fmoc chemistry

Three peptides were synthesised using the ABI 430A automated peptide synthesiser. Two were made using ABI Fmoc protocols, and one using the ABI Fastmoc protocol.

Angiotensin I (DRVYIHPFHL) and WJP-1 (NIRRLMDGEEPL) were made using a modified ABI Fmoc synthesis protocol (Applied Biosystems Inc., 1988). The chief modification of the standard ABI protocol was to extend the coupling time from one hour to two hours. The standard ABI DIC/HOBt activation protocol was used to form Fmoc-amino acyl-OBt esters for all couplings. All amino acids were single-coupled except Fmoc-Arg(Mtr), which was double-coupled using the standard ABI double coupling protocol.

The peptide KMP5 (YTRDLVYKNAPARPDIQKTCTF-NH_2) was synthesised using a modified ABI Fastmoc (Applied Biosystems Inc., 1990b) protocol, with HBTU activation of the Fmoc-amino acids. Two major changes were made to the standard Fastmoc cycles. DMF was used exclusively as the solvent for all washes and reactions, instead of DMF and NMP. Some of the solvent delivery times were altered to compensate for the difference in solvent viscosity between DMF and NMP. The second change was that the resin was washed ten times after completion of Fmoc removal instead of

five times. It was found that after five washes the smell of piperidine could still be detected in the resin. Seven or eight washes apparently removed all of the piperidine, but ten washes were used to provide a greater safety margin. The number of washes used to remove unreacted activated Fmoc-amino acid at completion of coupling was increased from seven to ten, again to provide a greater safety margin and to ensure quantitative removal of unreacted activated Fmoc-amino acid. Fmoc-amino acids were activated using the standard ABI Fastmoc HBTU activation cycles (Applied Biosystems Inc., 1990b). All Fmoc-amino acids were single coupled except Fmoc-Arg(Mtr), which was double coupled.

A standard set-up procedure was used for all automated peptide syntheses performed using the ABI 430A synthesiser, regardless of the synthesis protocol employed. The Fmoc-amino acyl-Perloza resin was weighed to provide 0.20 - 0.25 mmole of starting amino acid. The synthesiser was prepared for the synthesis according to the manufacturer's instructions (Applied Biosystems 1988, 1990b). Cartridges containing 1 mmole of the Fmoc-amino acids were loaded into the machine, and the synthesis was started. At the end of the synthesis the N-terminal Fmoc group was removed and the peptide-resin was dried under vacuum. The weight of dry peptide-resin was noted.

5.2.8 Cleavage of peptides from Perloza

Cleavage of peptides from the resin was achieved using one of the following cleavage reagents:

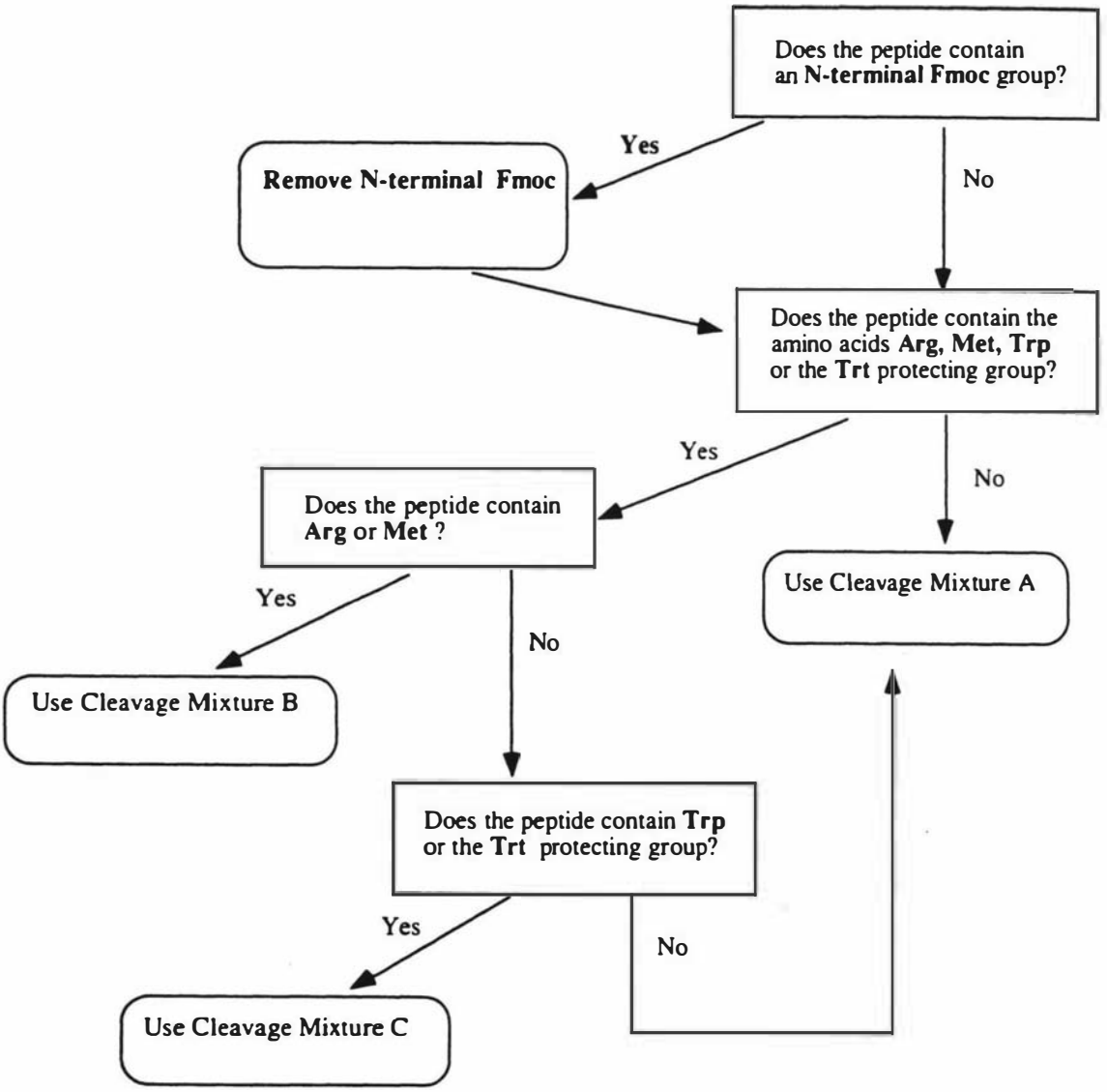
Reagent A 9.5 ml TFA, 0.5 ml water.

Reagent B 10 ml TFA, 0.75 g phenol, 0.5 ml water, 0.5 ml thioanisole, 0.25 ml 1,2-ethanedithiol.

Reagent C 10 ml TFA, 0.5 ml water, 0.5 ml 1,2-ethanedithiol.

The reagent required for a peptide cleavage depended on the peptide sequence and the amino acid side chain protecting groups employed. In this study the appropriate reagent to use for each peptide synthesised was determined using the flow chart given as Figure 5.4 below (from "Introduction to Cleavage Techniques", Applied Biosystems Inc., 1990a).

Figure 5.4 Cleavage reagent flow chart (from Applied Biosystems Inc., 1990a)



In general, 10 ml of cleavage reagent was used for up to 0.3 g of the dry peptide-Perloza resin. The peptide-resin was weighed into a vial, the cleavage reagent was added, and the suspension was stirred for up to 24 hours, depending on the side chain protecting groups present. In all cases the dried resin became highly swollen on treatment with TFA. In some cases the resin completely dissolved in the cleavage reagent. In these cases standard filtration of the resin from the peptide solution

could not be employed. Therefore a number of workup procedures were investigated. The procedure used for any given synthesis depended on the final state of the resin, although procedure 1 was generally found to be the most convenient, as it could be used regardless of the final state of the resin.

5.2.8.1 Peptide cleavage workup procedure 1

The peptide-resin / TFA suspension was added, with stirring, to 50 ml of water. In some cases, some of the TFA was removed in vacuo at 40°C prior to addition of the slurry to the water. The resin precipitated as a dough-like mass, which was collected by filtration and washed with water. If thiols were used as scavengers during the cleavage (Reagents B or C), the aqueous solution containing the peptide was extracted 3X 50 ml ether, then lyophilised.

5.2.8.2 Peptide cleavage workup procedure 2

TFA was removed from the peptide-resin suspension in vacuo at 40°C, and 50 ml ether was added to the resulting oily mass. The mixture was cooled to 4°C. After one hour the solid was collected by filtration and washed 2X 15 ml ether. The peptide was extracted from the solid using 5% acetic acid solution. The remaining resin was washed with 5% acetic acid solution, and the filtrates were pooled. If thiols were present the combined aqueous acetic acid solutions were extracted 3X 50 ml ether, then lyophilised.

5.2.8.3 Peptide cleavage workup procedure 3

The resin was separated from the TFA solution by filtration using glass wool. The resin was washed 1X 5 ml TFA, followed by 2X 10 ml DCM. The TFA and DCM were removed in vacuo at 40°C, and 50 ml water was added to the oily residue. The aqueous solution was extracted 3X 50 ml ether, then lyophilised.

Following cleavage, the remaining resin was dried in vacuo and weighed. A sample of the cleaved resin was subjected to amino acid analysis in order to determine the amount of peptide remaining on the resin. The results were used to calculate the peptide cleavage yield.

Where cleavage of the t-butyl protecting group from Cys(S-tBu) was necessary, the procedure of Van Wandelen et al (1989) was used. The aqueous solution of crude peptide from the cleavage was extracted extensively with ether to remove all thiols, then lyophilised. Mercuric acetate, 100 mg in 10 ml water, was added to 10 ml of a solution of the crude peptide (10 mg/ml) in 1% acetic acid. The reaction was left at room temperature for 90 minutes, then 0.5 ml of βME was added and the reaction heated at 45-50^{o}C for 1-2 hours. A precipitate formed as the reaction proceeded. The suspension was filtered through a bed of celite filter aid on a sintered glass funnel. The filtrate was extracted 3X 50 ml ether, then lyophilised.

5.3 RESULTS AND DISCUSSION

5.3.1 Synthesis of Fmoc-amino acyl-4-oxymethylphenoxyacetamido Perloza

In a series of preliminary experiments preformed Fmoc-amino acyl-4-oxymethylphenoxyacetic acid 2,4-dichlorophenyl esters were reacted with aminopropyl Perloza in the presence of pyridine catalyst (see Table 5.1). The results showed that useful levels of Fmoc-amino acids could be loaded onto aminopropyl Perloza using pyridine as catalyst.

Table 5.1 Synthesis of Fmoc-amino acyl-4-oxymethylphenoxyacetamido-propyl Perloza: pyridine catalyst

Amino acid	Initial resin amine substitution (mmole/g)	Reaction time (hours)	Amino acid substitution (mmole/g)		% Yield*
			a	b	
Gly	1.14	34	0.41	0.54	59
Gly	0.44	31	0.25	0.26	66
Leu	1.14	20	0.47	0.44	51
Leu	0.44	71	0.31	0.32	82
Lys	1.14	45	0.54	0.57	72
Lys	0.70	73	0.43	0.46	84
Phe	1.14	24	0.51	0.55	65
Val	0.44	43	0.27	0.31	79

a: amino acid analysis result, b: picrate titration result

* % Yield based on picrate titration result, see Equation 5.1.

The reaction yields given in Table 5.1 were based on the amino acid substitution calculated to result from quantitative reaction of aminopropyl Perloza with Fmoc-amino acyl-HMPA-2,4-dichlorophenyl esters. The quantitative reaction yields, using Fmoc-Gly-HMPA-2,4-dichlorophenyl ester as an example, were calculated using Equation 5.1 given below:

Equation 5.1 Calculation of % Yield for reaction of aminopropyl Perloza with Fmoc-Gly-HMPA-2,4-dichlorophenyl ester

$$\text{Fmoc-NH-CH}_2\text{-CO-O-CH}_2\text{-C}_6\text{H}_4\text{-O-CH}_2\text{-CO-O-C}_6\text{H}_3\text{Cl}_2(2,4) \;+\; \text{Perloza-NH}_2$$

$$\downarrow$$

$$\text{Fmoc-NH-CH}_2\text{-CO-O-CH}_2\text{-C}_6\text{H}_4\text{-O-CH}_2\text{-CO-NH-Perloza} \;+\; \text{HO-C}_6\text{H}_3\text{Cl}_2(2,4)$$

$$\downarrow \quad 20\% \text{ piperidine/DMF}$$

$$\text{H}_2\text{N-CH}_2\text{-CO-O-CH}_2\text{-C}_6\text{H}_4\text{-O-CH}_2\text{-CO-NH-Perloza}$$

If the initial weight of aminopropyl Perloza is 1.000 g, and the initial amine substitution is 1.14 mmole/g, then the expected final weight of the resin (after quantitative reaction with Fmoc-Gly-HMPA-2,4-dichlorophenyl ester followed by piperidine treatment to cleave the Fmoc protecting group) will be:

$$1.000 + (1.14 \times 10^{-3} \times 221)\ \text{g}$$

$$= 1.252\ \text{g}$$

However, there will be 1.14 mmole of glycine on the resin, therefore the expected glycine substitution level (after Fmoc cleavage) will be:

$$\frac{1.14\ \text{mmole}}{1.252\ \text{g}}$$

$$= 0.91\ \text{mmole/g}$$

If the actual substitution level (as determined by picrate titration, see Table 5.1) is 0.54 mmole/g, then the yield will be:

$$\frac{0.54\ \text{mmole/g}}{0.91\ \text{mmole/g}} \times 100\%$$

$$= 59\%$$

In another set of preliminary experiments, NMM and HOBt were used as catalysts instead of pyridine. The results of these experiments are given in Table 5.2. It appeared from a comparison of these results with those given in Table 5.1 that a combination of NMM and HOBt was more effective at catalysing the coupling reaction than was pyridine. Therefore, most couplings were carried out using NMM/HOBt as catalysts.

Table 5.2 Synthesis of Fmoc-amino acyl-4-oxymethylphenoxyacetamido-propyl Perloza: NMM/HOBt catalysts

Amino acid	Initial resin amine substitution (mmole/g)	Reaction time (hours)	Amino acid substitution (mmole/g)		% Yield*
			a	b	
Lys	0.70	8	0.46	0.44	81
Phe	1.14	16	0.76	0.76	90
Pro	0.44	43	0.27	0.37	94

a: amino acid analysis result

b: picrate titration result

* % Yield based on picrate titration result, see Equation 5.1 for calculation.

In a final series of experiments a standard coupling time of 5 hours, a three-fold excess of Fmoc-amino acyl-HMPA-2,4-dichlorophenyl ester, and NMM/HOBt catalysts, were used to anchor all 20 Fmoc-amino acids to aminopropyl Perloza (Table 5.3). Unreacted amine groups were acetylated using acetic anhydride and pyridine in DMF.

None of the reactions reported in Tables 5.1 - 5.3 were quantitative, regardless of the catalyst used or the time allowed for reaction. A possible explanation for the less than quantitative yields was that some of the resin-bound amine groups were not accessible to the bulky Fmoc-amino acyl-HMPA-2,4-dichlorophenyl esters. Although the coupling yields were not quantitative, the Fmoc-amino acid substitution levels obtained for all 20 amino acids were at a useful level for peptide synthesis.

Table 5.3 Synthesis of Fmoc-amino acyl-4-oxymethylphenoxyacetamido-propyl Perloza using standardised conditions: NMM and HOBt catalysts

Amino acid	Initial resin amine substitution (mmole/g)	Amino acid substitution (mmole/g)		% Yield*
		a	b	
Ala	0.79	0.47	0.52	78
Arg	0.87	0.34	0.37	63
Asn	0.87	0.41	0.45	63
Asp	0.83	0.39	0.49	75
Cys	0.77	dec.	0.54	86
Gln	0.76	0.44	0.49	79
Glu	0.81	0.36	0.53	84
Gly	0.69	0.43	0.52	87
His	0.87	0.33	n.d.	56
Ile	0.64	0.36	0.42	78
Leu	0.64	0.35	0.40	74
Lys	0.87	0.42	0.52	80
Met	0.83	0.23	0.47	70
Phe	0.76	0.50	0.47	81
Pro	0.81	0.52	0.65	97
Ser	0.73	0.32	0.47	70
Thr	0.76	0.44	0.52	85
Trp	0.68	n.d.	0.46	84
Tyr	0.69	0.32	0.43	78
Val	0.83	0.41	0.53	78

a: amino acid analysis result

b: picrate titration result

* % Yield based on picrate titration result (except His), see Equation 5.1 for calculation.

The amino acid substitution levels were determined, after cleavage of the Fmoc group, both by picrate titration and by amino acid analysis of an acid hydrolysate. Many of the amino acid substitution levels results as determined by the two analytical methods differed by a relatively

small amount, up to 0.06 mmole/g. However, some of the amino acid substitution results showed considerable differences between the two analytical methods. The differences between the results derived from the two analytical methods may have been due to a number of factors. For example, amino acid may have been occluded in the resin during acid hydrolysis of the amino acyl-Perloza for amino acid analysis, resulting in an amino acid analysis result lower than that of the picrate titration. Some amino acids could have been destroyed during the acid hydrolysis. For example, it is known that serine, threonine, and tyrosine are partially destroyed during acid hydrolysis (Stewart and Young, 1984). Some of the hydrolyses may not have been quantitative, although this was considered unlikely considering the acid-labile HMPA linker had been employed. Finally, mechanical losses of amino acid may have occurred during the hydrolysis and drying procedure.

An alternative reason for the differences in the results of the two analytical methods may have been that a positively charged species was generated on the resin, during the coupling reaction, by an unknown side reaction. If the postulated charged species bound picrate, which was also eluted on treatment with triethylamine solution, then it would be expected that the picrate titration result would be higher than the amino acid analysis result. To test this hypothesis, two Fmoc-amino acyl-HMPA Perloza resins were subjected to picrate titration. Picrate titration of Fmoc-Val and Fmoc-Glu resins (Table 5.3) did not indicate that a charged species was present.

The greatest differences between the picrate titration and amino acid analysis results were for glycine (Table 5.1), proline (Table 5.2), glutamic acid, aspartic acid, lysine, methionine, proline, serine, tyrosine, and valine (Table 5.3). Serine and tyrosine are known to be partially destroyed during acid hydrolysis (Stewart and Young, 1984), and this possibly accounted for the disagreement between the results of the two analytical methods for these two amino acids. Proline was low for two results (Tables 5.2 and 5.3), and this suggested a poor recovery of proline in the amino acid analysis method. The discrepancy between the results for the Glu and Val resins was investigated further by determining the Fmoc substitution using the absorbance at 301 nm of the fulvene-piperidine adduct derived after piperidine treatment (Section

5.2.3). The Fmoc substitution of the Val resin was 0.52 mmole/g using this method, which may be compared to the picrate titration result of 0.53 mmole/g. Similarly, the Fmoc substitution of the Glu resin as determined by the A_{301} method was 0.48 mmole/g, compared to the picrate titration of 0.53 mmole/g. The picrate titration showed good agreement with the fulvene-piperidine A_{301} method. The results, taken in combination, suggested that in some cases the resin amino acid substitution as determined by amino acid analysis was not the true result. The picrate titration results were used for calculation of the amount of Fmoc-amino acyl Perloza to be used in peptide syntheses. If anything, use of the picrate titration result would mean that less amino acid would be present during a synthesis if the resin-bound amino acid substitution as determined by amino acid analysis was in fact the true result. This was not envisaged as leading to problems, as it would only mean that a greater excess of activated Fmoc-amino acid over resin-bound amino acid would be being used during the synthesis.

5.3.2 Reaction of Fmoc-Gly-OBt with underivatised Perloza

In this study most of the Fmoc-amino acids were activated by forming their HOBt esters in DMF using DIC. The extent of reaction of activated Fmoc-glycine with the carbohydrate support was investigated. Fmoc-glycine was chosen because this is the least sterically hindered of the Fmoc-amino acids, and therefore may be expected to be the most reactive.

Fmoc-Gly-OBt was formed in DMF using the standard activation protocol used during peptide synthesis. The Fmoc-glycine-OBt ester was recycled through unfunctionalised Perloza resin for one hour. The resin was then washed with DMF and the Fmoc group was cleaved. A ninhydrin assay of the resin showed an amine substitution of 0.005 mmole/g. This was very low, and indicated that negligible reaction of Fmoc-amino acid-OBt esters with Perloza would be expected. Any peptides formed directly on the carbohydrate matrix would not be cleaved under the acidic conditions used, and would therefore not be expected to compromise peptide quality. However, significant amounts of matrix-bound peptide would consume activated Fmoc-amino acids, possibly leading to lower synthesis efficiencies. The result of this experiment suggested that it was likely that only very low levels of peptides would be formed on the

Table 5.4 Synthesis conditions for peptides made using Perloza and Fmoc chemistry, Biolynx continuous flow peptide synthesiser

Peptide	Sequence	Synthesis scale(mmole)	Excess of activated aa	Coupling time per Fmoc-aa
1 ACP 65-74	VQAAIDYING	0.20	5X	1 hr
2 Angio II	DRVYIHPF	0.25	4X	1 hr
3 JPP1	HPHPHLSFMA IPPK	0.11	4X	2 hr
4 Angio I	DRVYIHPFHL	0.25	4X	2-3 hr
5 ACTH 4-11	MEHFRWGK	0.25	4X	2-3 hr
6 JPP4	HLSFMAIPPK	0.10	4X	2 hr
7 LHRH	pGluHWSYGL RPG-NH_2	0.25	4X	2 hr
8 KSP2	CYEPFWQDEEK	0.25	4X	1 hr
9 KMP4(Cys tBu)	YTRDLVYKNP ARPKIQKT C(tBu)TF-NH_2	0.20	5X	1 hr
10 KMP5(Cys tBu)	YTRDLVYKNP ARPDIQKT C(tBu)TF-NH_2	0.20	5X	1 hr
11 KMP4	YTRDLVYKNP ARPDIQKTCTF-NH_2	0.20	5X	1 hr

carbohydrate backbone of Perloza during a synthesis under the Fmoc-amino acid activation conditions used. Also, as discussed in section 3.3.6 of Chapter 3, it would be expected that many of the reactive hydroxyls of Perloza would have reacted with acrylonitrile during the initial functionalisation of the resin. Therefore, it would be expected that less than 0.005 mmole/g of Fmoc-amino acid would couple to the functionalised support per synthesis cycle, because many of the reactive hydroxyls would have already reacted with acrylonitrile during the initial functionalisation of the resin.

5.3.3 Synthesis of peptides on Perloza using Fmoc methodology: Biolynx semimanual continuous flow peptide synthesiser

A number of peptides were synthesised using the LKB Biolynx 4175 semi-manual peptide synthesiser. Details of the syntheses are given in Tables 5.4 and 5.5.

Table 5.5 Cleavage conditions and yields for peptides made using Perloza and Fmoc chemistry, Biolynx continuous flow peptide synthesiser

Peptide	Purified yield	Cleavage time (hours)	Workup method (Section 5.2.8)	Cleavage yield
1 ACP 65-74	30%	4	1	86%
2 Angio II	45%	23	1	98%
3 JPP1	30%	3	3	n.d.
4 Angio I	37%	18	3	89%
5 ACTH 4-11	47%	8	3	90%
6 JPP4	14%	10	1	66%
7 LHRH	28%	21	1	94%
8 KSP2	39%	2	1	88%
9 KMP4(Cys tBu)	9%	6	1	91%
10 KMP5(Cys tBu)	25%	6	1	100%
11 KMP4	4%	7	2	92%

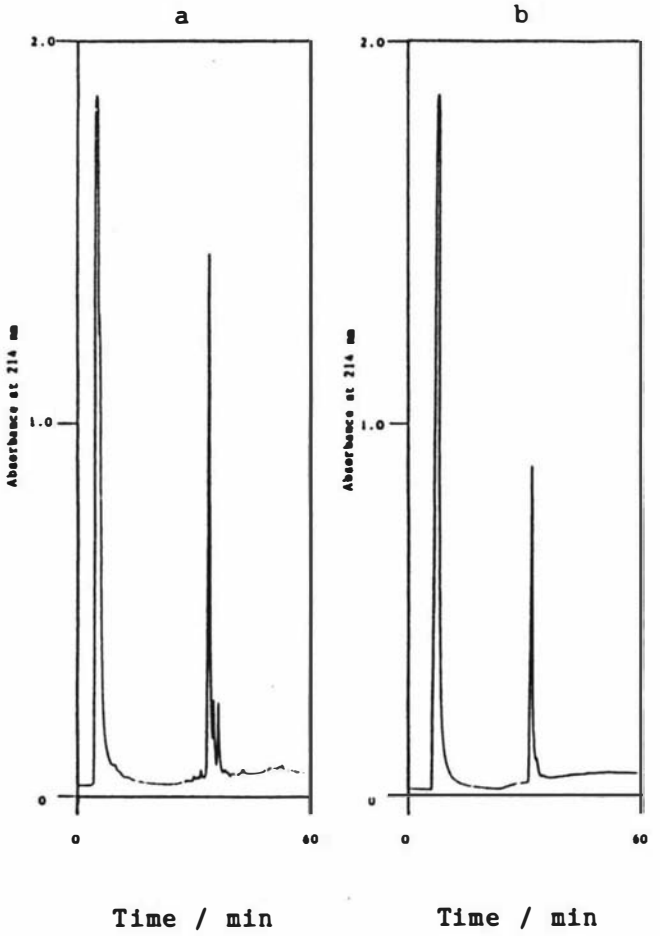
All peptides were cleaved with reagent B except 1 (ACP 65-74, Reagent A), and 8 (KSP2, Reagent C).

5.3.3.1 ACP 65-74

Val-Gln-Ala-Ala-Ile-Asp-Tyr-Ile-Asn-Gly

No problems were encountered during the synthesis of this peptide. HPLC traces of the crude and purified peptide are given in Figure 5.5. Amino acid analysis of the purified peptide gave the expected ratios: Asp 2.03 [2], Glu 0.95 [1], Gly 1.00 [1], Ala 2.07 [2], Val 0.91 [1], Ile 1.87 [2], Tyr 0.91 [1].

Figure 5.5 HPLC traces of a) crude, b) purified, ACP 65-74

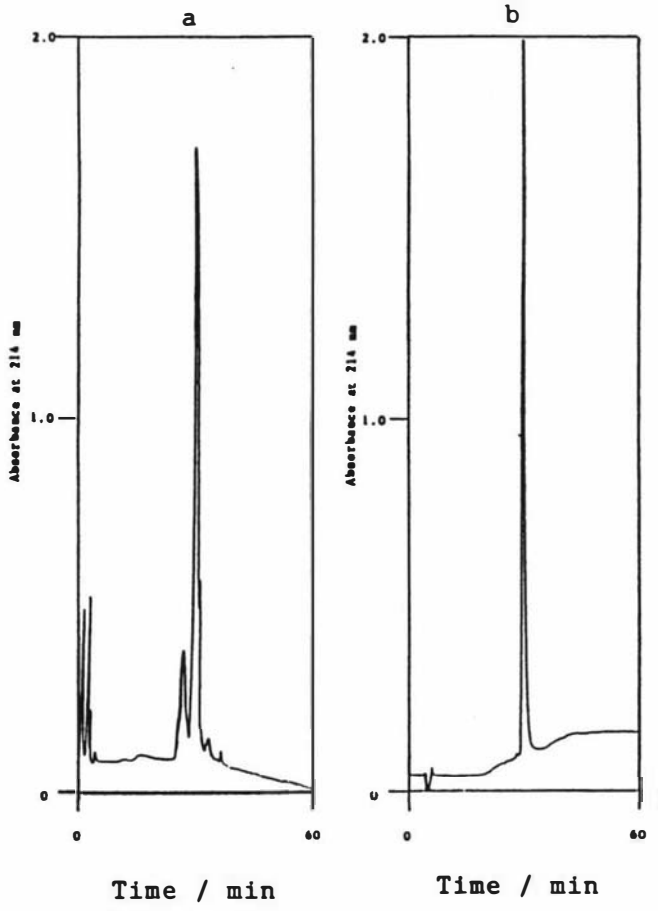


5.3.3.2 Angiotensin II

Asp-Arg-Val-Tyr-Ile-His-Pro-Phe

No problems were encountered during the synthesis of this peptide, but cleavage for 3 hours resulted in two peaks on HPLC. In this case it was concluded that the later eluting peak was Angiotensin II with the Mtr group still attached to the N^G of arginine, because longer cleavage times gave a single major peak. HPLC traces of the crude and purified peptide are given in Figure 5.6. The purified peptide coeluted on HPLC with an authentic sample. Amino acid analysis of the purified peptide gave the expected ratios: Asp 1.02 [1], Pro 0.96 [1], Val 1.00 [1], Ile 0.85 [1], Tyr 0.93 [1], Phe 0.99 [1], His 0.85 [1], Arg 0.97 [1].

Figure 5.6 HPLC traces of a) crude, b) purified, Angiotensin II

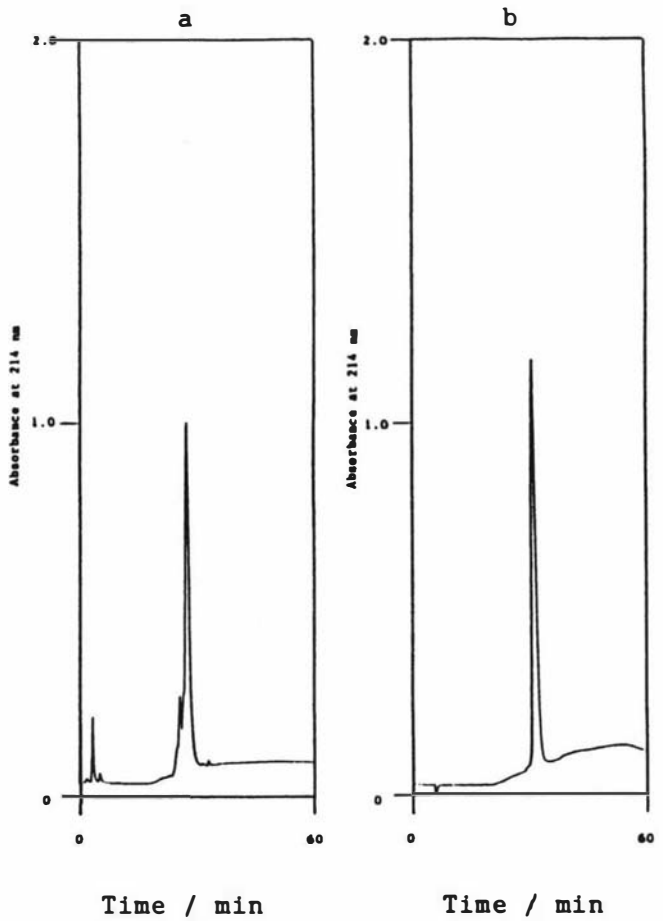


5.3.3.3 JPP1

His-Pro-His-Pro-His-Leu-Ser-Phe-Met-Ala-Ile-Pro-Pro-Lys

No problems were encountered during the synthesis of this peptide, although previous attempts to synthesise it using standard ABI Boc protocols were not successful (Gibson, 1991). HPLC traces of the crude and purified peptide are given in Figure 5.7. Amino acid analysis of the purified peptide gave the expected ratios: Ser 0.93 [1], Pro 3.86 [4], Ala 1.00 [1], Met 0.75 [1], Ile 0.94 [1], Leu 1.02 [1], Phe 0.95 [1], Lys 1.00 [1], His 2.74 [3].

Figure 5.7 HPLC traces of a) crude, b) purified, JPP1

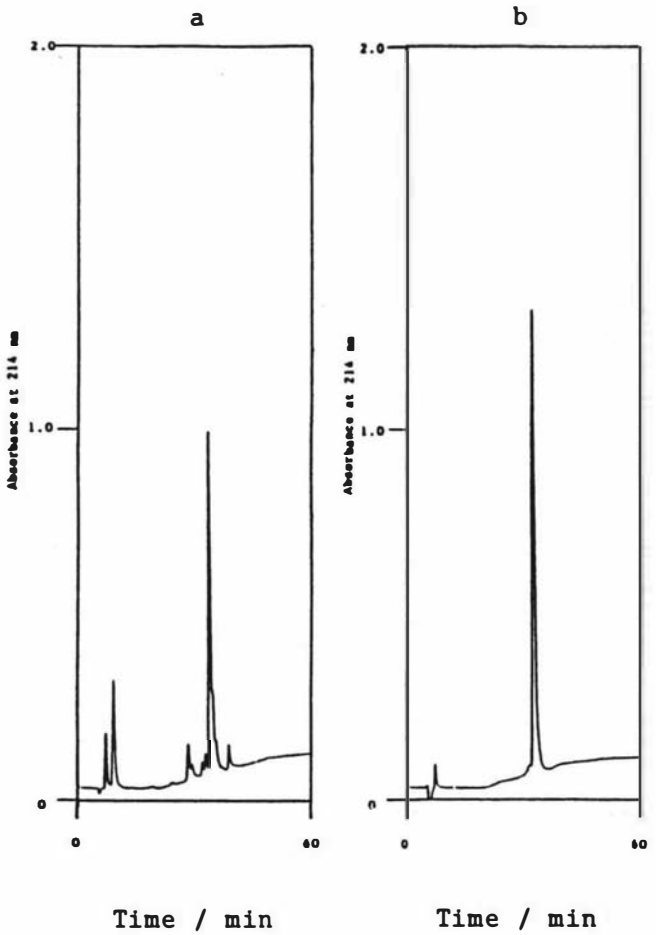


5.3.3.4 Angiotensin I

Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu

No problems were encountered during the synthesis of this peptide. HPLC traces of the crude and purified peptide are given in Figure 5.8. The purified peptide coeluted on HPLC with an authentic sample. Amino acid analysis of the purified peptide gave the expected ratios: Asp 0.98 [1], Pro 0.95 [1], Val 1.00 [1], Ile 0.86 [1], Leu 0.99 [1], Tyr 0.94 [1], Phe 0.95 [1], His 1.84 [2], Arg 0.94 [1].

Figure 5.8 HPLC traces of a) crude, b) purified, Angiotensin I



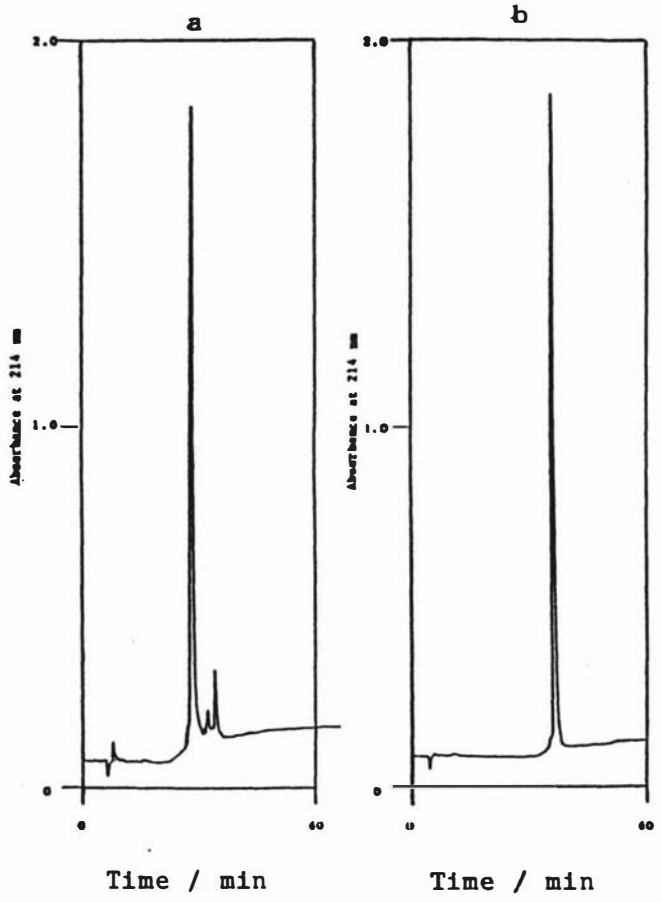
5.3.3.5 ACTH 4-11

Met-Glu-His-Phe-Arg-Trp-Gly-Lys

No problems were encountered during the synthesis of this peptide. HPLC traces of the crude and purified peptide are given in Figure 5.9. The purified peptide coeluted on HPLC with an authentic sample. Amino acid analysis gave the expected ratios, except for Trp, which was destroyed during acid hydrolysis: Glu 1.09 [1], Gly 1.00 [1], Met 0.92 [1], Phe 0.96 [1], Lys 1.04 [1], His 0.98 [1], Arg 0.95 [1], Trp n.d. [1].

It was particularly significant that the purified peptide coeluted with an authentic sample, because the sequence includes tryptophan, which could not be determined by amino acid analysis using acid hydrolysis. Tryptophan is readily alkylated by Mtr from arginine if the correct scavengers are not present (Sieber, 1987). If alkylation of tryptophan had occurred the synthetic ACTH 4-11 so formed would not have been expected to coelute with the authentic sample. These results indicated that an acceptable solid phase synthesis of a potentially problematic peptide, using Perloza as support and Reagent B for peptide cleavage, had been achieved.

Figure 5.9 HPLC traces of a) crude, b) purified, ACTH 4-11

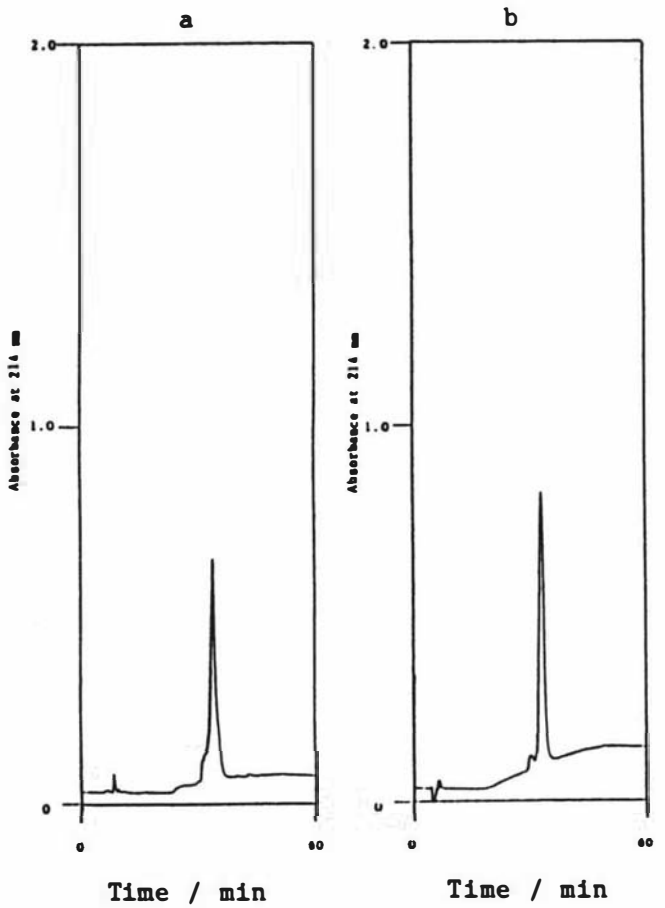


5.3.3.6 JPP4

His-Leu-Ser-Phe-Met-Ala-Ile-Pro-Pro-Lys

No problems were encountered during the synthesis of this peptide. The cleavage yield of this peptide was lower than all of the others (see Table 5.5), and this was also reflected in the yield of purified peptide. The most likely cause of the low cleavage yield was that during this cleavage a modified work up procedure was used, involving removal of TFA in vacuo prior to addition of water to the resin. This could possibly have resulted in occlusion of peptide in the cellulose precipitate. The peptide was probably not extracted from the cellulose precipitate by the washing method employed. Greater attention was paid to washing of the cellulose precipitate during subsequent cleavages of other peptides after this low yield was discovered. No attempt was made to repeat this synthesis. HPLC traces of the crude and purified peptide are given in Figure 5.10. Amino acid analysis of the purified peptide gave the expected ratios: Ser 0.86 [1], Pro 1.96 [2], Ala 1.00 [1], Met 0.84 [1], Ile 1.10 [1], Leu 1.09 [1], Phe 1.04 [1], Lys 1.11 [1], His 1.03 [1].

Figure 5.10 HPLC traces of a) crude, b) purified, JPP4

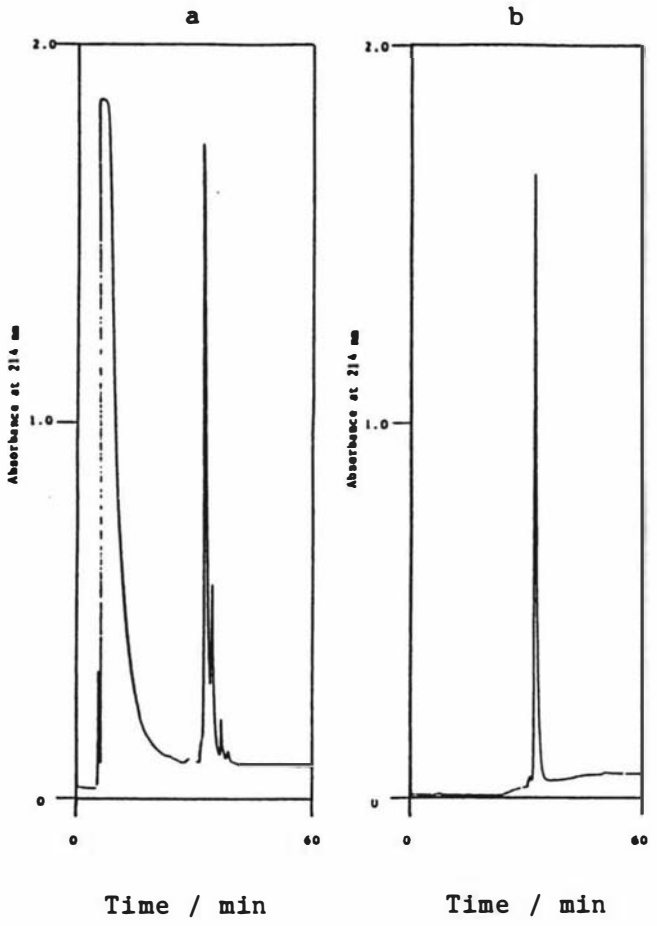


5.3.3.7 LHRH

pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2

No problems were encountered during the synthesis of this peptide amide using the Novabiochem amide-linker. HPLC traces of the crude and purified peptide are given in Figure 5.11 The synthetic peptide coeluted on HPLC with an authentic sample. This peptide also contained tryptophan, as well as arginine which was protected by the Mtr group. A satisfactory synthesis was achieved, which further validated the use of Reagent B and Perloza for synthesis of tryptophan-containing peptides. In addition, the successful use of Perloza as a support for synthesis of a peptide amide was demonstrated. Amino acid analysis of the purified peptide gave the expected ratios, except for Trp, which was destroyed by acid hydrolysis: Ser 0.93 [1], Glu 1.08 [1], Pro 1.08 [1], Gly 2.00 [2], Leu 1.03 [1], Tyr 1.03 [1], His 1.00 [1], Arg 0.99 [1], Trp n.d. [1].

Figure 5.11 HPLC traces of a) crude, b) purified, LHRH

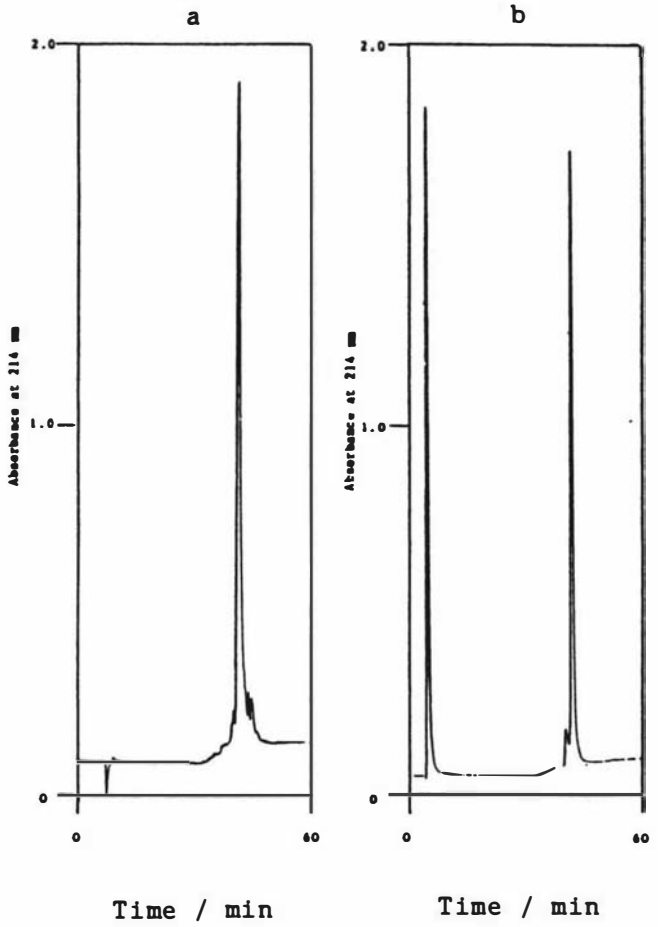


5.3.3.8 KSP2

Cys-Tyr-Glu-Pro-Phe-Trp-Gln-Asp-Glu-Glu-Lys

No problems were encountered during the synthesis of this peptide. However, some problems were encountered in the cleavage of the Cys S-tBu protecting group. The Cys-deprotected peptide was eventually obtained in reasonable yield. HPLC traces of the crude and purified peptide are given in Figure 5.12. Amino acid analysis of the purified peptide gave the expected ratios except for Trp and Cys, which were destroyed by acid hydrolysis, and Pro, which gave high results for two separate analyses: Asp 1.10 [1], Glu 4.08 [4], Pro 1.46 [1], Cys 0.27 [1], Tyr 1.00 [1], Phe 1.00 [1], Lys 1.14 [1], Trp n.d. [1]. No reason could be found for the unexpectedly high proline result, unless the peptide was not homogeneous. However, the purified peptide appeared to be homogeneous on HPLC.

Figure 5.12 HPLC traces of a) crude, b) purified, KSP2

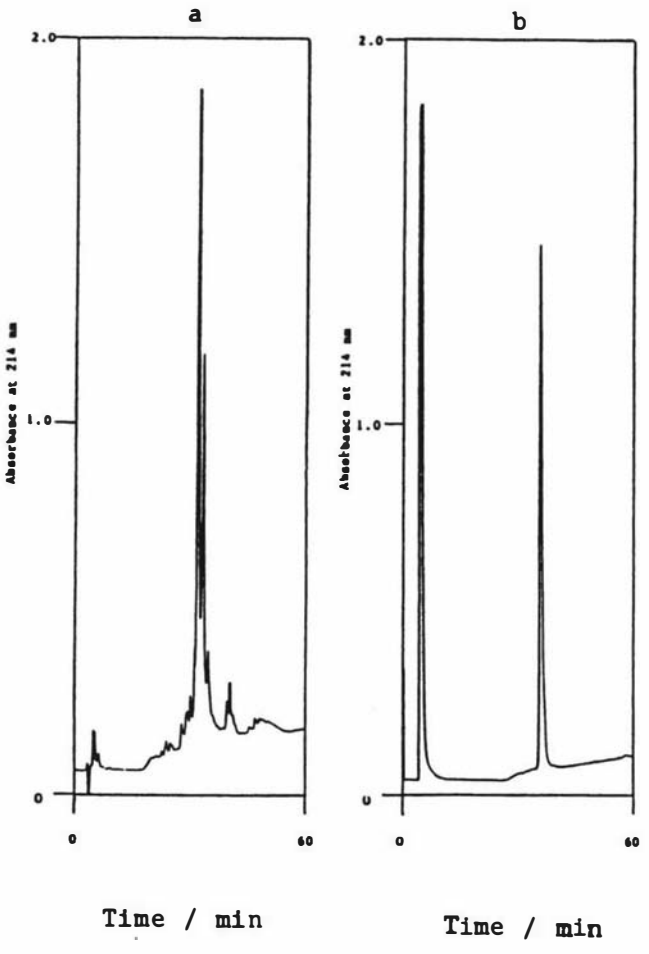


5.3.3.9 KMP4 (Cys-tBu)

Tyr-Thr-Arg-Asp-Leu-Val-Tyr-Lys-Asn-Pro-Ala-Arg-Pro-Lys-Ile-Gln-Lys-Thr-Cys(S-tBu)-Thr-Phe-NH_2

This peptide was originally required with the cysteine protected by the t-butyl group. One problem was encountered during the synthesis of this peptide. HPLC traces of the crude and purified peptide are given in Figure 5.13 HPLC analysis of the crude peptide showed two significant peaks. Both peaks were isolated by preparative HPLC. The later eluting peak was shown by amino acid analysis to contain one less arginine than expected. Sequencing of the deletion peptide from the N-terminus showed that the third amino acid from the N-terminus was [Arg^3]. This result indicated that the coupling of [Arg^{12}] had not gone to completion. The yield of purified peptide was low at 9%. Amino acid analysis of the purified peptide gave the expected ratios: Asp 1.94 [2], Thr 2.55 [3], Glu 0.98 [1], Pro 1.85 [2], Ala 1.00 [1], Cys 0.80 [1], Val 1.17 [1], Ile 1.18 [1], Leu 1.09 [1], Tyr 1.74 [2], Phe 1.05 [1], Lys 2.88 [3], Arg 1.76 [2].

Figure 5.13 HPLC traces of a) crude, b) purified, KMP4 (Cys-tBu)

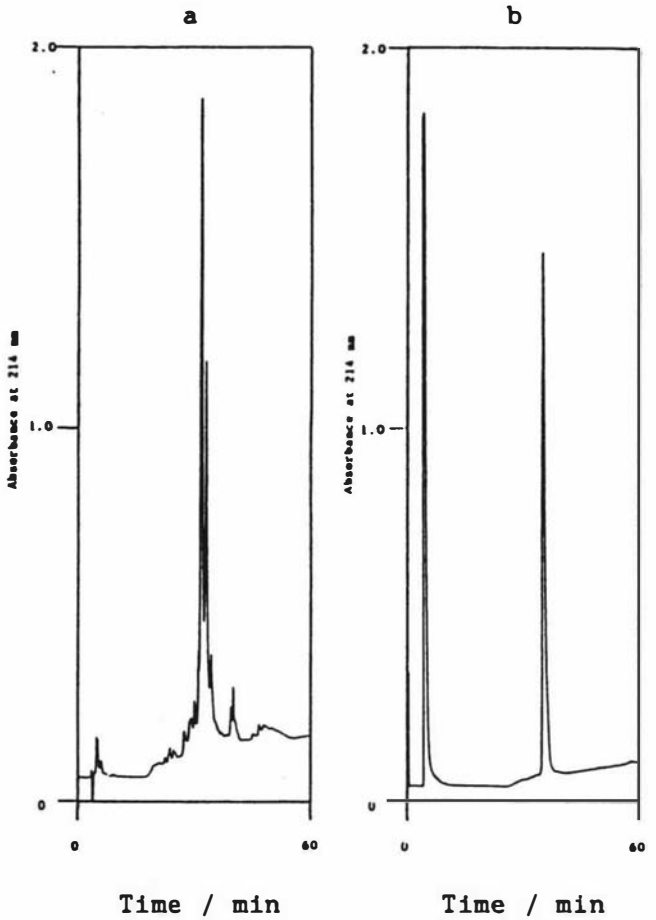


5.3.3.10 KMP5 (Cys-tBu)

Tyr-Thr-Arg-Asp-Leu-Val-Tyr-Lys-Asn-Pro-Ala-Arg-Pro-Asp-Ile-Gln-Lys-Thr-Cys(S-tBu)-Thr-Phe-NH_2

This peptide was also originally required with the cysteine protected by the t-butyl group. The only difference between KMP5 and KMP4 was that KMP5 contained [Asp^{14}] instead of the [Lys^{14}] of KMP4. HPLC analysis of the crude peptide (Figure 5.14) again revealed two significant peaks. Because of the sequence similarity of the two peptides it was likely that the later eluting peak was again an arginine deletion peptide. The composition of the minor peak was not determined. HPLC traces of the crude and purified peptide are given in Figure 5.14. Amino acid analysis of the purified peptide gave the expected ratios: Asp 3.01 [3], Thr 2.71 [3], Glu 0.95 [1], Pro 1.94 [2], Ala 1.00 [1], Cys 0.86 [1], Val 0.78 [1], Ile 0.90 [1], Leu 0.98 [1], Tyr 1.82 [2], Phe 1.01 [1], Lys 2.00 [2], Arg 1.90 [2]. The yield of this peptide was satisfactory, 25%, especially when compared to the poorer yield of 9% obtained for the almost identical peptide KMP4 (Cys tBu) (section 5.3.3.9).

Figure 5.14 HPLC traces of a) crude, b) purified, KMP5 (Cys-tBu)



5.3.3.11 KMP4

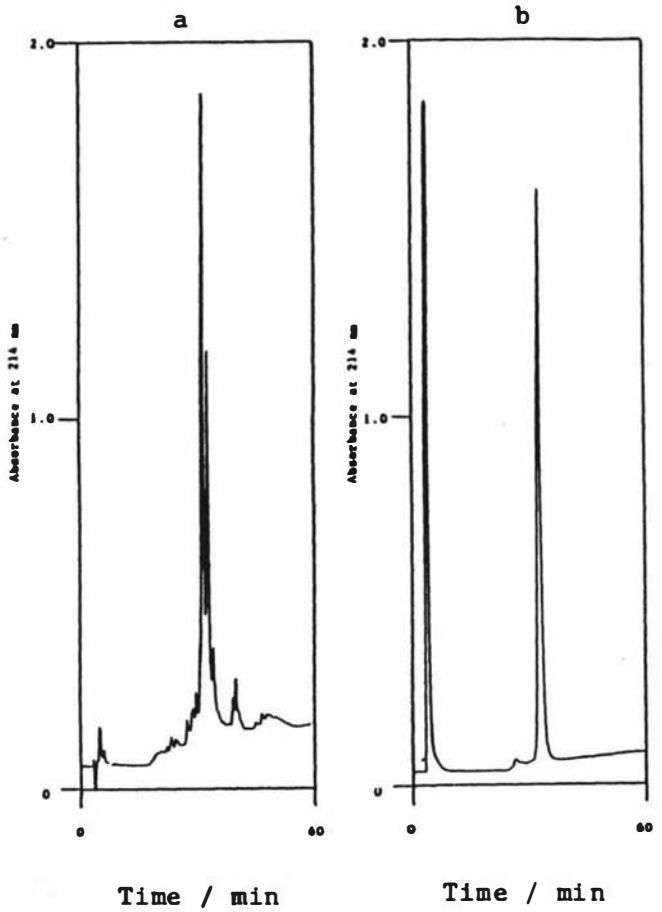
Tyr-Thr-Arg-Asp-Leu-Val-Tyr-Lys-Asn-Pro-Ala-Arg-Pro-Lys-Ile-Gln-Lys-Thr-Cys-Thr-Phe-NH_2

The t-butyl protection was cleaved from the cysteine side chain thiol of crude KMP4 (Cys-tBu) to give KMP4. The yield of the Cys deprotected peptide was low, only 4%. However, considering the number of manipulations required to remove the t-butyl protection from the cysteine, and also that the procedure was not optimised, it was not unexpected. It would probably be better to synthesise the peptide using acid labile Fmoc-Cys(S-Trt) if the peptide with the free thiol were required in future. HPLC traces of the crude and purified peptide are given in Figure 5.15. Amino acid analysis* of the purified peptide gave the expected ratios: Asp 1.93 [2], Thr 2.80 [3], Glu 1.07 [1], Pro 1.90 [2], Ala 1.00 [1], Cys 1.13**[1], Val 0.99 [1], Ile 1.00 [1], Leu 0.98 [1], Tyr 1.81 [2], Phe 1.18 [1], Lys 3.01 [3], Arg 1.68 [2].

*analysed by Dr. Paul Davis, Malaghan Institute, Wellington, NZ

** determined as cysteic acid

Figure 5.15 HPLC traces of a) crude, b) purified, KMP4



5.3.4 Synthesis of peptides on Perloza using Fmoc methodology: ABI 430A automated batchwise peptide synthesiser

Three peptides were synthesised using the ABI 430A automated peptide synthesiser. Details of the syntheses are given below in Tables 5.6 and 5.7.

Table 5.6 Synthesis conditions for peptides made using Perloza and Fmoc chemistry, ABI 430A automated batchwise peptide synthesiser

Peptide	Sequence	Synthesis scale(mmole)	Excess of activated aa	Coupling time per Fmoc-aa
1 Angio I	DRVYIHPFHL	0.25	4X	2 hr
2 WJP1	NIRRLMDGEE PL	0.25	4X	2 hr
3 KMP5	YTRDLVYKNA PARPDIQKTC TF-NH_2	0.20	5X	30 min

Table 5.7 Cleavage conditions and yields for peptides made using Perloza and Fmoc chemistry, ABI 430A automated batchwise peptide synthesiser

Peptide	Purified yield	Cleavage time (hours)	Workup method (Section 5.2.8)	Cleavage yield
1 Angio I	30%	21 hr	1	94%
2 WJP1	26%	20 hr	1	83%
3 KMP5	16%	8 hr	2	84%

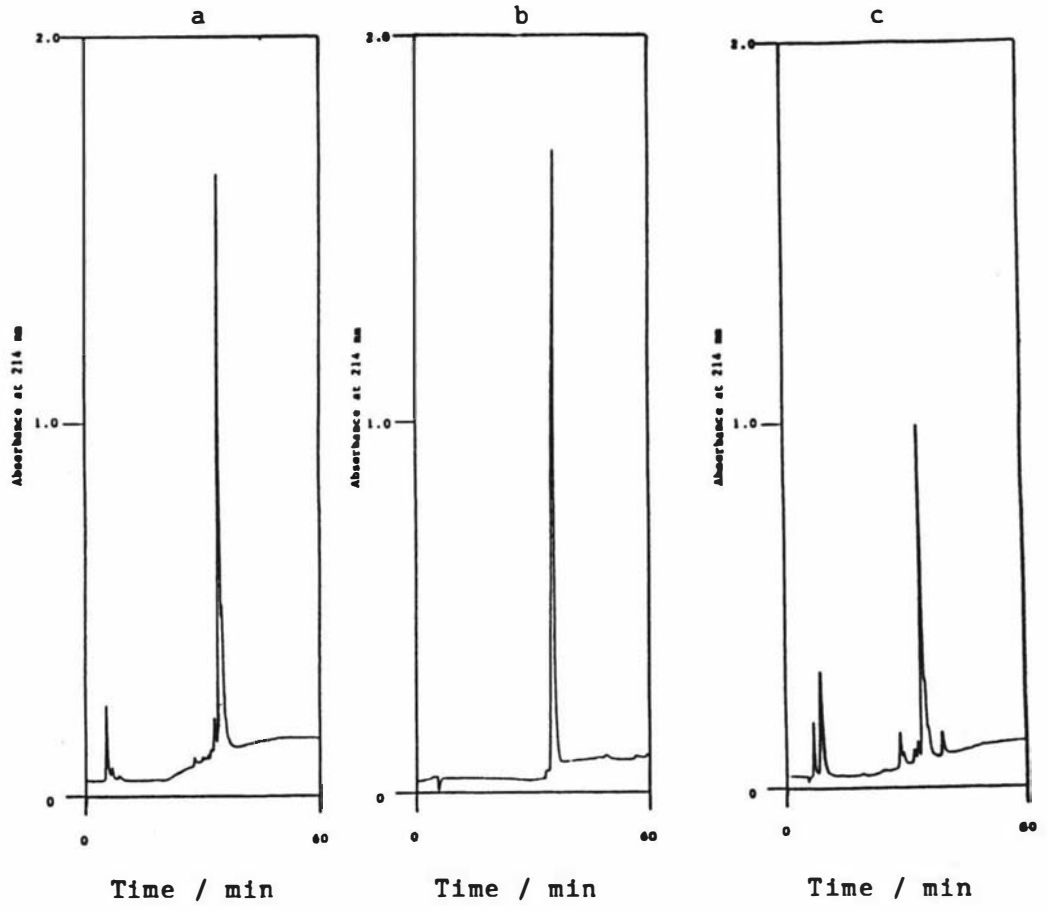
All peptides were cleaved with Reagent B.

5.3.4.1 Angiotensin I

Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu

No problems were encountered during the synthesis of this peptide. HPLC traces of the crude and purified peptide are given in Figure 5.16. The peptide coeluted on HPLC with an authentic sample. A trace of the crude peptide resulting from the synthesis using the Biolynx continuous flow peptide synthesiser (Section 5.3.3.4) is also given in Figure 5.16, for comparison. The HPLC traces of the crude peptides made using the different synthesisers were similar, and indicated, in this case, that the peptide quality did not depend much on the synthesiser used. The purified peptide was obtained in 30% yield, which compares favourably with the 37% yield obtained for the continuous flow synthesis. The difference in yields was probably due more to variation in the individual preparative HPLC runs than to differences in the quality of the crude peptide. Amino acid analysis of the purified peptide gave the expected ratios: Asp 1.13 [1], Pro 1.05 [1], Val 1.00 [1], Ile 0.81 [1], Leu 1.13 [1], Tyr 0.98 [1], Phe 1.09 [1], His 1.94 [2], Arg 1.07 [1].

Figure 5.16 HPLC traces of a) crude (ABI), b) purified (ABI), c) crude (Biolynx), Angiotensin I



5.3.4.2 WJP1

Asn-Ile-Arg-Arg-Leu-Met-Asp-Gly-Glu-Glu

No problems were encountered during the synthesis of this peptide. HPLC traces of the crude and purified peptide are given in Figure 5.17. The purified peptide trace shows a minor side peak which eluted a little later than the main peak. It was difficult to separate the two peaks using preparative HPLC. Amino acid analysis of the purified peptide gave the expected ratios: Asp 2.00 [2], Glu 2.10 [2], Pro 1.06 [1], Gly 2.00 [2], Met 1.05 [1], Ile 0.85 [1], Leu 2.01 [2], Arg 1.66 [2].

Figure 5.17 HPLC traces of a) crude, b) purified, WJP1

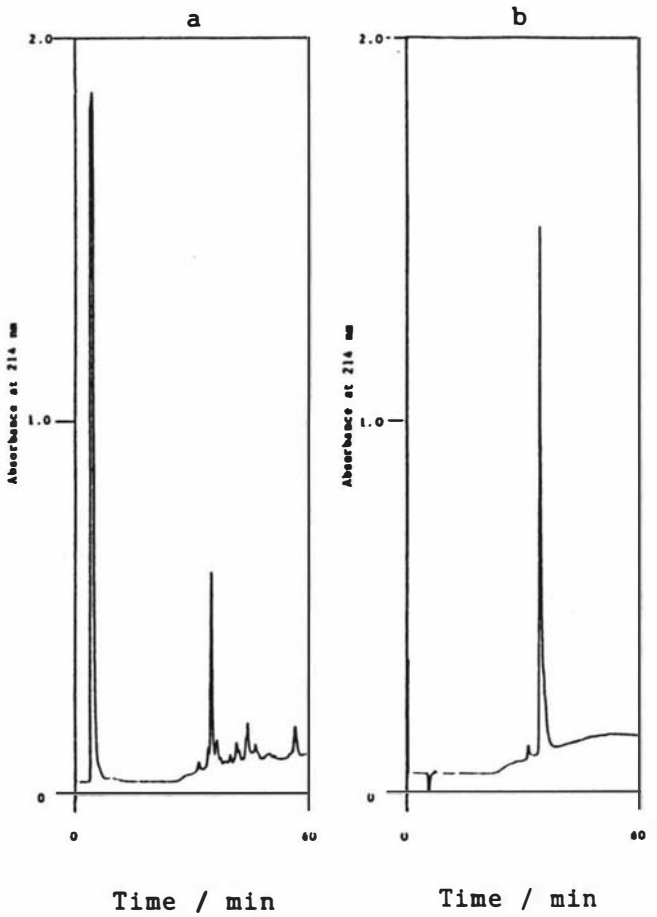
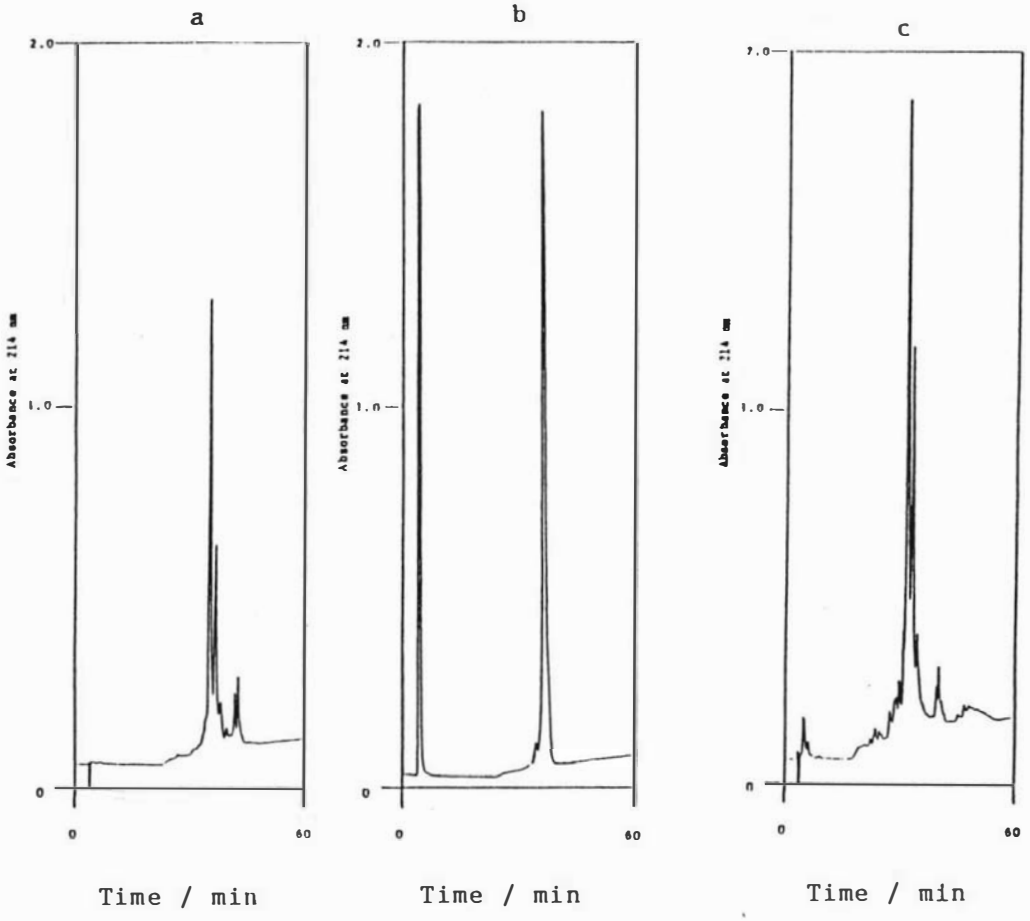


Figure 5.18 HPLC traces of a) crude (ABI), b) purified (ABI), c) crude (Biolynx), KMP5



5.3.4.3 KMP5

Tyr-Thr-Arg-Asp-Leu-Val-Tyr-Lys-Asn-Pro-Ala-Arg-Pro-Asp-Ile-Gln-Lys-Thr-Cys-Thr-Phe-NH_2

HPLC traces of the crude and purified peptide are given in Figure 5.18. The crude peptide showed two significant peaks. A trace of the same crude peptide resulting from the synthesis using the Biolynx continuous flow peptide synthesiser (Section 5.3.3.10) is also given in Figure 5.18, for comparison. The peptide made using the Biolynx also showed two significant peaks on HPLC. The crude peptide made using the Biolynx appeared to be of similar quality to that made using the ABI 430A. The S-tBu group was cleaved from the cysteine using the procedure given above. Amino acid analysis of the purified peptide gave the expected ratios: Asp 3.04 [3], Thr 2.46 [3], Glu 1.08 [1], Pro 1.87 [2], Ala 1.00 [1], Cys 0.54 [1], Val 1.13 [1], Ile 1.23 [1], Leu 1.11 [1], Tyr 2.06 [2], Phe 1.30 [1], Lys 2.28 [2], Arg 2.07 [2]. The yield of the purified peptide was 16%, which was considered reasonable taking into account all of the manipulations and losses which accompanied cleavage of the t-butyl protection from the cysteine thiol side chain. The yield may be compared to that of KMP4, which was 4%. The two peptides differ only by one amino acid (KMP4 [Lys^{14}], KMP5 [Asp^{14}]) and it was expected that each would be obtained in similar yield. The results of the syntheses showed that this not the case.

5.3.5 Peptide synthesis on Perloza using Fmoc methodology: a summary

No major problems were encountered during any of the peptide syntheses using the Fmoc methodology with Perloza. However, a number of workup procedures had to be developed for use with Perloza. The standard methods given in the literature (Van Wandelen et al, 1989; Applied Biosystems Inc., 1990a) assume that the cleaved peptide can be separated from the support by filtration. This was not usually possible with Perloza because the resin formed a highly swollen gel in TFA, which was very slow to filter. The most effective workup procedure in this study appeared to be to slowly pour the cleavage mixture into 50 ml of vigorously stirred water. Using this procedure, the aqueous solution was then easily and quickly filtered from the support. The aqueous solution containing the crude peptide was extracted with ether to remove thiol and phenol scavengers, then lyophilised. If short hydrophobic peptide cleavages are worked up using this procedure, care must be taken to ensure that the peptide is present in the aqueous layer rather than in the ether layer.

One other difficulty which arose was in cleaving the t-butyl protecting group from the thiol side chain of cysteine. The number of manipulations required during Cys (S-t-butyl) cleavage resulted in relatively low yields for those peptides (KSP2, KMP4, KMP5). Higher yields of purified cysteine-containing peptides would probably be obtained if the cysteine thiols were to be protected by the acid labile Trt group. Use of the Fmoc-Cys(S-Trt) derivative would mean the mercuric acetate S-t-butyl removal would not have to be undertaken, with its accompanying losses of material.

The crude HPLC traces of most of the arginine containing peptides showed the presence of later eluting contaminant peaks. In one case (KMP4 (Cys S-tBu), (Section 5.3.3.9), the later eluting peak was identified as an Arg deletion peptide. Some of the other arginine-containing peptides also showed a main product peak with minor later eluting peaks (for example see Figure 5.11 (LHRH)), which may have also been arginine deletion peptides. The results, although mainly circumstantial, suggest that work must be carried out to improve Fmoc-Arg(Mtr) coupling yields when using Perloza for SPPS. The poor coupling of arginine is not a

problem that is confined solely to Perloza, as this amino acid is known to give poor coupling yields with other supports (Atherton et al, 1983b; see also Harrison et al, 1988).

The conditions required for peptide cleavage always resulted in destruction or serious degradation of the non cross-linked Perloza resin. This was not unexpected, nor of concern, in this study. However, it would be of concern if peptides synthesised directly on aminopropyl Perloza for use as affinity matrices had amino acids with protected side chains. It would not be possible to deprotect the side chains, using the standard deprotection reagents used in this study, without also compromising or destroying the useful properties of the matrix. Although it may be possible to increase the acid stability of Perloza by cross-linking with epichlorohydrin, this was not investigated in this study. The results of an investigation into the synthesis of peptide-ligands directly onto aminopropyl Perloza, for use in affinity chromatographic processes, are reported in Chapter 6. It was found in that study that it was possible to cleave amino acid side chain protecting groups from peptides bound to Perloza, without degrading the properties of the support. Therefore, cross-linked Perloza was not seen as being essential for the affinity chromatographic applications studied in Chapter 6.

5.4 CONCLUSIONS

1 HMPA-linked Fmoc-amino acids could be coupled to aminopropyl Perloza, via their Fmoc-amino acyl-4-oxymethylphenoxyacetic acid 2,4-dichlorophenyl esters, to give useful amino acid substitution levels for all 20 amino acids.

2 Perloza can be used as a support for synthesising peptides, up to at least 21 amino acid residues in length, in acceptable purity and yield using Fmoc chemistry. Standard cleavage reagents could be employed for peptide cleavage, but standard workup procedures to separate the resin from the peptide had to be modified to allow for the highly swollen Perloza resin.

3 Both batchwise and continuous flow column peptide synthesisers may be used for SPPS with Perloza as a support using Fmoc chemistry. Little difference was seen in the quality of two peptides made using the two different synthesisers.

CHAPTER 6

USE OF PEPTIDE-PERLOZA FOR AFFINITY CHROMATOGRAPHIC PROCESSES

CHAPTER 6 USE OF PEPTIDE-PERLOZA FOR AFFINITY CHROMATOGRAPHIC PROCESSES

6.1 INTRODUCTION

It was demonstrated in Chapters 3-5 that Perloza was a viable support for solid phase peptide synthesis. The results of the studies reported in Chapters 3-5 were used as a basis for an investigation into the synthesis of peptide-ligands directly onto aminopropyl Perloza. The overall aim of this study was to investigate the synthesis and use of peptide-ligand Perloza resins for affinity chromatographic processes.

Affinity chromatography is a powerful method for purifying proteins. The technique makes use of the unique biological specificity of the protein-ligand interaction. The ligand is covalently bound to an insoluble support, which is packed into a chromatographic column. The solution containing the protein to be purified is passed through the column. In principle only the protein to be purified will have appreciable affinity for the ligand and will therefore bind to it. All other proteins will pass through the column. The specifically bound protein may then be eluted by altering the solution composition to favour dissociation of the protein-ligand complex (Lowe and Dean, 1974). The ligand bound to the support may be a simple organic molecule (for example, 4-phenylbutylamine (to interact with chymotrypsin-like enzymes, Stevenson and Laudman, 1971)), or a more complex biological macromolecule, for example, anti-human IgG antibodies (Phillips et al, 1984).

Some applications of affinity chromatography (from Lowe and Dean, 1974) include:

1) Protein purification	Enzymes
	Antibodies
	Binding proteins
	Complementary proteins
	Repressor proteins

2) Separative procedures Cells and viruses
Denatured and chemically modified proteins from native proteins
Nucleic acids and nucleotides

3) Concentration of dilute protein solutions

4) Storage of otherwise unstable proteins in immobilised form

5) Investigation of kinetic sequences and mechanisms

The affinity chromatographic method offers rapid, essentially one step purification of protein from other contaminants. Yields are often high, possibly because of protection of proteins from denaturation during isolation by stabilisation of the tertiary structure. Although the technique appears straightforward,

> *...successful application of the method depends largely on how closely the experimental conditions chosen permit the ligand interaction characteristic of the components in free solution.* (Lowe and Dean, 1974).

A large number of variables must be taken into account when choosing an affinity method. These include, but are not limited to, the properties of the support, the ligand chosen, the length and type of spacer arm, the nature of the ligand-macromolecule interaction, the affinity of the macromolecule for the ligand, the method of ligand immobilisation to the support, ligand concentration, and method of elution. Detailed discussions of the affinity chromatographic technique are given by Lowe and Dean (1974), Turkova (1978), Lowe (1979), Dean et al (1985), Mohr and Pommerening (1985), and Carlsson et al (1989). Other useful references include Ruoslahti (1976), Epton (1978), Sundaram and Eckstein (1978), and Lidisch et al (1990).

Perloza beaded cellulose possesses many of the properties required of an affinity support in that it:

1) forms a loose porous network which permits unimpaired movement of large macromolecules.

2) has uniform, spherical rigid beads with good flow properties.

3) does not interact with proteins in general so there is minimal non-specific adsorption.

4) has an abundant supply of chemical groups which can be functionalised to allow covalent attachment of a variety of ligands.

5) is mechanically and chemically stable to conditions of coupling, adsorption and elution.

6) can be crosslinked for increased stabilty for large scale processing.

Perloza beaded cellulose has been used successfully as a support for biomolecule separations by affinity chromatographic techniques. Examples of the use of Perloza for affinity chromatographic separations are given by Stamberg, (1988); Chemopetrol, (1988); Gemeiner et al, (1989); and Haggarty et al (1990).

One of the many uses of synthetic peptides is as ligands for affinity chromatographic purposes. Peptides may be bound to activated carbohydrate supports using any of the standard methods for coupling amine-containing ligands. Commonly used methods for activating carbohydrate supports for coupling peptides or proteins include: N-hydroxysuccinimide esters of carboxyl-containing supports (Cuatrecasas and Parikh, 1972), cyanogen bromide activation (Mains and Eipper, 1976), hydroxybenzotriazole (HOBt) esters of carboxyl-containing supports (Pohl et al, 1984), and carbonyldiimidazole (CDI) activation (Kuyas et al, 1990). One of the potential problems with this approach can occur if the peptide contains one or more lysine residues. In this case the lysine N^{ε} amine as well as the N-terminal amine can react with the activated support. This would introduce a distribution of the peptide bound to the support at two different points in the sequence. If the N^{ε} amine of the lysine were essential for binding to the target molecule, only that population of peptide bound at the N terminal amine would be effective as an affinity ligand. This problem may be controllable by maintaining careful control of pH during the coupling reaction, if the peptide to be immobilised contains only one lysine. It would be more

difficult to direct the attachment point of the peptide if it contained more than one lysine. In some cases it may be possible to selectively protect amine groups of the peptide to be coupled to the support (see below: Robinson et al, 1976; Kuyas et al, 1990). Another potential problem with peptides containing more than one amine group is that cross linking of the matrix through the two amine groups could occur.

The commonly encountered methods for coupling peptides to supports rely on reaction of peptide amine groups with the activated support. Very few methods for coupling peptide-ligands to supports, such that the peptide amino-terminus is directed into the aqueous phase, appear to have been reported.

Robinson et al (1976) synthesised [8-Lysine] vasopressin (sequence: Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Lys-Gly-NH_2) with the N-terminal amino group blocked by acetone to form a 2,2, dimethyl-4-imidazolidinone compound. They coupled the Nα-amino protected peptide, via the lysine N^{ε}-amine group, to cyanogen bromide activated Sepharose 4B. Yamashiro et al (1967) synthesised the same peptide derivative, and found that, to regenerate [Lysine-8] vasopressin, heating at 90°C in 0.25% acetic acid for 30 minutes was required. Robinson et al (1976) could not deprotect the support-bound [Lysine-8] vasopressin, using the conditions reported by Yamashiro et al, without risk of damaging the agarose support. Therefore, they washed the peptide extensively with dilute acid (3 days, 0.5M acetic acid) to liberate the N-terminal amino group. Robinson et al noted that

> *...the capacity of the agarose-LVP derivative increased somewhat at first, suggesting that complete removal of acetone had not been achieved in the initial washing step.*

which indicated that care had to be taken to ensure complete removal of the Nα-amino protection when using this approach. The [Lysine-8] vasopressin-Sepharose 4B was used for affinity purification of neurophysins.

Kuyas et al (1990) synthesised Boc-Gly-Pro-Arg-Pro-Lys-OMe using classical solution peptide synthesis techniques. The peptide was then coupled to a CDI activated resin support (Fractogel TSK AF-CDI 650, Merck) via the N^{ε}-amino group of the lysine. The Boc group was cleaved for one hour with neat TFA, resulting in direction of the peptide amino-terminus into the aqueous phase. The peptide-matrix was used for the affinity purification of human fibrinogen and its derivatives. As well as achieving directed orientation of the peptide amino-terminus into the aqueous phase, the method used by Kuyas et al to couple a protected peptide to a support was a means of overcoming the problem of coupling peptides with more than one amine group, as discussed above. This procedure for immobilising peptides in order to direct the N-terminal amino group into the aqueous phase is practical if the matrix is not degraded by neat TFA. However, not all matrices can withstand neat TFA. It was demonstrated in Chapter 4, for example, that the flow properties of Perloza are seriously degraded after a one and a half hour exposure to 95% TFA. In addition, Kuyas et al used classical solution techniques to synthesise the protected peptide. Such a synthesis of a selectively protected peptide may be difficult using the more commonly employed SPPS methodology.

Fassina et al (1992) developed a synthetic procedure for immobilisation on preactivated affinity supports of peptide-ligands requiring free α-amino groups to recognise their targets properly. The peptide-ligand was assembled by SPPS on an octa-branched heptalysine core through a polyglycine spacer. The peptide was coupled to a preactivated support. Following immobilisation, only a limited number of peptide chains were covalently linked to the solid phase, leaving the remainder facing the aqueous phase. The procedure was applied to the design, synthesis, and oriented immobilisation of a multimeric peptide-ligand (Met-Tyr-Phe) for affinity purification of bovine neurophysin.

The established methods of coupling peptide-ligands to affinity matrices in order to direct the peptide carboxyl-terminus into the aqueous phase are by and large satisfactory. However, only relatively few studies have been reported (see above) in which synthetic peptides were coupled to supports in such a way that the amino-terminus was oriented into the aqueous phase. One means of directing the amino-terminus of a peptide-

ligand into the aqueous phase would be to synthesise the peptide directly onto an amine-functionalised affinity chromatographic support using established SPPS methodology. In addition to achieving the desired orientation into the aqueous phase of the amino-terminus of the peptide-ligand, a number of other advantages were envisaged if a peptide-ligand were to be synthesised directly onto a chromatographic support:

i) the peptide would be attached at a known point.
ii) high peptide-ligand substitutions would be possible, if required.
iii) cross linking would not be possible.

However, a number of disadvantages to the strategy of synthesising a peptide-ligand directly onto an affinity matrix may be envisaged. One of the features of currently used methods for immobilising peptides is that the peptide can be purified to homogeneity and characterised before it is coupled to the matrix. However, if a peptide is synthesised directly onto a support via a non-cleavable covalent linkage, it is impossible to directly estimate the amount of target peptide-ligand present. This is because it would be expected that deletion and termination peptides would also be present, as well as the target peptide-ligand. An indirect method of assessing the integrity of a support-bound peptide is amino acid analysis, where the amino acid ratios can be compared with the theoretical ratios for the target sequence. However, some amino acids are partially (for example Cys, Ser, Thr, Tyr) or completely (Trp) destroyed during acid hydrolysis. It would not always be possible to tell whether a low amino acid analysis result was due to destruction of an amino acid during hydrolysis, or due to a poor amino acid coupling reaction during the synthesis of the peptide onto the support. Consequently, some uncertainty could exist as to the integrity of a matrix-bound peptide when using amino acid analysis for the assessment. An investigation of the applicability to Perloza of different methods of hydrolysis for amino acid analysis, for example basic hydrolysis to preserve tryptophan, was not made.

An alternative method of estimating the integrity of a matrix-bound peptide would be to simultaneously synthesise the peptide on the same matrix but with the C-terminal amino acid anchored via a cleavable

linker. If it were valid to assume that the amino acid coupling yields would be similar for both matrices, then cleavage and characterisation of the cleavable peptide would give an indication of the relative amount of target peptide-ligand synthesised on the non-cleavable matrix. This approach was used to assess the integrity of one of the peptide-ligand-supports synthesised in this study.

Possibly the best method for verifying peptide integrity would be to synthesise the peptide on a matrix bearing a cleavable linker between it and the first amino acid. This strategy would require that the peptide-linkage not be cleaved during removal of side chain protecting groups. A small sample of the peptide-matrix could be cleaved and the product characterised to determine the amount of target peptide present. Desirable characteristics of a potential peptide-linker for such an application are that it:

i) be chemically stable to any of the buffers used in the affinity purifications.
ii) give minimal non-specific binding of proteins.
iii) not require a cleavage method that would give rise to contaminating side products, that is, products that would give a false impression of peptide integrity.

A linker which could possibly fulfil these requirements might be one of the photolabile linkers (Barany et al, 1987). However, this was not investigated during this study.

One of the reasons for wanting to ensure that peptide-ligand integrity is maintained is that deletion and termination peptides would be formed during a peptide synthesis on the matrix. The formation of incomplete sequences would mean there would be less of the target peptide-ligand on the support; that is, the yield of target peptide would be lower than expected. Whether the presence of incomplete peptides would affect an affinity separation is another question. Incomplete peptides could bind non-target proteins, or they could bind the target protein, or they could be totally inert. If non-target proteins were bound by the incomplete peptides, could not be removed by a washing step, and were eluted along with the target protein, then the presence of incomplete peptides could not be tolerated.

Some control of the integrity of a peptide-ligand synthesised onto a support would be possible during the synthesis. The amount of deletion peptide could be kept low if coupling reactions were close to quantitative. Poor coupling reactions could be identified using the ninhydrin assay, and the amino acid in question could be recoupled before proceeding on to the next amino acid in the peptide sequence.

Solid phase peptide synthesis is almost always undertaken from the C to the N terminus of the peptide. Although it is possible to synthesise peptides from the N to the C terminus, two problems were inherent in the methodology as originally applied: racemisation, and excessive loss of peptide chains because of side reactions (Erickson and Merrifield, 1976). More recently, Patel et al (1990) have used an N to C terminal peptide synthesis strategy to synthesise a number of tripeptides directly onto an agarose gel bearing a spacer with a carboxyl terminus. The C-terminal amino acid in their study was arginal, and the N to C terminal peptide synthesis was necessary in order to result in direction of the aldehyde moeity into the aqueous phase. The peptide-agarose gel matrices were used to investigate the affinity purification of tissue plasminogen activator (tPA). Synthesis of peptides onto the matrix from the N to C terminus results in the peptide carboxyl terminus being presented to the aqueous phase, whereas the usual solid phase peptide synthesis methodology, as envisaged for use in this study, would result in presentation of the peptide amino terminus to the aqueous phase.

In addition to the work of Patel et al (1990) discussed above, the use of peptides synthesised directly onto supports for affinity type applications has been reported by a number of other groups. In these examples the peptides were synthesised onto the supports using the standard C to N terminal synthesis strategy, in contrast to the N to C terminal synthesis strategy used by Patel et al.

Smith et al (1977) synthesised Trp-Ala-Lys-Val-Glu-Ala-Asp-Val-Ala-Gly-Gly, a known antigenic determinant of sperm whale myoglobin, directly onto a benzhydrylamine functionalised high porosity polystyrene-divinylbenzene polymer. The Boc methodology was used, and side chain protecting groups were cleaved using 2M HBr in glacial acetic acid, at room temperature, for one hour. The polystyrene matrix was stable to

the side chain cleavage reagent. The peptide-matrix conjugate was shown by radioimmunoassay (RIA) to be able to adsorb antibodies specific to the peptide.

Eisele et al (1984) synthesised the ligand testosterone 17β-hemisuccinyl -tetra-glycine directly onto aminoethyl cellulose. The tetra-glycine spacer arm was synthesised by repetitively coupling Boc-glycine, using 1M HCl in glacial acetic acid for Boc cleavage. Testosterone 17β-hemi-succinate was then coupled to the Nα-amino group of the support-bound N-terminal glycine. The affinity resin was tested and found to be able to bind androgen receptors. In this case the tetra-glycine functioned as a spacer arm rather than as a ligand, but the example still serves to demonstrate the feasibility of synthesising a peptide directly onto a support for affinity chromatographic applications.

Geysen et al (1984) synthesised a series of hexapeptides, plus a dipeptide spacer, onto polyethylene pins using Boc methodology. Side chain protecting groups were cleaved using boron tris trifluoroacetate in TFA. The peptides were overlapping sequences from an immunogenic region of the coat protein of foot and mouth disease virus (type O_1). The peptide-pins were successfully used as the solid phase in an enzyme linked immunosorbent assay (ELISA) to determine the immunogenic epitope. However, the pins would not be very suitable for preparative scale affinity purifications.

Frank et al (1991) used cellulose paper as a support to synthesise a series of decapeptides to map the epitope of an immunogenic region of the human cytomegalovirus (CMV). Peptides were synthesised onto a diglycine spacer linked directly to the support via an acid stable ester bond. The Fmoc chemistry was used for these syntheses. Amino acid side chain protecting groups were cleaved using 20% TFA, 1% triisobutylsilane in DCM for 20 minutes. The support-linked peptides were successfully used as the solid phase for an ELISA to determine the immunogenic epitope. This methodology does not appear to have been applied for affinity purification processes.

It was shown in all of these examples that it was possible for biological macromolecules to bind to peptides which were synthesised

directly onto a support. However, most of these examples were used for small scale assay-type applications, as opposed to larger scale affinity purification processes. The work described by Eisele et al appeared to be the exception, in that they intended to use their matrix for isolation of androgen receptors. However, in their case the peptide they synthesised onto the support (tetraglycine) was not a ligand, but rather a spacer arm. With this limited number of examples as precedents, it was decided to attempt to synthesise peptide-ligands directly onto aminopropyl Perloza, and to test the peptide-Perloza conjugates as supports for affinity chromatographic processes.

The results reported in Chapter 3 indicated that it was possible to use Boc chemistry for the synthesis of short peptides on Perloza if protic deprotection reagents were avoided. In that study a dioxane solution of the aprotic Lewis acid, boron trifluoride, was used to cleave the Boc Nα-amino protecting group. Amino acid side chain protecting groups used in Boc chemistry are usually cleaved with strong anhydrous acid, for example liquid hydrogen fluoride. It was found during this study (data not shown) that liquid HF dissolves Perloza. Boc chemistry would therefore be feasible for synthesis of resin-bound peptide-ligands either if none of the amino acids was trifunctional, or if side chain protecting groups could be used which were labile to mild cleavage conditions. For example, it should be possible to protect the N^{ε} amino group of lysine using the acid stable Fmoc group. Boc-Lys (N^{ε} Fmoc) could be coupled to the matrix bound peptide, and the Fmoc side chain protection cleaved, using piperidine, at the end of the synthesis. However, no syntheses of this type have been reported.

Successful use of the Fmoc method for SPPS of peptides on Perloza was demonstrated in Chapters 4 and 5. The side chain protection of trifunctional amino acids used for the Fmoc method are labile to milder acid (for example 95% TFA) than those used for the Boc method. However, it was shown in Chapters 4 and 5 that concentrated TFA solutions degrade Perloza. It would be necessary to develop a reagent milder than concentrated TFA in order to cleave the side chain protecting groups from a peptide-resin synthesised by the Fmoc method. The reagent would have to be able to cleave all of the different side chain protecting groups used in the Fmoc method for SPPS. Currently, the most difficult

to cleave side chain protecting group used for Fmoc SPPS is the arginine N^G-Mtr group. The cleavage reagent would have to be able to cleave this group while preserving the flow properties of the peptide-support. In addition, the reagent should not cleave the peptide-ligand from the support. It was demonstrated in Chapter 3, for example, that treatment of Boc-valine-amidopropyl Perloza with 10% sulphuric acid in dioxane resulted in loss of valine from the support as determined by picrate titration.

In addition to direct C to N terminal synthesis of a peptide-ligand onto aminopropyl Perloza, an unequivocal approach to coupling a peptide-ligand to a solid support, via a stable thioether bond, was conceived. In this strategy, the N or C-terminus of a peptide could be directed into the aqueous phase to take part in binding of the target protein.

The peptide immobilisation strategy involved synthesis of a peptide bearing a free thiol group, by including cysteine in the peptide sequence. The cysteine could be coupled at the peptide C- or N-terminus, with the cysteine located at the opposite end to the portion of the peptide required for binding the target protein. If both the C and N terminus of the peptide were required for binding the target protein, then it may be possible to locate the cysteine in the middle of the peptide sequence. Once the peptide had been synthesised and purified, it would be coupled to α-bromoacetamido Perloza (Chapter 3), at pH 8-9, to anchor the peptide to the support via a stable thioether bond. At neutral to slightly alkaline pH, reaction of bromo-resin is primarily with thiol groups. At higher pH values, reaction with amine groups is preferred (Lowe and Dean, 1974). This peptide immobilisation strategy was inspired by the work of Robey and Fields (1989) and Kolodny and Robey (1990). Kolodny and Robey conjugated peptides containing a bromoacetyl moiety (made using the Boc methodology), to the free thiols of reduced bovine serum albumin (BSA), in 0.1M $NaHCO_3$ solution, to yield peptide-protein conjugates with the peptides linked to the carrier protein via stable thioether bonds. The envisaged use of the peptide-protein conjugates was for immunisation of animals in order to generate antibodies to the bound peptide. In this study, the approach of Kolodny and Robey was reversed by locating a thiol group on the peptide, and reacting the peptide with a bromine-containing solid support in order to anchor the peptide-ligand to the support via a stable thioether bond.

Halide substituted supports have generally been used to couple ligands via amine or tyrosine hydroxyl groups (Cuatrecasas, 1971; Lowe and Dean, 1974), although reaction of a thiol-containing ligand with bromoacetamidoalkyl agarose has been noted (Lowe, 1979).

The thioether bond generated by the coupling reaction is very stable. Acid hydrolysis of peptides, coupled via reaction of a cysteine thiol with an α-bromoacetamido moiety, generates stable S-carboxymethyl cysteine, which may be quantitated by amino acid analysis (Kolodny and Robey, 1990). Therefore, hydrolysis of a peptide-resin (where the peptide is bound to the resin via a thioether linkage) followed by amino acid analysis for S-carboxymethyl cysteine could be used as a method to determine the substitution of the peptide on the support. The amino acid analysis results of the other peptide amino acids could, of course, also be used to assess the substitution level of the peptide-ligand.

The thioether method of attachment of a peptide to a support could have several advantages. The peptide would be purified and fully characterised (HPLC, amino acid analysis; sequencing if required) before it was coupled to the support. High coupling yields were envisaged based on the yields obtained by Kolodny and Robey (1990) in coupling bromoacetyl peptides to the thiols of reduced bovine serum albumin. The point of attachment of the peptide to the support would be unambiguous. That is, at the slightly alkaline pH used, coupling would be expected to be almost totally via the cysteine sulphydryl. The cysteine could be located anywhere in the peptide, which would enable direction of any desired region of the peptide into the aqueous phase. Leakage of the peptide-ligand should not occur because of the chemical stability of the thioether bond. In principle, if a peptide-ligand had to contain a free cysteine thiol for binding the target protein, a differential thiol-protection strategy could be employed to accomplish this. For example, using Fmoc chemistry, all thiols except the anchoring thiol could be protected by the S-acetamidomethyl (Acm) group, which is not cleaved during TFA-mediated peptide cleavage. The peptide could then be coupled to α-bromoacetamido Perloza via the free thiol, and the S-Acm protecting groups cleaved using the procedure of Van Wandelen et al (1989).

A possible disadvantage of this strategy may be encountered in the instance where a cysteine had to be located in the middle of a peptide in order to direct both the N and C termini of the peptide into the aqueous phase. Location of a cysteine in the middle of the peptide may alter the peptide conformation, with the result of lower or no binding affinity of the target protein for the altered peptide conformation. However, at present no better method of directing the orientation of both the C- and N-termini of a support-bound peptide-ligand appears to have been proposed.

Two model proteins, the proteolytic enzyme chymosin, and antibodies to luteinising hormone-releasing hormone (LHRH), were used to test whether peptide-ligand-Perloza resins, with the peptide-ligands synthesised directly onto aminopropyl Perloza, would be useful as matrices for affinity purification of biomolecules.

Bovine chymosin (rennin) is an aspartic protease of molecular weight 30700 (Raymond and Bricas, 1979) from the fourth stomach of suckling calves (Foltmann, 1981). Chymosin preferentially cleaves *K*-casein at a single Phe-Met bond with little non-specific proteolysis, inducing instability in milk micelles and leading to clotting. This makes chymosin a commercially valuable enzyme in the production of cheese (Strop et al, 1990). Chymosin is now produced from recombinant DNA sources as well as being isolated from a natural source (calf stomachs).

The peptide Val-dLeu-Pro-Phe-Phe-Val-dLeu is a specific inhibitor of aspartate proteinases of the pepsin type, of which chymosin is an example (Pohl et al, 1984; Strop et al, 1990). Strop et al (1990) coupled this peptide to the hydroxybenzotriazole-activated carboxyl group of 6-aminohexanoic acid functionalised Sepharose 4B, to give an affinity matrix for purification of Val^{111}-Phe site mutated calf chymosin expressed in E. Coli. Strop et al optimised the method of Pohl et al (1984) to purify the recombinant chymosin.

Because the inhibitor peptide used by Strop et al for chymosin purification did not contain any side chain functional groups, it was decided to attempt to synthesise Val-dLeu-Pro-Phe-Phe-Val-dLeu onto aminopropyl Perloza using the Boc methodology. The SPPS protocols

reported in Chapter 3 were used for the synthesis, except that the Boc-amino acids were activated as their symmetrical anhydrides rather than as their HOBt esters.

Luteinising hormone-releasing hormone (LHRH) (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2) is a decapeptide responsible for stimulating the secretion of luteinising hormone (LH) and follicle stimulating hormone (FSH) (Matsuo et al, 1971; Schally et al, 1971). Polyclonal antibodies to LHRH are usually generated by conjugating the peptide to a carrier molecule such as bovine serum albumin, and then using the peptide-protein conjugate to immunise animals (for example, see Pique et al, 1978). Serum from the immunised animals, containing the antibodies, may then be subjected to established procedures in order to concentrate the antibody. For example, Fraser et al (1986) used sodium sulphate precipitation to isolate the gamma globulin fraction from serum containing antibodies to LHRH. The antibodies contained in the gamma globulin fraction were used to study the effect of immunoneutralisation on follicular development, the LH surge and luteal function in the stumptailed macaque monkey (*Macaca arctoides)*. Affinity purification of antibodies to LHRH using the peptide as an affinity ligand does not appear to have been reported.

Two approaches to purification of antibodies to LHRH using a peptide-resin conjugate as an affinity matrix were considered. The first was to synthesise LHRH directly onto aminopropyl Perloza using Fmoc chemistry, and to develop a mild reagent to cleave side chain protecting groups. The second approach was to synthesise an analogue of LHRH (Ac-Cys-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2) using standard Fmoc solid phase peptide synthesis techniques. The peptide was then coupled to α-bromoacetamido Perloza, via the thiol of the cysteine, to anchor the peptide to the support via a thioether bond. The ability of both peptide-resin conjugates to bind antibodies to LHRH was investigated.

6.2 MATERIALS AND METHODS

6.2.1 Chemicals and equipment

Recombinant chymosin solution was a gift from Genencor, S.F., USA. Commercial food grade chymosin was from NZ Co-op Rennet Co. Ltd., Eltham, NZ. Ovine antiserum to LHRH was a gift of Dr. K.P. McNatty, MAF, Wallaceville, NZ. Lamb serum was a gift of Life Technologies Ltd., NZ. Boc-L-proline was from Bachem (USA). Tri-sodium citrate, citric acid, D-leucine, and di-tert butyl pyrocarbonate were from Sigma (USA). Reagent grade sodium formate, and analytical reagent grade formic acid, were from May and Baker (NZ). Reagent grade tri-n-butyl phosphine, and trimethylsilylbromide (TMSBR), were from Aldrich (USA). Reagent grade ammonium sulphate, ethylenediaminetetraacetic acid (EDTA) sodium azide, and analytical reagent grade sodium chloride, were from Ajax Chemicals, Sydney, Australia. Reagent grade succinic anhydride was from Riedel-de Haen (Germany). Analytical reagent grade sodium bicarbonate was from Merck (Germany). Reagent grade m-cresol was from BDH (NZ) Ltd. Fmoc-Cys(Trt) was from ABI, USA. All other reagents were from sources given in previous chapters.

The absorbance of effluent from chromatographic runs was monitored using a Cecil Instruments CE 212 variable wavelength UV monitor. Absorbance traces were recorded using a Sekonic SS 250F Recorder. Disposable polystyrene columns, 2 ml, were from Pierce Chemical Co., Rockford, Illinois (USA). Dialyses were performed using Serva 36/32 dialysis membrane, MW cutoff 10-15 kDa. A DuPont Instruments Sorvall model RC-5B centrifuge was used to centrifuge serum samples after ammonium sulphate precipitation. An MSE model Microcentaur micro centrifuge was used to centrifuge samples from the peptide cleavage experiments reported in Section 6.2.8.4.

SDS-PAGE gels were run using a Pharmacia PhastSystem. All gels were run using the instructions given in the Pharmacia PhastSystem instruction manual. **All samples were reduced** using the method given in PhastSystem Separation Technique File No. 110. Gels were run using the method given in PhastSystem Separation Technique File No. 111. Pharmacia Phastgels, 12.5% or 20% homogeneous, were used for running all of the SDS-PAGE gels

reported in this Chapter. Gels were developed using the sensitive silver staining method given in PhastSystem Development Technique File No. 210. Reagents used for the silver staining of the gels were from the following sources. Analytical reagent grade silver nitrate was from May and Baker. Reagent grade glutaric dialdehyde solution, 25% by weight, was from Aldrich. Reagent grade glycerol and 40% formalin solution were from Scientific Supplies Ltd., NZ. Analytical reagent grade sodium carbonate was from Merck. Bromophenol blue solution was from BDH, NZ. Molecular weight standards (carbonic anhydrase (29 kDa), egg albumin (45 kDa), bovine albumin (66 kDa), phosphorylase b (97.4 kDa), β-galactosidase (116 kDa) and myosin (205kDa)) were from Sigma. Bovine albumin, IgG free and containing low endotoxin levels, was from Immunochemical Products (ICP) Ltd., NZ.

6.2.2 Synthesis of Boc-D-leucine

D-leucine (5.07 g, 38.6 mmole) and NaOH (2 g, 50 mmole) were dissolved in 100 ml water. Dioxane, 60 ml, was added, followed by 10.0 g (45.9 mmole) di tert-butyl pyrocarbonate. The solution was stirred for three hours, then another 10 g di-tert butyl pyrocarbonate was added. The solution was stirred for a further 18 hours. Tlc ($CHCl_3$:MeOH 9:1, ninhydrin visualisation) indicated that reaction had gone to completion. Dioxane was removed in vacuo, and the solution was extracted 2X 100 ml ethyl acetate. The solution was acidified with 10% sulphuric acid, and extracted 3X 100 ml ethyl acetate. The ethyl acetate solutions were combined, washed twice with 25 ml water, and twice with 25 ml saturated NaCl solution. The ethyl acetate was removed in vacuo, and the residue was recrystallised from ethyl acetate : n-hexane. The yield of Boc-D-leucine was 8.0 g, 89%. Melting point: 82 - 84°C (lit. 84°C, Pettit, 1970).

6.2.3 Synthesis of Val-dLeu-Pro-Phe-Phe-Val-dLeu-acetamidopropyl Perloza resin for binding chymosin

Water wet aminopropyl Perloza 100 Medium (0.541 g dry, amine substitution 0.37 mmole/g) was transferred to a Biolynx reaction column. The resin was washed with dioxane and then with DMF. The peptide Val-dLeu-Pro-Phe-Phe-Val-dLeu was synthesised on the resin using the

standard Boc chemistry reported in Chapter 3, except that the Boc-amino acids were activated as their symmetrical anhydrides rather than as their HOBt esters. The scale of the synthesis was 0.20 mmole. A 2.5X excess of Boc amino acid anhydride was used for each coupling. Single couplings were performed for one hour, at a flow rate of 5 ml/min.

Boc-amino acid symmetrical anhydrides were preformed in solution. Boc-amino acid, 1 mmole, was dissolved in 4 ml DCM. DCC solution, 0.5M in DCM, 1 ml, was added and the solution was stirred for 30 minutes. A precipitate of dicyclohexylurea (DCU) formed. The DCM was removed in vacuo, the residue was dissolved in 4 ml DMF, and the DCU precipitate was removed by filtration. The Boc-amino acyl anhydride solution was loaded onto the reaction column. N-methyl morpholine (NMM), 100 µl, was added with the activated Boc-amino acid to neutralise the BF_3 salt of the resin bound amine.

A residual amine substitution of 23 µmole/g was found by ninhydrin assay after the first amino acid, dLeu, was coupled to the aminopropyl resin. The remaining amine groups were acetylated for 30 minutes at a flow rate of 5 ml/min using a solution of acetic anhydride 0.5 ml, pyridine 1.0 ml, DMF 3 ml. The resin was washed with DMF after acetylation. All other couplings followed the standard Biolynx protocol reported in Chapter 3. Samples of the peptide-resin were taken for picrate titration and amino acid analysis at completion of the synthesis. A sample was also analysed by the ninhydrin assay to determine the amine substitution.

It was anticipated that harsh conditions for column sterilisation and regeneration, such as 0.1M NaOH solution, would possibly be employed. To determine the stability of the peptide-resin to 0.1M NaOH, it was washed for 30 minutes with 0.1M NaOH, then with water. A sample was taken for amino acid analysis and the result compared to that found prior to NaOH washing. In addition to its anticipated use for column washing, the first NaOH wash may also have served to cleave any amino acids or peptides esterified directly to the carbohydrate backbone (Vlasov et al, 1973).

6.2.4 Preliminary experiments to test for binding of chymosin to Val-dLeu-Pro-Phe-Phe-Val-dLeu-acetamidopropyl Perloza resin

Commercial food grade chymosin was dialysed against 10mM citrate buffer, pH 5.5 at 4°C for 24 hours, and then lyophilised (Burton, 1991).

Buffers used for the affinity chromatography experiments with chymosin were:

Buffer A: 30mM formate, pH 4.0, with 15% dioxane
Buffer B: 30mM formate, pH 4.0, with 13% dioxane
Buffer C: 30mM formate, pH 6.5, with 33% dioxane and 1mM EDTA.

Buffer D: 10mM citrate, pH 4.4
Buffer E: 10mM citrate, pH 6.0

The peptide-resin (Section 6.2.3) was transferred to a 10 X 150 mm glass column and washed with Buffer A. Lyophilised chymosin, 4.4 mg, was dissolved in 10 ml of buffer A and loaded onto the column at a flow rate of about 1 ml/min. The absorbance of the effluent was monitored at 280 nm. The column was washed with Buffer B until the absorbance had returned to the baseline. The column was then washed with Buffer C, which was the buffer used by Strop et al (1990) to elute chymosin. The A_{280} trace showed no increase in absorbance on washing with Buffer C. Fractions were analysed for chymosin using a milk clotting assay (Section 6.2.4.1). Chymosin activity was only found in the breakthrough fraction, none was found in the Buffer C elution fraction.

6.2.4.1 Milk clotting assay for chymosin

Test solution, 1 ml, and pasteurised milk, 4 ml were incubated at 40-45°C. Clotting of the milk within 30 minutes was taken to indicate the presence of chymosin.

6.2.5 Succinylation of the peptide-resin

It was reported in section 6.2.4 that chymosin did not bind to the peptide-resin. In that study the peptide had a free amino terminus

directed into the aqueous phase, whereas the peptide-ligand used by Strop et al (1990) had the carboxyl end of the peptide directed into the aqueous phase. The peptide-resin was reacted with succinic anhydride in order to introduce a carboxyl group at the end of the peptide.

The peptide-resin was washed with dioxane, then with DMF. Succinic anhydride, 0.200 g, 10 eq, was added and reacted with the peptide-resin for 70 hours. After 70 hours a ninhydrin assay of the peptide-resin gave an amine substitution of 27 µmole/g. Therefore 1 ml of pyridine was added as catalyst and the reaction was continued for 18 hours. The resin was washed with DMF. The unreacted amine substitution as determined by ninhydrin assay was 4 µmole/g, which indicated 98.5% reaction. The peptide-resin was transferred to a 10 mm X 150 mm glass column, and washed with dioxane, water, and Buffer A.

6.2.6 Binding of chymosin to the succinylated peptide-resin

Lyophilised food grade chymosin, 3.1 mg, was dissolved in 10 ml Buffer A and loaded onto the column. The peptide-resin was washed with 20 ml of Buffer B, then Buffer C, the elution buffer, was applied. Assay of the fractions for milk clotting activity showed that activity was confined to the elution (buffer C) fraction. The result of this preliminary experiment showed that the succinylated peptide-resin could be used as an affinity matrix for binding and elution of chymosin using the binding and elution conditions reported by Strop et al (1990).

6.2.7 Isolation of chymosin from a solution containing contaminating proteins

6.2.7.1 Separation of chymosin using Buffers A, B, and C

A solution of chymosin derived from a recombinant source (gift of Genencor Inc.) was dialysed (but not lyophilised) as described in section 6.2.4, then stored at 4°C. Ten ml of the solution was diluted with 10 ml water. Formate buffer, 1M, pH 4.0, 1 ml, was added. The pH of the solution was 4.0. The solution was loaded onto the peptide-resin column at a flow rate of about 1 ml/min. The column was washed with Buffer B until the absorbance at 280 nm had returned to the baseline.

The chymosin was eluted with Buffer C. The column was then washed with 0.1M NaOH, followed by Buffer A. The fractions were assayed for chymosin activity using the milk clotting assay. Samples of the fractions were taken for analysis by SDS-PAGE (a Pharmacia PhastGel, 20% homogeneous, was used for the analysis).

6.2.7.2 Separation of chymosin using Buffers D and E

The peptide-resin column was washed from Buffer A to Buffer D. Buffer D, 1 ml, was added to 10 ml of the recombinant chymosin solution. The chymosin was loaded onto the column at a flow rate of about 1 ml/min, and the column was washed with Buffer D until the absorbance at 280 nm had returned to the baseline. The chymosin was eluted with Buffer E. The column was then washed with 0.1M NaOH, followed by Buffer D. The fractions were assayed for chymosin activity using the milk clotting assay. Samples of the fractions were taken for analysis by SDS-PAGE.

6.2.8 Synthesis of pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-Perloza 500 Medium (LHRH-Perloza 500, Resin 1)

The method of synthesising a peptide-ligand directly onto aminopropyl Perloza was applied to the synthesis of a peptide-resin intended to purify antibodies specific to the decapeptide luteinising hormone-releasing hormone (LHRH, sequence: pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2). Sheep were immunised with LHRH conjugated to BSA to generate the antibodies to LHRH (McNatty, 1992). Serum from the immunised sheep was a gift of Dr. K.P. McNatty.

Solid phase peptide synthesis of LHRH by Fmoc methodology required side chain protection of a number of amino acids. The N^{im} of His was protected by the Trt group, the hydroxyls of Ser and Tyr were protected as their tBu ethers, and the N^{G} of Arg was protected by the Mtr group. Two peptide-resins were synthesised. The first had the LHRH peptide sequence bound directly to aminopropyl Perloza. The second peptide-resin had a cleavable amide-linker (see Chapter 5) inserted between the peptide and the support. Treatment of the cleavable peptide-resin with acidic reagents would furnish LHRH (see Chapter 5, Section 5.2.3.7). It was proposed to treat the cleavable peptide-resin with a number of

acidic peptide cleavage reagents, and to analyse the products by HPLC. The reagent which was most efficient at cleaving the peptide would be tested at lower dilutions to determine whether it was still effective at cleaving LHRH. If it was still effective at low concentrations it would then be tested with the non-cleavable peptide-resin to determine:

i) whether it cleaved side chain protecting groups.
ii) whether it gave significant resin degradation.
iii) whether it cleaved the peptide from the support.

Once a suitable reagent had been identified it would be used to cleave the side chain protecting groups from the amino acids of the resin-bound LHRH.

6.2.8.1 Synthesis of pGlu-His(Trt)-Trp-Ser(tBu)-Tyr(tBu)-Gly-Leu-Arg(Mtr)-Pro-Gly-Perloza 500 Medium

Water-wet aminopropyl Perloza 500 medium (0.484 g dry, amine substitution 0.31 mmole/g) was washed to dioxane and then to DMF. The total amine available was 0.15 mmole. The resin was transferred to a Biolynx reaction column. A separate column was loaded with 1.73 g of DMF wet p-[(R,S)-α-[1-(9H-Fluoren-9yl)-methoxyformamido]-2,4-dimethoxybenzyl]-phenoxyacetamido Perloza 200 Medium resin (amide-linker resin, amine substitution 0.26 mmole/g, 0.05 mmole amine available). The Fmoc group was removed from the amide-linker resin using 20% piperidine in DMF. The Biolynx reaction column containing the aminopropyl Perloza 500 resin was connected to the Biolynx peptide synthesiser. The reaction columns were in series.

Fmoc-amino acids were activated for coupling using the method of Beyermann et al (1991). Fmoc-amino acid, 1 mmole, was dissolved in 3 ml DCM. HOBt.H_2O, 1.1 mmole, was added. Not all of the HOBt dissolved. The suspension was stirred, and 1.05 mmole DIC was added. Most of the HOBt dissolved, but within 30 seconds a voluminous precipitate of Fmoc-amino acyl-OBt ester formed. After ten minutes 1-2 ml DMF was added to dissolve the precipitate. DIEA, 1 mmole, and the Fmoc-amino acyl-OBt ester solution were added to the resin, and recycled for one hour at 3 ml/min. A standard Biolynx Fmoc peptide synthesis protocol was used

(see Chapter 5). After the first Fmoc-amino acid (Fmoc-glycine) was coupled, samples of the Fmoc-Gly-amidopropyl resin were taken for ninhydrin assay and amino acid analysis. The ninhydrin assay showed a residual amine substitution of 19 µmole/g. Unreacted amine groups were acetylated for 30 minutes using a solution of 1 ml acetic anhydride, 0.05 g DMAP, and 3 ml DMF. Following acetylation, the resin was washed with DMF and the synthesis was continued. After the third amino acid, arginine, was coupled, samples of the aminopropyl resin were taken for amino acid analysis, picrate titration, and an experiment to investigate cleavage of the Arg N^G-Mtr protecting group from the Arg(Mtr)-Pro-Gly-Perloza. The synthesis was then continued to completion. Pyroglutamic acid, the terminal residue, required 3 ml DMF to completely dissolve the activated derivative, but otherwise coupled adequately. Samples of both cleavable and non-cleavable peptide-resin were taken for amino acid analysis.

6.2.8.2 Reaction of Perloza 100 Medium with Fmoc-Gly-OBt

The method used in Section 6.2.8.1 to form Fmoc-amino acyl-OBt active esters results in a very reactive species (Beyermann et al, 1991). This method of activating Fmoc-amino acids had not been used in the other studies reported in this thesis. An investigation was carried out to determine whether significant amounts of Fmoc-amino acyl-OBt ester, formed using the method of Beyermann et al, would react with underivatised Perloza.

Water-wet Perloza 100 Medium, 3.37 g (0.337 g dry), was washed with ethanol followed by DMF. The resin was transferred to a Biolynx reaction column, which was then fitted to the Biolynx synthesiser. Fmoc-glycine-OBt active ester was pre-formed using the method of Beyermann et al (1991) given in Section 6.2.8.1. The Fmoc-glycine-OBt solution was circulated through the Perloza at 3 ml/min for one hour. The Fmoc group was cleaved using 20% piperidine in DMF and the glycine substitution was determined using the methods given below.

The amine substitution of the glycyl-Perloza was determined by picrate titration. Dried samples were also hydrolysed in 0.3 ml of 0.5M NaOH solution for 55 minutes, cooled, and dried under vacuum. The dried

samples were suspended in 5 ml 0.2M pH 2.2 citrate buffer to extract the glycine into solution for amino acid analysis. In addition, a sample of the glycyl-Perloza was hydrolysed for amino acid analysis using standard acid hydrolysis procedures (see Chapter 3, Section 3.2) to serve as a comparison of the two hydrolysis methods. A sample of the resin was also dried and sent for elemental analysis.

6.2.8.3 Cleavage of Mtr from Arg(Mtr)-Pro-Gly-Perloza

A sample of Arg(Mtr)-Pro-Gly-Perloza resin (see Section 6.2.8.1) was washed with DCM. Excess DCM was removed by filtration and the peptide-resin was transferred to a vial. DCM, 2 ml, was added. The resin was stirred, and 100 µl thioanisole, 200 µl TFA, and 100 µl TMSBR were added. The resin was stirred for 15 minutes, then filtered and washed sequentially with DCM, ethanol, water, 0.5M NaOH, and distilled water until the effluent was neutral to pH paper. The amine substitution was determined by picrate titration. Following determination of the amine substitution the dried resin was subjected to amino acid analysis.

6.2.8.4 Reagents for cleavage of side chain protecting groups from pGlu-His(Trt)-Trp-Ser(tBu)-Tyr(tBu)-Gly-Leu-Arg(Mtr)-Pro-Gly-Perloza 500 Medium

The approach to this problem was to analyse, by HPLC, the product resulting from cleavage of small samples of cleavable LHRH-amide linker-resin. The retention times of the HPLC peaks were compared to that of LHRH previously synthesised (Chapter 5, Section 5.3.3.7). The reagents tested were:

Reagent 1: TMSBR (1 ml, 10%), thioanisole (1 ml, 10%), EDT (0.25 ml, 2.5%), TFA (1 ml, 10%), DCM (6.75 ml, 67.5%).

Reagent 2: TFA (9 ml, 90%), TMSBR (0.5 ml, 5%), thioanisole (0.5 ml, 5%), EDT (0.3 ml, 3%), anisole (0.2 ml, 2%).

Reagent 3: TFA (8 ml, 80%), TMSBR (0.5 ml, 5%), thioanisole (0.5 ml, 5%), EDT (0.5 ml, 5%), m-cresol (0.5 ml, 5%).

Small samples of DCM-wet LHRH-amide linker-resin were treated with the cleavage reagents, and 1 ml samples were withdrawn at intervals. The 1 ml samples were added to 3 ml ether (a precipitate formed), 1 ml water was added, the samples were shaken, and the aqueous layer was extracted. The aqueous layer was transferred to an Eppendorf tube and centrifuged at 13000 rpm for 5 minutes using an MSE centrifuge. The clear aqueous layer was then subjected to HPLC analysis. The absorbance at 280 nm was used for the following calculations. The ratio of the height of the peak appearing at 45 minutes (presumed to be LHRH with Mtr still attached to the Arg) to the height of the peak appearing at 31 minutes (LHRH) was calculated. Comparing the peak height ratios for different reagents gave an indication of the effectiveness of the reagent in cleaving the Mtr group from Arg(Mtr). Of the reagents tested, Reagent 3 was found to give the fastest cleavage of the Arg(Mtr) group. Reagent 3 was diluted and the experiment was repeated with the lower dilutions of the reagent. The cleavage time was increased to compensate for the lower concentration of active species.

The concentrations tested were:

Reagent 4: DCM (8 ml, 80%), TFA (1.6 ml, 16%), TMSBR (0.1 ml, 1%), thioanisole (0.1 ml, 1%), EDT (0.1 ml, 1%), m-cresol (0.1 ml, 1%)

Reagent 5: DCM (9 ml, 90%), TFA (0.8 ml, 8%), TMSBR (0.05 ml, 0.5%), thioanisole (0.05 ml, 0.5%), EDT (0.05 ml, 0.5%), m-cresol (0.05 ml, 0.5%).

Treatment of LHRH-amide linker-resin with Reagent 4 for three hours gave almost quantitative cleavage of amino acid side chain protecting groups to generate LHRH, as determined by HPLC analysis of the peptide cleavage product. The next question to be answered was whether the reagent would be suitable for cleaving the amino acid side chain protecting groups of support-bound LHRH. The most significant considerations were whether the low concentration of TFA and TMSBR would significantly degrade the flow properties of LHRH-amidopropyl Perloza, and whether the reagent would give cleavage of peptide from the support.

6.2.8.5 Cleavage of side chain protection from pGlu-His(Trt)-Trp-Ser(tBu)-Tyr(tBu)-Gly-Leu-Arg(Mtr)-Pro-Gly-Perloza 500 Medium using Reagent 4: Experiment 1

It was noted in Section 3.3.1 of Chapter 3 that treatment of Boc-valine-amidopropyl Perloza with the acidic Boc cleavage reagent 10% sulphuric acid in dioxane gave loss of valine from the support as determined by picrate titration. It was assumed that if the acidic Reagent 4 gave cleavage of the peptide from the support, it would be possible to determine the extent of cleavage of peptide by picrate titration of the support-bound Arg N^G and His N^{im} groups.

The final peptide substitution of the peptide-resin, from the amino acid analysis result, was 0.20 mmole/g (see Section 6.2.8.1). Complete removal of side chain protecting groups would result in a final peptide substitution of 0.23 mmole/g because of loss of the two tBu, one Mtr, and one Trt side chain protecting groups of Ser, Tyr, Arg, and His respectively. The reason for the apparent increase of the peptide substitution is illustrated in Equation 6.1.

Equation 6.1 Calculation of the expected peptide substitution of LHRH-Perloza 500 after cleavage of side chain protecting groups

If the amino acid side chain protecting groups of side chain protected LHRH-amidopropyl Perloza are cleaved (initial weight of protected LHRH-Perloza: 1.000 g, peptide substitution: 0.20 mmole/g), then the loss in weight on cleavage of the side chain protecting groups would be:

$$0.20 \times 10^{-3} \times \{56 + 56\ (2x\ tBu - 2H) + 242\ (Trt - 1H) + 212\ (Mtr - 1H)\}$$
$$= 0.113\ g.$$

There would still be 0.20 mmole of peptide on the resin, so the peptide substitution would be (after base wash):

$$\frac{0.20}{1 - 0.113}\ \text{mmole/g}$$

$$= 0.23\ \text{mmole/g}$$

DCM-wet pGlu-His(Trt)-Trp-Ser(tBu)-Tyr(tBu)-Gly-Leu-Arg(Mtr)-Pro-Gly-Perloza 500 medium, 0.70 g, was transferred to a vial. Reagent 4, 10 ml, was added, and the LHRH-Perloza was mixed using the Rototorque. Samples of LHRH-Perloza were removed for picrate titration and amino acid analysis after 2 and 3 hours reaction.

6.2.8.6 Cleavage of side chain protection from pGlu-His(Trt)-Trp-Ser(tBu)-Tyr(tBu)-Gly-Leu-Arg(Mtr)-Pro-Gly-Perloza 500 Medium using Reagent 4: Experiment 2

DCM-wet pGlu-His(Trt)-Trp-Ser(tBu)-Tyr(tBu)-Gly-Leu-Arg(Mtr)-Pro-Gly-Perloza 500 medium, 2.17 g, was treated for 3 hours with 30 ml of Reagent 4. The resin was washed 5X dioxane, water, 10% triethylamine in 50% aqueous ethanol, and finally with water. Samples were taken for picrate titration and amino acid analysis.

The results of the experiments reported in Sections 6.2.8.5 and 6.2.8.6 suggested that, within experimental error, quantitative side chain deprotection had been accomplished without significant cleavage of the peptide-ligand from the support. In addition, the peptide-resin was easy to filter and the flow properties did not appear to be impaired. The deprotected peptide-resin from Section 6.2.8.6 will be referred to as Resin 1.

The picrate titration and amino acid analysis results of Resin 1 suggested that the required ligand was present. In addition, HPLC analysis of the peptide cleaved from the LHRH-amide linker-resin showed a major peak which had the same retention time as authentic LHRH. This suggested that the peptide bound to the resin was of similar quality, assuming that the amino acid coupling yields were the same for both peptide syntheses. The next step was to determine whether the LHRH-Perloza could bind antibodies to LHRH.

6.2.9 Binding of Antibodies to LHRH to Resin 1 and their later elution

6.2.9.1 Ammonium sulphate precipitation of immunoglobulins from ovine serum

Ammonium sulphate precipitation was used for preliminary separation of immunoglobulins from ovine serum (Livingston, 1974; Harris and Angal, 1989).

Ammonium sulphate, 2.561 g, was added over 10 minutes to 13 ml stirred ovine serum at 0°C. The weight of ammonium sulphate used was the quantity calculated to give 50% saturation at 0°C. The solution was stirred at 0°C for one hour, then centrifuged at 3000X gravity for 30 min at 4°C. The supernatant was decanted off and the precipitate redissolved in 10 ml 10mM Tris buffer, pH 7.5. The solution was dialysed against 10mM pH 7.5 Tris buffer for 24 hours at 4°C. The final volume of the dialysed solution was 23 ml.

6.2.9.2 Affinity isolation of antibodies using Resin 1

Buffers used in this study were:

Buffer 1 10mM Tris, pH 7.5
Buffer 2 10mM Tris, pH 7.5, 500mM NaCl
Buffer 3 10mM citrate, pH 3.0
Buffer 4 5M guanidine hydrochloride, pH 1.5

Dialysed solution containing antibodies to LHRH (Section 6.2.9.1) 2 ml, was diluted with 20 ml buffer 1, and filtered through a 0.22 µm filter. The solution was loaded onto the affinity column, which had been equilibrated with buffer 1. The breakthrough fraction was eluted using buffer 1. The column was then washed with buffer 2 to elute material bound via ionic interactions. The column was washed again with buffer 1. The column was then washed with buffer 3, followed by washing with buffer 4 to elute the antibodies. The column was then washed with buffer 1, followed by a wash with 0.1M NaOH solution, and a final wash with buffer 1. The elution fractions from buffers 3 and 4 were immediately neutralised with 100mM Tris buffer, pH 8.0. The fractions

from the column washes with buffers 2 and 4 were dialysed vs buffer 1 at 4°C (2X 30 minutes, 2X 1 hour, 1X 16 hours). The fractions were reduced before analysis by SDS-PAGE (a Pharmacia PhastGel, 12.5% homogeneous, was used for the analysis). Samples were also subjected to RIA to determine the presence of antibodies to LHRH.

6.2.9.3 RIA to determine the presence of antibodies to LHRH

The assay described in this Section was carried out by Mr. Stan Lun, MAF, Wallaceville, NZ. The following is a transcript of the methodology employed

> *The tracer used to detect binding was synthetic 'native' LHRH made by Peninsular Laboratories Inc, Belmont CA USA. It was iodinated with ^{125}I using a Lactoperoxidase/Glucose Oxidase method and purified on a QAE Sephadex A25 column. Samples were diluted accordingly with Assay buffer (20mM Phosphate buffered saline, pH 7.4 containing 0.5% Bovine serum albumin, 1mM Bacitracin and 0.01% Triton X-100. 100 µl of the diluted samples were incubated with approx 15000 counts/min of the tracer in a total volume of 300 µl for 20 hours at room temperature. One ml of 15% Polyethylene glycol 8000 was then added along with 25 µl filler serum and the tubes mixed well. The tubes were then centrifuged at 3000g for 25 min and the supernatant removed by aspiration. 'Bound counts' in the pellet were determined with a Packard gamma counter.*
> (Lun, 1992).

6.2.9.4 Binding of serum components to Resin 1

Lamb serum, 2 ml, was diluted with 20 ml of buffer 1. The serum was filtered through a 0.22 µm filter, and loaded onto the affinity column. The column was subjected to the same set of washes given in Section 6.2.9.2. Fractions were collected for analysis by SDS-PAGE.

6.2.10 Synthesis of Perloza-S-(Ac)-Cys-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 (Resin 2)

It appeared from previous studies into the nature of the LHRH Ag-Ab interaction that the C-terminal glycine amide of LHRH had to be present

in order for the antibodies to bind to LHRH (Singh, 1985, 1986). The C-terminal glycine of Resin 1 was bound to the support, and therefore not available for binding to the antibodies. A novel method for coupling a fragment of LHRH (containing the 7 C-terminal amino acids) to Perloza via a thioether bond was investigated. The aim of the study was to synthesise an analogue of LHRH containing cysteine at the N-terminus, and to investigate conditions for reaction of the analogue with α-bromoacetamido Perloza to anchor the peptide via a thioether bond. The cysteine was placed at the N-terminus in order to direct the LHRH C-terminal glycine amide into the aqueous phase for binding antibodies.

6.2.10.1 Synthesis of α-bromoacetamido Perloza 500 Medium

Aminopropyl Perloza 500 Medium, amine substitution 0.56 mmole/g (1.79 g dry, 1 mmole amine), was washed with dioxane and then with DMF. α-Bromoacetic anhydride was prepared by dissolving α-bromoacetic acid (0.58 g, 4 mmole) in 6 ml DCM, then adding 2.1 mmole DIC (328 µl) and stirring for 10 minutes. A white precipitate formed after 30 seconds, this was probably the α-bromoacetic anhydride. After 10 minutes 10 ml of DMF was added, the precipitate dissolved. The α-bromoacetic anhydride solution was added to the aminopropyl Perloza and reaction was continued for one hour. After 35 minutes ninhydrin analysis showed a residual amine substitution of 1.3 µmole/g. The resin was washed with DMF, ethanol and dioxane. A sample was taken for elemental analysis.

6.2.10.2 Blocking of unreacted α-bromoacetamido groups

If a cysteine-containing peptide did not react in 100% yield with α-bromoactamido Perloza then it would be necessary to block remaining α-bromoacetamido groups to avoid unwanted coupling of thiol-containing proteins during chromatography. A study was carried out to investigate various methods for blocking resin-bound α-bromoacetamido groups.

6.2.10.2.1 Reaction of α-bromoacetamido Perloza 500 Medium with 2-mercaptoethanol

α-Bromoacetamido Perloza (0.104 g dry, 0.052 mmole Br) was washed with water and then with 0.1M $NaHCO_3$ solution. To the resin was added 1 ml

0.1M $NaHCO_3$ solution and 7.3 µl (0.104 mmole, 2 eq) 2-mercaptoethanol. Samples were taken for elemental analysis 15 and 60 minutes after addition of the βMe.

6.2.10.2.2 Reaction of α-bromoacetamido Perloza 500 Medium with ethanolamine

α-Bromoacetamido Perloza (0.317 g dry, 0.165 mmole Br), was washed with water and then with 0.1M $NaHCO_3$ solution. To the resin was added 3 ml 0.1M $NaHCO_3$ and 20 µl (0.330 mmole, 2 eq) ethanolamine. The resin was reacted for one hour, then the amine substitution was determined by picrate titration. A sample of unreacted α-bromoacetamido Perloza was also titrated to serve as a control.

6.2.10.2.3 Reaction of α-bromoacetamido Perloza 500 Medium with 0.1M NaOH solution

α-Bromoacetamido Perloza, 1.12 g wet with dioxane, was washed with water. NaOH solution, 0.1M, 20 ml, was added to the resin. Samples of resin were taken at intervals, washed with water, and then washed and dried for elemental analysis.

6.2.10.2.4 Reaction of α-bromoacetamido Perloza 500 Medium with 0.1M Na_2CO_3 solution

α-Bromoacetamido Perloza, 1.18 g wet with dioxane, was washed with water. Na_2CO_3 solution, 0.1M, 20 ml, was added to the resin. Samples were taken at intervals, washed with water, and then washed and dried for elemental analysis.

6.2.10.3 Solid phase synthesis of Ac-Cys-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2, an analogue of LHRH

The sequence of the LHRH analogue to be synthesised for coupling to α-bromoacetamido Perloza was Ac-Cys-Ser-Tyr-Gly-Leu-Arg Pro-Gly-NH_2. The peptide was synthesised on Perloza 200 Medium amide-linker resin, amine substitution 0.26 mmole/g, using the LKB Biolynx peptide synthesiser. The coupling cycles used were reported in Chapter 5. The

scale of the synthesis was 0.162 mmole. Fmoc-amino acids were activated for coupling using the method of Beyermann et al (1991) given in Section 6.2.8.1. A 6.2 X excess of activated Fmoc-amino acid was coupled. The thiol of the Cys was protected with the TFA-labile Trt group. The N-terminal amino acid (Cys) was acetylated for 30 minutes, after Fmoc cleavage, with acetic anhydride 1 ml (10.6 mmole), DIEA 1.92 ml (11 mmole), DMF 2 ml. The peptide-resin was washed with DMF, DCM, ether, and dried in vacuo.

6.2.10.3.1 Peptide cleavage # 1

Dry peptide-resin, 224 mg, was treated with 12 ml Reagent B (Chapter 5, Section 5.2.6) for 14.5 hours. The cleavage mixture was worked up using workup procedure 2, reported in Section 5.2.8.2 of Chapter 5. The peptide was purified by HPLC to yield 15 mg of peptide. A sample of the purified peptide was taken for amino acid analysis. The remainder of the purified peptide was used in a trial experiment to test whether the peptide thiol would react with α-bromoacetamido Perloza.

6.2.10.3.2 Peptide cleavage # 2

The remainder of the peptide-resin, 707 mg, was treated with 22 ml of Reagent B for 16.5 hours, then worked up as described for cleavage # 1. The peptide was purified by HPLC to give 51.8 mg of purified peptide. A sample of the purified peptide, 2.0 mg, was taken for amino acid analysis.

6.2.10.4 Reaction of Ac-Cys-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 with α-bromoacetamido Perloza

6.2.10.4.1 Reaction of Ac-Cys-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 with α-bromoacetamido Perloza: Experiment 1

Dioxane-wet α-Bromoacetamido Perloza (0.0114 g dry, 0.0118 mmole Br) was washed with water. The resin was transferred to a vial containing 14.1 mg (0.0158 mmole) of the peptide (Ac-Cys-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2). $NaHCO_3$ solution, 1 ml, was added. The reaction was mixed (Rototorque) for one hour, then filtered. The peptide-resin was washed

with water. The peptide substitution was determined by picrate titration of the arginine guanidine group, and also by amino acid analysis of the dried peptide-resin resulting from the picrate titration. A sample of the dried peptide-resin from the picrate titration was also subjected to elemental analysis for sulphur and bromine.

6.2.10.4.2 Reaction of Ac-Cys-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 with α-bromoacetamido Perloza: Experiment 2

α-Bromoacetamido Perloza 500 medium (0.0572 g dry, 0.0286 mmole Br) was washed from dioxane to distilled water. The peptide (Ac-Cys-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2), 49.1 mg (0.055 mmoles), was added to the resin. $NaHCO_3$ solution, 0.1M, 1 ml, was added, followed by 54 µl 0.22 mmole tri-n-butyl phosphine in 146 µl 2-propanol. The reaction was mixed (Rototorque) for 2 hours. After 2 hours βMe, 4 µl, 0.0572 mmoles, was added to block unreacted α-bromoacetamido groups, and the reaction was mixed for a further hour. The peptide-resin was collected by filtration, and washed sequentially with 50% aqueous ethanol, dioxane, ethanol, and isopropanol until the odours of βMe and tri-n-butyl phosphine were no longer present. The peptide-resin was then washed with water. Samples of dried peptide-resin were taken for elemental and amino acid analyses. This peptide-resin (referred to as Resin 2) was tested for binding and elution of antibodies to LHRH.

6.2.11 Affinity isolation of antibodies to LHRH using Resin 2

An identical procedure to that given in Section 6.2.9.2 was used. All buffers used were the same as those given in section 6.2.9.2. Dialysed solution containing antibodies to LHRH (Section 6.2.9.1) 2 ml, was diluted with 20 ml buffer 1, and filtered through a 0.22 µm filter. The solution was loaded onto the affinity column, which had been equilibrated with buffer 1. The breakthrough fraction was eluted using buffer 1. The column was then washed with buffer 2 to elute material bound via ionic interactions. The column was washed again with buffer 1. The column was then washed with buffer 3, followed by washing with buffer 4 to elute the antibodies. The column was then washed with buffer 1, followed by a wash with 0.1M NaOH solution, and a final wash with buffer

1. The elution fractions from buffers 3 and 4 were immediately neutralised with 100mM Tris buffer, pH 8.0. The fractions from the column washes with buffers 2 and 4 were dialysed vs buffer 1 at 4°C (2X 30 minutes, 2X 1 hour, 1X 16 hours). The fractions were reduced before analysis by SDS-PAGE (a Pharmacia PhastGel, 12.5% homogeneous, was used for the analysis). Samples were also subjected to RIA to determine the presence of antibodies to LHRH.

6.2.11.1 Binding of serum components to Resin 2

Lamb serum, 2 ml, was diluted with 20 ml of buffer 1. The serum was filtered through a 0.22 µm filter, and loaded onto the affinity column. The column was subjected to the same set of washes given in Section 6.2.9.2. Fractions were collected for analysis by SDS-PAGE.

6.3 RESULTS AND DISCUSSION

6.3.1 Chymosin purification using Val-dLeu-Pro-Phe-Phe-Val-dLeu-Perloza

Val-dLeu-Pro-Phe-Phe-Val-dLeu, a specific inhibitor of aspartic proteinases, was synthesised directly onto aminopropyl Perloza. The peptide-Perloza was tested for use as an affinity matrix for chymosin purification.

Because the peptide contained no side chain protected amino acids, the peptide-resin was synthesised using the Boc methodology reported in Chapter 3, except that the Boc-amino acids were activated as their symmetrical anhydrides. At completion of the synthesis the amine substitution determined by picrate titration was 0.26 mmole/g. The theoretical amine substitution, based on an initial amine substitution of 0.37 mmole.g, was 0.28 mmole/g. The picrate titration result showed reasonable agreement with the theoretical result, thus providing an initial indication that the synthesis had proceeded satisfactorily.

Amino acid analysis of the peptide-resin gave the expected ratios: Pro 0.9 [1], Val 1.8 [2], Leu 2.0 [2], Phe 1.9 [2], based on Leu = 2.00. After a 30 minute wash with 0.1M NaOH the ratios were: Pro 1.0 [1], Val 1.7 [2], Leu 2.0 [2], Phe 2.0 [2]. The ratios were similar to those found before the wash with NaOH, and suggested the peptide was stable to this reagent. The peptide-ligand substitution calculated from the amino acid analysis result was 0.25 mmole/g, which showed good agreement with the picrate titration result of 0.26 mmole/g and the theoretical result of 0.28 mmole/g. In both cases, pre and post wash, the valine ratio was slightly low, which may indicate that valine had not coupled as efficiently as the other amino acids. Considering that single couplings of only a 2.5X excess of activated Boc-amino acid were employed, the amino acid analysis results were encouraging. The close agreement between the theoretical and experimentally found amino acid ratios suggested that a significant amount of the peptide bound to the resin was the desired ligand.

One consequence of washing the peptide-resin with NaOH is that any amino acids or incomplete peptides bound directly to the support may be cleaved by alkaline hydrolysis (Vlasov et al, 1973).

An initial attempt to bind and elute chymosin using the peptide-resin with the buffers and washing sequence reported by Strop et al (1990) was unsuccessful. The main difference between the peptide-resin synthesised in this study and that used by Strop et al was that they coupled the peptide to the support via its amino terminus, thus leaving the C-terminal carboxyl group directed into the chymosin solution. It was postulated that the free amino terminus of the peptide-resin used in this study may have prevented binding of the chymosin to the peptide-ligand. The resin was therefore succinylated to introduce a carboxyl group at the end of the peptide.

The peptide-resin was able to bind chymosin following introduction of the carboxyl group by succinylation. The chymosin could then be eluted using the conditions given be Strop et al (1990). This experiment, using commercial food grade chymosin, served to demonstrate that it was possible to synthesise a peptide-ligand directly onto Perloza, and to use the peptide-resin to bind and later release a biological macromolecule. The next step was to test whether the peptide-resin affinity matrix could be used to isolate chymosin from a crude solution containing a number of contaminant proteins.

A dialysed solution containing chymosin from a recombinant source (gift of Genencor, SF, USA) was used in this study. As well as chymosin, the solution contained a number of contaminant fungal proteins. Two purification runs were undertaken.

In run A the buffers reported by Strop et al (1990) were used. In run B the buffers were changed to eliminate dioxane (a known carcinogen). The elution profiles of purification runs A and B are given as Figures 6.1 and 6.2 respectively. A milk clotting assay of the fractions derived from the purification runs revealed that chymosin activity was confined to the fractions derived from the elution buffers.

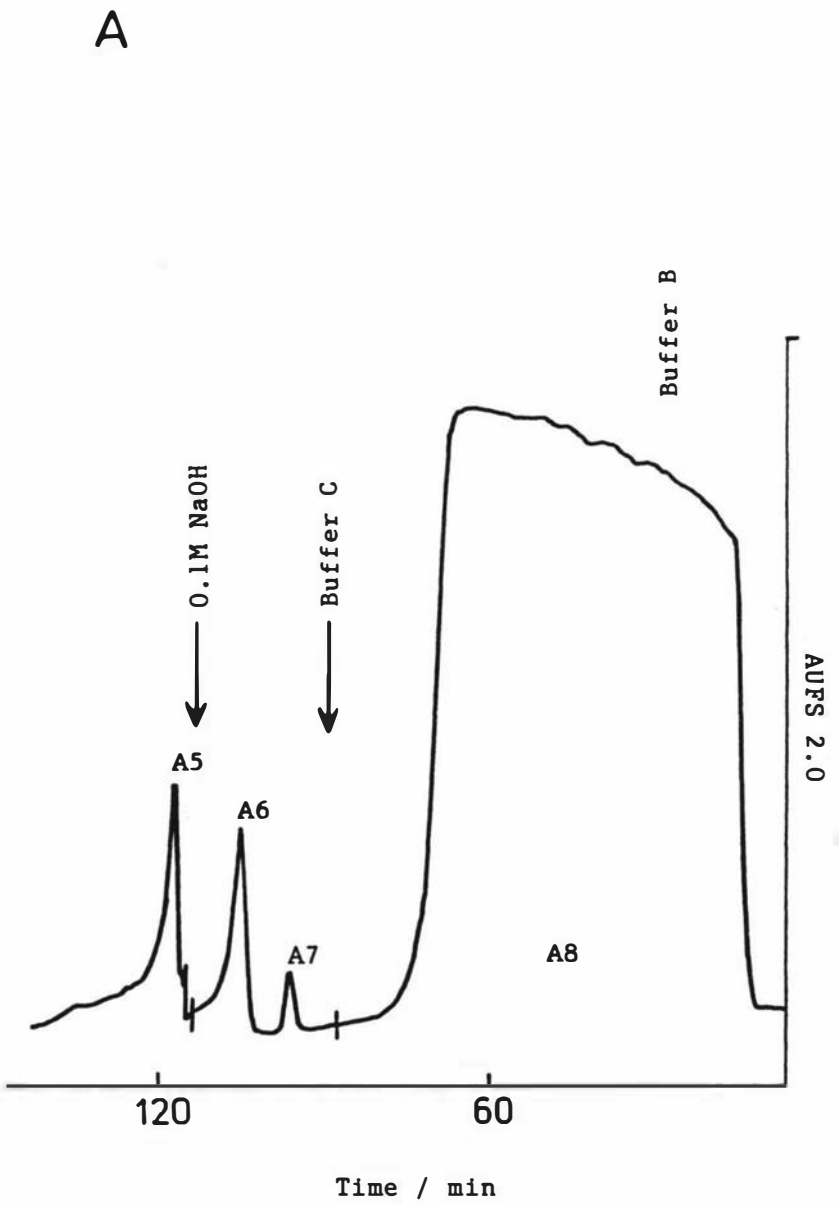
Samples of the fractions from the purifications runs were taken and concentrated 10X in vacuo. The solutions were prepared for SDS-PAGE.

The final concentration of the solutions analysed by SDS-PAGE was 5X the initial concentration. Proteins were visualised using the sensitive silver stain. A photograph of the gel is given as Figure 6.3. Lanes 5-8 of Figure 6.3 correspond respectively to samples taken from the peaks numbered A5-A8 in Figure 6.1 (run A). Lane 4 of Figure 6.3 was the crude chymosin solution applied to the affinity column at the start of each purification run. Lanes 3 and 2 of Figure 6.3 correspond to samples taken from peaks B3 and B2 of Figure 6.2 (run B). Lane 1 was a dilution of peak B2 (Figure 6.2, run B).

The milk clotting assay showed that chymosin activity was confined to peak A6 of run A (Figure 6.1) and peak B2 of run B (Figure 6.2). No chymosin milk clotting activity was found in the breakthrough fractions of runs A and B (peak A8 of figure 6.1 and peak B3 of figure 6.2 respectively). Neither was milk clotting activity found in the first peak to elute from run A (peak A7, Figure 6.1) after application of buffer C, the chymosin elution buffer. The SDS-PAGE gel (Figure 6.3) showed a single band in lane 6 (peak A6, run A, buffer C chymosin elution fraction), and one dark and one very faint band in lane 2 (peak B2, run B, buffer E chymosin elution fraction). Lanes 6 and 2 (the chymosin elution fractions of runs A and B respectively) of Figure 6.3 may be compared to the crude chymosin solution run in lane 4. Comparison of lanes 6 and 2 with lane 4 shows considerable purification of chymosin from the original solution, as evidenced by the almost total lack of contaminants in elution buffer fractions run in lanes 6 and 2 of Figure 6.3.

It was found in this study that chymosin did not bind to the peptide-ligand when it contained a free N-terminal amine group. However, succinylation of the peptide-ligand gave a peptide-support which was successfully used for the isolation of chymosin. In this instance the synthesis of the ligand directly onto aminopropyl Perloza using standard C to N terminal SPPS methodology was probably not the method of choice for immobilisation of the ligand. However, the results of this preliminary study still served to prove that an affinity matrix made by synthesising a peptide-ligand directly onto Perloza could be used to isolate and purify the target enzyme.

Figure 6.1 Chymosin purification using buffers A, B, and C

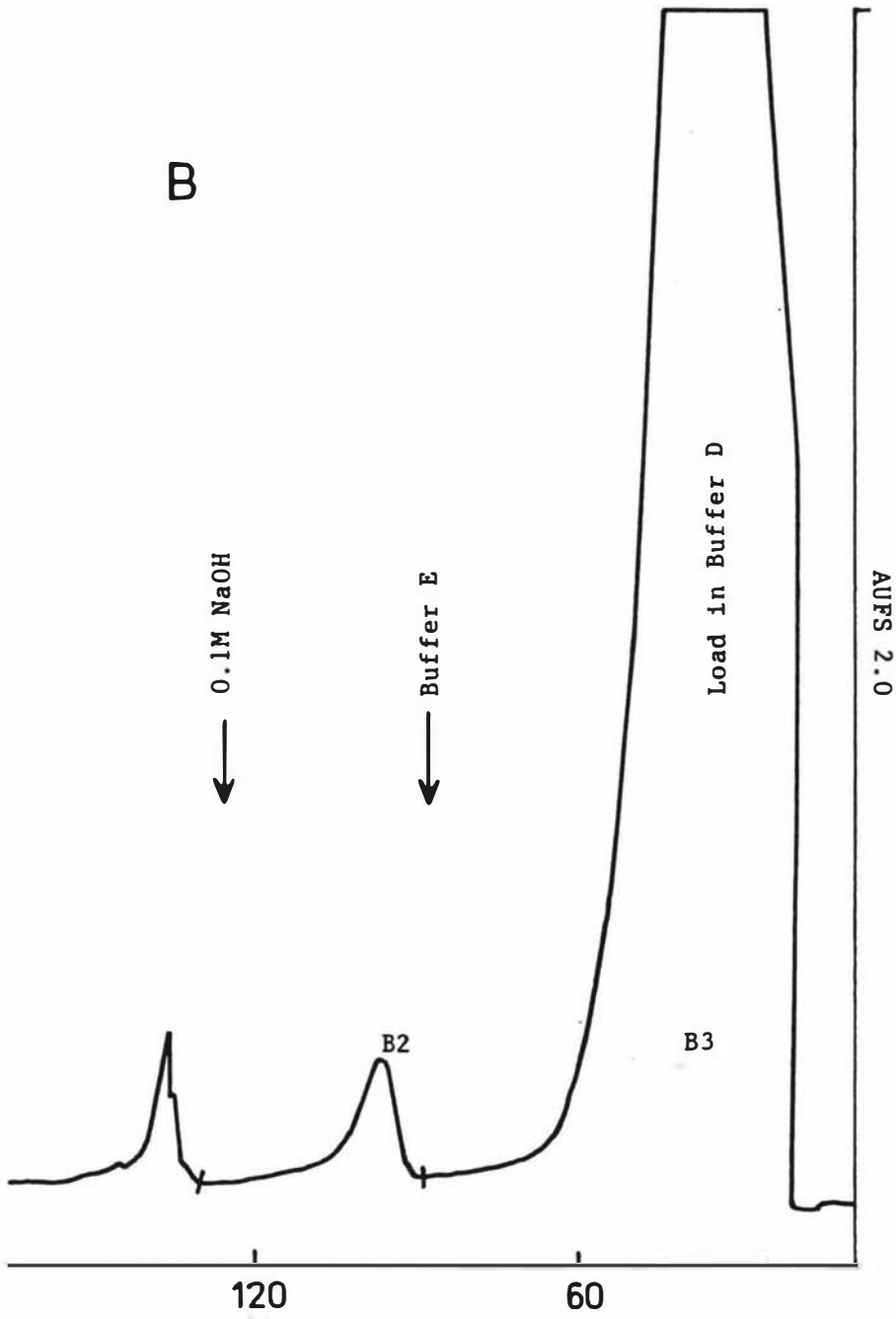


Buffer A: 30mM formate, pH 4.0, 15 % dioxane (loading buffer)

Buffer B: 30mM formate, pH 4.0, 13 % dioxane

Buffer C: 30mM formate, pH 6.5, 33% dioxane, 1mM EDTA

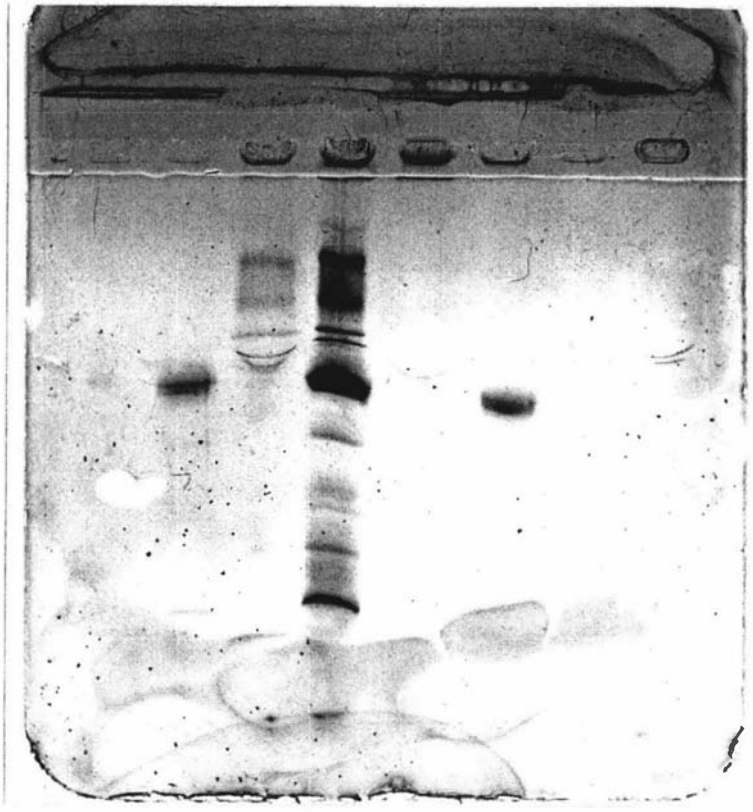
Figure 6.2 Chymosin purification using buffers D and E



Buffer D: 10mM citrate, pH 4.4 (loading buffer)

Buffer E: 10mM citrate, pH 6.0

Figure 6.3 SDS-PAGE of fractions from chymosin purification runs



1 2 3 4 5 6 7 8

Lane 1: Dilution of peak B2, Figure 6.2 (buffer E chymosin elution fraction)

Lane 2: Peak B2, Figure 6.2 (buffer E chymosin elution fraction)

Lane 3: Peak B3, Figure 6.2 (buffer D breakthrough fraction)

Lane 4: Crude chymosin solution prior to affinity purification

Lane 5: Peak A5, Figure 6.1 (NaOH wash fraction)

Lane 6: Peak A6, Figure 6.1 (buffer C chymosin elution fraction)

Lane 7: Peak A7, Figure 6.1 (buffer C, first peak to elute)

Lane 8: Peak A8, Figure 6.1 (buffers A and B breakthrough fraction)

6.3.2. Reaction of Perloza with Fmoc-Gly-OBt

Unfunctionalised Perloza was reacted with Fmoc-glycine-OBt ester. The glycine substitution was 0.24 mmole/g by picrate titration, 0.20 mmole/g by elemental analysis for nitrogen, 0.24 mmole/g by amino acid analysis of an alkaline hydrolysate, and 0.25 mmole/g by amino acid analysis of an acid hydrolysate. The results of the different analytical methods showed good agreement except for the elemental analysis for nitrogen, which was slightly lower than the others. The glycine substitution level of 0.24 mmole/g indicated that a significant amount of the Fmoc-Gly-OBt ester had reacted with the unmodified Perloza. This result may be compared with that found in Section 5.3.2 of Chapter 5. In Chapter 5 Fmoc-Gly-OBt was formed by reaction in DMF, whereas in this study it was formed by reaction in DCM. The glycine substitution from the result reported in Chapter 5 was 0.005 mmole/g, whereas in this study it was 0.24 mmole/g. From a comparison of these two results it appeared that Fmoc-Gly-OBt formed in DCM was significantly more reactive than Fmoc-Gly-OBt formed in DMF. All Fmoc-amino acid couplings reported in this Chapter were via Fmoc-amino acyl-OBt esters formed in DCM. However, it was not known whether significant coupling of Fmoc-amino acids to functionalised Perloza would occur, as it was expected that many of the reactive cellulose hydroxyl groups would have reacted with acrylonitrile during the initial functionalisation of the resin, making them unavailable for reaction with Fmoc-amino acyl-OBt esters. The results of this study suggested that significant amounts of incomplete peptides may be able to form on the carbohydrate backbone. However, it was proposed that washing the peptide-resin with NaOH solution would probably cleave any peptide bound to the carbohydrate backbone (Vlasov et al, 1973; Orlowska et al, 1975).

6.3.3 Synthesis of pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-Perloza 500 Medium (LHRH-Perloza 500, Resin 1)

6.3.3.1 Treatment of Arg(Mtr)-Pro-Gly-Perloza 500 with TMSBR/thioanisole/TFA in DCM

Resin 1, pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-Perloza 500 Medium, was made by synthesising the peptide (LHRH) directly onto aminopropyl

Perloza (initial amine substitution 0.31 mmole/g). A sample of the peptide-resin was taken after addition of the third amino acid (arginine) and cleavage of the Fmoc group. Picrate titration gave an amine substitution of 0.28 mmole/g (theory: 0.27 mmole/g). A portion of the resin was hydrolysed for amino acid analysis. The amino acid ratios were as expected: Pro 1.0 [1], Gly 1.0 [1], Arg 1.0 [1]. The peptide substitution calculated from the amino acid analysis result was 0.27 mmole/g (theory: 0.27 mmole/g). The peptide-resin was analysed after coupling of arginine to ensure that the coupling had gone in high yield. Fmoc-Arg(Mtr) couplings can be slower than couplings of other Fmoc-amino acids (Atherton et al, 1983b) and do not always go to completion (Eberle et al, 1986; Harrison et al, 1988). The amino acid analysis result showed that the arginine coupling had been nearly quantitative. A sample of the Arg(Mtr)-Pro-Gly-resin was treated with a solution of 8.3% TFA, 4.2% TMSBR, 4.2% thioanisole, in DCM for 15 minutes. The aim of this experiment was to determine whether the reagent would cleave the Mtr group from the N^G of arginine, but leave the flow properties of the resin unimpaired. If the Mtr group had been quantitatively cleaved the expected amine substitution based on the picrate titration result would be 0.60 mmole/g. The found result was 0.38 mmole/g. This suggested that either not all of the N^G-Mtr had been cleaved, or that some of the peptide had been cleaved from the resin. Amino acid analysis of the treated resin gave the amino acid ratios: Pro 0.9 [1], Gly 1.0 [1], Arg 0.6 [1]. The peptide substitution based on this amino acid analysis was 0.22 mmole/g, which may be compared to the pre-treatment substitution of 0.27 mmole/g. These results suggested that the reagent had cleaved some of the arginine at the Arg-Pro bond, and also that some of the peptide had been cleaved from the resin. The expected amine substitution based on the amino acid analysis result was, to a first approximation, 0.36 mmole/g ([0.22 + 0.6 X 0.22] mmole/g), assuming that all of the N^G-Mtr had been cleaved from the arginine. This result may be compared to the found result of 0.38 mmole/g, and suggested that the Mtr group had indeed been cleaved by a combination of TFA, TMSBR, and thioanisole.

The results of this experiment suggested that a solution of 8.3% TFA, 4.2% TMSBR, 4.2% thioanisole, in DCM, was able to rapidly cleave the arginine N^G-Mtr protecting group in a short (15 minutes) time. In addition, the flow properties of the resin did not appear to have been

significantly impaired after the treatment. The cleavage reagent also appeared to give cleavage of peptide from the resin, as well as cleavage of the Arg-Pro bond. However, despite the drawbacks found in this preliminary experiment, a combination of TFA/TMSBR/thioanisole appeared to have some potential as a reagent for cleavage of amino acid side chain protecting groups from a peptide-ligand synthesised onto Perloza using the Fmoc methodology. Therefore, further development of the reagent was undertaken in a later study (see Section 6.3.3.3).

6.3.3.2 Analysis of side chain protected LHRH-Perloza resins

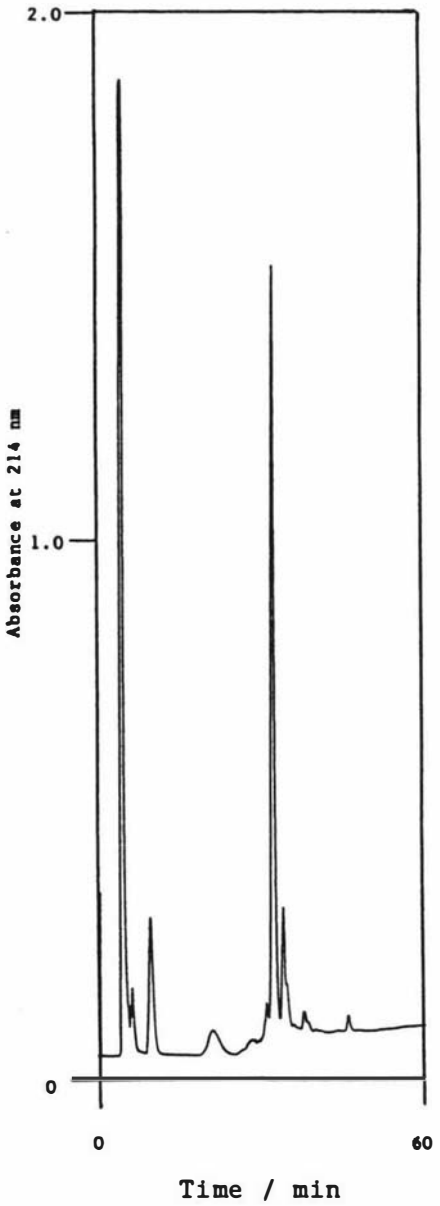
The peptide synthesis was completed and samples of both the cleavable-linked and non-cleavable peptide-resins were taken for hydrolysis and amino acid analysis. The amino acid analysis results are given in Table 6.1. Comparison of the two sets of results shows very similar amino acid ratios for the two resins except for arginine, where the non-cleavable result was significantly lower. The low result for arginine found for the non-cleavable resin was not a cause for concern because the ratio found directly after coupling of the arginine (Section 6.3.3.1) had indicated nearly quantitative coupling of this amino acid. The difference between the arginine results for the two peptide-resins may have been due to random experimental variation in the Arg analysis result for the non-cleavable peptide-resin.

Table 6.1 Amino acid analyses of LHRH-peptide-resins

Amino acid	Expected	Found	
		Cleavable	Non-cleavable
Ser	1	0.79	0.78
Glu	1	1.03	0.98
Pro	1	1.07	1.09
Gly	2	2.00	2.00
Leu	1	0.84	0.81
Tyr	1	0.78	0.74
His	1	0.84	0.80
Arg	1	1.01	0.83
Trp	1	n.d.	n.d.

The similarity between the amino acid analysis results for the cleavable and non-cleavable peptide-resins (Table 6.1) suggested that the amino acid coupling yields were similar for both syntheses. If it is assumed that the coupling yields were identical for the two peptide-resins, then HPLC analysis of the crude cleaved peptide would give an indication of the integrity of resin-bound peptide ligand. Figure 6.4 is a HPLC trace of crude cleaved peptide derived from the cleavable peptide-resin. The trace shows a major peak at 31 minutes with a minor contaminant peak at 33 minutes. The HPLC retention time of the major peptide peak was identical to that of authentic LHRH. A combination of the amino acid analysis results and HPLC analysis of the crude cleaved peptide suggested that a substantial proportion of the resin-bound peptide was the required ligand.

Figure 6.4 HPLC trace of crude cleaved LHRH from amide-linker resin



The resin-bound peptide substitution levels based on amino acid analysis results were 0.18 mmole/g for the cleavable peptide-resin (theory 0.18 mmole/g) and 0.20 mmole/g for the non-cleavable peptide-resin (theory 0.20 mmole/g. The peptide substitution levels, as determined by amino acid analyses, suggested that little or no peptide had formed directly onto the carbohydrate matrix, even though highly active Fmoc-amino acyl-OBt esters were used for the couplings (Section 6.3.2). This in turn suggested that many of the reactive carbohydrate hydroxyls had been occupied during the initial derivatisation of the resin with acrylonitrile.

6.3.3.3 Cleavage of side chain protecting groups from pGlu-His(Trt)-Trp-Ser(tBu)-Tyr(tBu)-Gly-Leu-Arg(Mtr)-Pro-Gly-Perloza 500 Medium

The next study was an investigation of the cleavage of side chain protecting groups from the peptide bound to the resin. A study of cleavage reagents containing TFA/TMSBR/thioanisole with combinations of other scavengers was undertaken. TMSBR/TFA/thioanisole in combination with other scavengers was used because it had previously been shown to be an effective reagent for cleavage of the Mtr group from N^G of arginine (Yajima et al, 1988; Fischer et al, 1992).

Portions of the cleavable peptide-amide linker-resin were treated with different cleavage reagents. Samples of the solutions were taken at intervals, added to ether, and water was added. The water layer was separated and centrifuged prior to HPLC analysis. Mulholland and Hague (1992) developed a capillary zone electrophoresis (CZE) method for monitoring deprotection of Arg(Mtr) containing peptides as an alternative to HPLC monitoring because it was thought that multiple ether extractions were necessary to prepare samples for HPLC. However, it was found in this study that a single ether extraction followed by centrifugation was all that was required to prepare the samples for analysis by HPLC.

Two major peaks were usually seen on HPLC, one with a retention time of 31 minutes (same retention time as authentic LHRH), with another at 45 minutes. The 45 minute peak was found to decrease in height with time

relative to the 31 minute peak. This suggested that the 45 minute peak was LHRH with the Mtr protecting group still on the N^G of the arginine. In order to evaluate the relative effectiveness of the reagents tested, the ratio of the height of the 45 minute peak to the height of the 31 minute peak was compared. The reagent which gave the lowest peak height ratio was then taken as being the most effective at cleaving the Mtr group. The results of this study of Reagents 1 - 3 are given in Table 6.2.

Table 6.2 Treatment of LHRH-amide linker-resin with cleavage reagents

Reagent*	Time (min)	Ratio: 45 min peak height / 31 min peak height
1	5	5.00
	10	4.00
	60	0.50
2	45	0.66
3	5	0.32
4	30	0.40
	60	0.18
	120	0.05
	180	0.01
5	60	0.68
	120	0.39
	180	0.21

* Refer to Section 6.2.8.4 for reagent composition

The results given in Table 6.2 indicated that Reagent 3 was the most effective of the three reagents tested. Reagent 3 contained 80% TFA, which, based on the results reported in Chapters 4 and 5, was likely to degrade the Perloza resin. Reagent 3 was therefore diluted to final TFA concentrations of 16% and 8%. The dilutions were named Reagents 4 and 5 respectively. The exposure time of the peptide-amide linker-resin to

Reagents 4 and 5 was increased in comparison to that used for Reagents 1-3 in order to compensate for the lower concentration of the cleavage reagents. Samples of the cleavable peptide-resin was treated with Reagents 4 and 5 for three hours. HPLC analysis of the solutions showed that after three hours Reagent 4 had cleaved almost all of the Mtr from the N^G of arginine (see Table 6.2). Reagent 5 was not as effective as Reagent 4 at cleaving the N^G-Mtr group after 3 hours (Table 6.2).

All of the reagents tested gave a product which on HPLC had the same retention time as authentic LHRH. This was significant because the tryptophan of LHRH can be readily alkylated by Mtr from arginine unless appropriate scavengers are used (Sieber, 1987). If the tryptophan had been alkylated the retention time on HPLC would have been expected to differ from that of authentic LHRH.

LHRH was a useful model peptide to use in this study because it contained the alkylation-sensitive tryptophan residue in company with the difficult to cleave Arg N^G-Mtr group. In addition, the sometimes difficult to cleave tBu ether of serine (Ramage et al, 1991) was present. It would be expected that if a cleavage reagent was found to be suitable for removing the side chain protecting groups employed in this study of LHRH, then it should be usable at least for most short (<15 residues) peptide-ligands.

Reagent 4 was selected for cleavage of amino acid side chain protecting groups from the non-cleavable LHRH-resin. The reagent performance was evaluated in two ways. Two of the amino acids of LHRH bound to Perloza, His and Arg, had side chains which could be titrated with picric acid. Since the peptide substitution was known from the amino acid analysis results (0.20 mmole/g, Section 6.3.3.2), the amine substitution expected on quantitative side chain cleavage could be calculated. Allowance had to be made in the calculation for loss of weight due to removal of side chain protecting groups. The final peptide substitution expected on quantitative side chain cleavage (assuming no cleavage of peptide from the resin) would be 0.23 mmole/g (for calculation see Equation 6.1, Section 6.2.8.5). The expected titration result would be twice the expected peptide substitution level, that is, 0.46 mmole/g (one His, one Arg, no N-terminal amine).

In a preliminary experiment, side chain protected Resin 1 was treated with Reagent 4 for three hours. Samples of the LHRH-resin were taken after 2 and 3 hours for picrate titration and amino acid analysis (see Table 6.3). After both 2 and 3 hours the amine substitution by picrate titration was 0.46 mmole/g, which indicated quantitative side chain deprotection with insignificant cleavage of peptide from the support.

The peptide substitution, after treatment of the peptide-resin with Reagent 4, as determined by amino acid analysis, was slightly higher than expected (0.28 mmole/g by titration compared to 0.23 mmole/g by calculation). However, the picrate titration result was as expected from calculation. Considering the good agreement between the picrate titration result and the calculated amine substitution, the higher than expected amino acid analysis result may have been due to an error in the calibration of the amino acid analyser for that day.

Table 6.3 Amino acid analyses of LHRH-Perloza after treatment with Reagent 4

Amino acid	Expected	Initial	2hr reaction	3hr reaction
Ser	1	0.78	0.80	0.80
Glu	1	0.98	0.93	0.92
Pro	1	1.09	0.93	0.90
Gly	2	2.00	2.00	2.00
Leu	1	0.81	0.95	0.91
Tyr	1	0.74	0.74	0.73
His	1	0.80	0.84	0.81
Arg	1	0.83	0.82	0.84
Trp	1	n.d.	n.d.	n.d.

Peptide substitution based on Gly result:

2 hr 0.27 mmole/g

3 hr 0.28 mmole/g

Expected by calculation: 0.23 mmole/g

Following the success of the preliminary experiment, the bulk of the non-cleavable LHRH-Perloza was treated with Reagent 4 for 3 hours to cleave the amino acid side chain protecting groups. The amine substitution as determined by picrate titration was 0.44 mmole/g (theory 0.46 mmole/g), and indicated substantial removal of amino acid side chain protecting groups. The LHRH-Perloza 500 peptide-resin (Resin 1) was then used to study binding and elution of antibodies to LHRH (see Section 6.2.1, gift of Dr. K.P. McNatty, MAF, Wallaceville, NZ).

6.3.4 Use of Resin 1 for isolation of antibodies to LHRH

Buffers used in this study were:

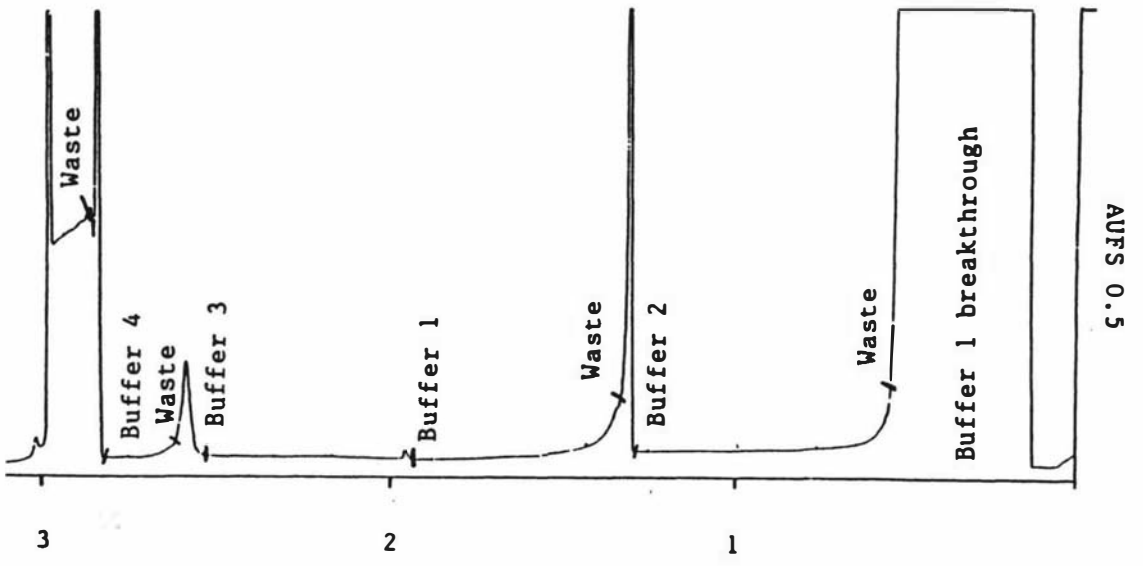
Buffer 1 10mM Tris, pH 7.5
Buffer 2 10mM Tris, pH 7.5, 500mM NaCl
Buffer 3 10mM citrate, pH 3.0
Buffer 4 5M guanidine hydrochloride, pH 1.5

A copy of the A_{280} trace of the antibody affinity purification is given as Figure 6.5. Samples from this run were analysed by SDS-PAGE, a photograph of the gel is given as Figure 6.6. Antibodies consist of two types of polypeptide chains (the heavy chain, molecular weight approximately 55000, and the light chain, molecular weight approximately 25000) held together by disulphide bonds. Because the samples applied to the SDS-PAGE gels were reduced using βME, both the heavy and light chains were expected to be seen on the gel. Lanes 5 and 6 of Figure 6.6 are of the buffer 2 and buffer 3 washes respectively. Both of these fractions show the presence of immunoglobulin heavy chain, although the light chain does not appear to have been stained, even though RIA indicated that antibodies to LHRH were present in the samples run in these lanes. Lane 7 was the fraction from the elution buffer, that is, buffer 4, and lane 8 was the fraction from a wash of the column with 0.1M NaOH. These fractions also contained heavy chain immunoglobulin, but again the light chain does not appear to have stained. Fractions represented by lanes 5-8 all contained a band, heavier than the immunoglobulin heavy chain, that was of the same weight as albumin (run in lane 2). RIA of the fractions run in lanes 5-7 indicated that antibodies to LHRH were present in all of the fractions. However, the fractions represented by

lanes 6 and 7 in Figure 6.5 (lane 6:- buffer 3 (10mM citrate, pH 3.00): lane 7:- buffer 4 (5M guanidine.HCl, pH 1.5)) appeared to contain mainly immunoglobulin heavy chain, with some heavier contaminant protein, which had a similar weight to albumin. The results of this preliminary experiment (not optimised) showed that it was possible to construct a peptide-ligand onto Perloza for the affinity isolation of antibodies specific to the peptide-ligand.

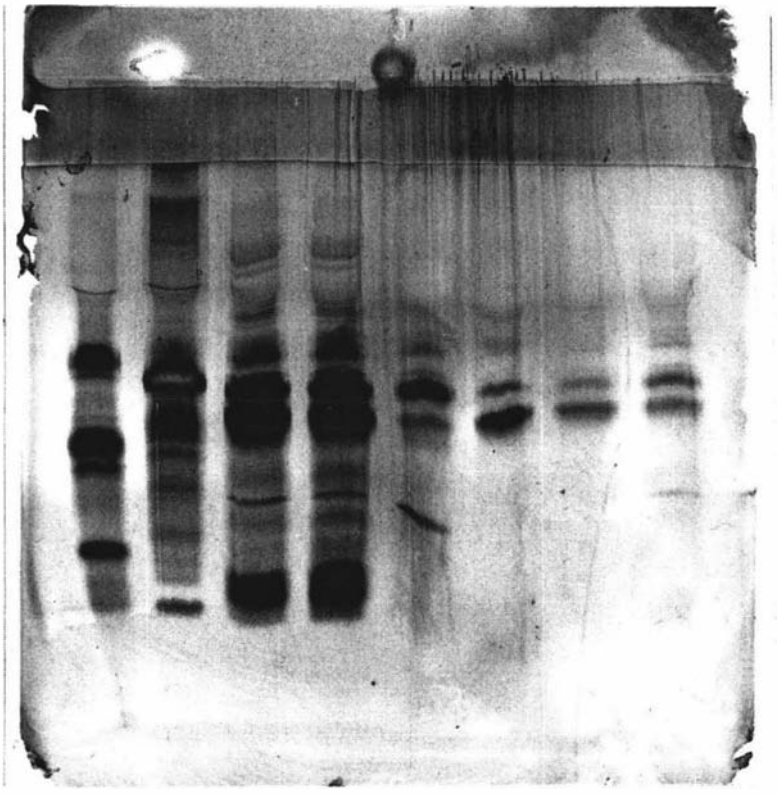
RIA (performed by Mr. S. Lun, MAF, Wallaceville, NZ) of the fractions from the purification run (Figure 6.5) showed that antibodies to LHRH were present in every fraction, including the breakthrough peak. Because the antibodies were from a polyclonal source, the presence of antibodies to LHRH in every fraction may have been due to different populations of antibody, each with different affinity for the LHRH ligand, being present in the serum. Alternatively, the column capacity may have been exceeded, or the flow rate of the serum through the column may have been too fast to allow time for full interaction of the antibodies with the immobilised peptide-ligand.

Figure 6.5 **A_{280} trace of purification of antibodies to LHRH using Resin 1**



Buffer 1: 10mM Tris, pH 7.5
Buffer 2: 10mM Tris, pH 7.5, 500mM NaCl
Buffer 3: 10mM citrate, pH 3.0
Buffer 4: 5M guanidine hydrochloride, pH 1.5

Figure 6.6 SDS-PAGE gel of fractions from affinity purification of antibodies to LHRH using Resin 1

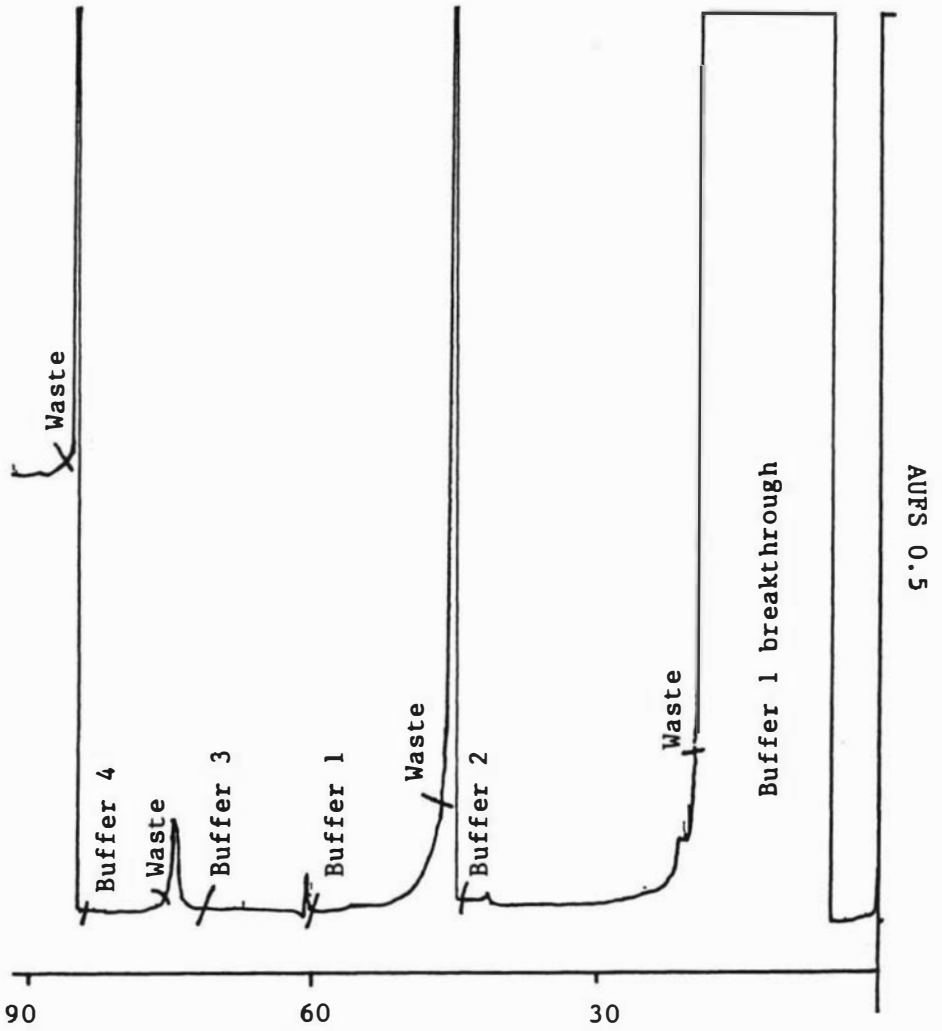


Lane 1: Molecular weight standards
Lane 2: ICP bovine albumin
Lane 3: IgG serum fraction loaded onto column
Lane 4: Buffer 1 (10mM Tris, pH 7.5) breakthrough fraction
Lane 5: Buffer 2 (10mM Tris, pH 7.5, 500mM NaCl) eluate
Lane 6: Buffer 3 (10mM citrate, pH 3.0) eluate
Lane 7: Buffer 4 (5M guanidine hydrochloride, pH 1.5) eluate
Lane 8: 0.1M NaOH eluate

6.3.4.1 Binding of serum components to Resin 1

The purpose of this experiment was to determine whether any components of serum would bind to the peptide-resin. The lamb serum used in this study was not subjected to ammonium sulphate precipitation in order to give a "worst case" indication of non-specific binding. Lamb serum was diluted and run through Resin 1 using the same set of washes employed for the antibody isolation run. A copy of the A_{280} trace is given as Figure 6.7. Samples of the fractions were analysed by SDS-PAGE, a photograph of the gel is given as Figure 6.8. Lanes 6 and 7 of Figure 6.8 represent the elution buffer (buffer 4, 5M guanidine hydrochloride, pH 1.5) and 0.1M NaOH washes of the peptide-resin. The gel shows that significant amounts of protein had bound to the peptide-resin, which were only eluted using relatively harsh conditions (lane 6; buffer 4 {5M guanidine.HCl, pH 1.5}: lane 7: 0.1M NaOH). The major protein present appeared to have the same weight as albumin (run in lane 1). From the results given in the previous Section (see Figure 6.6, lanes 6 and 7), it appeared that albumin was also eluted when the column, with antibodies to LHRH bound (as shown by RIA), was washed with buffer 4 and 0.1M NaOH. From the results given in these two Sections (6.3.4, 6.3.4.1), it appeared that there was some non-specific binding of a serum component (possibly albumin) to the peptide-resin, as well as binding by the antibodies to LHRH.

Figure 6.7 A_{280} **trace of experiment to determine binding of serum components to Resin 1**



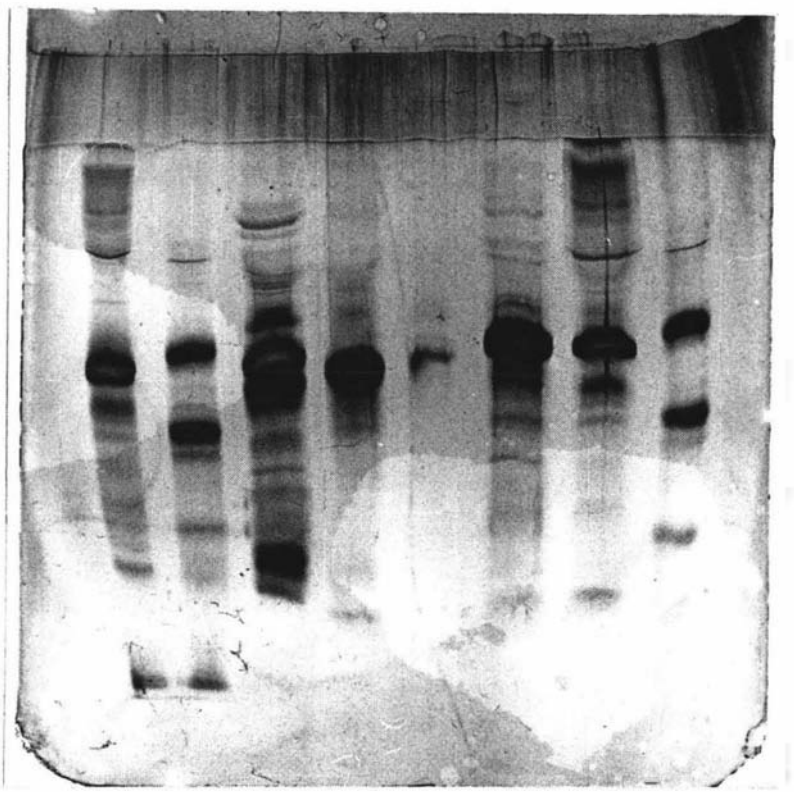
Buffer 1: 10mM Tris, pH 7.5

Buffer 2: 10mM Tris, pH 7.5, 500mM NaCl

Buffer 3: 10mM citrate, pH 3.0

Buffer 4: 5M guanidine hydrochloride, pH 1.5

Figure 6.8 SDS-PAGE gel of serum components bound to Resin 1



Lane 1: ICP bovine albumin
Lane 2: Molucular weight standards
Lane 3: Lamb serum loaded onto column
Lane 4: Buffer 2 (10mM Tris, pH 7.5, 500mM NaCl) eluate
Lane 5: Buffer 3 (10mM citrate, pH 3.0) eluate
Lane 6: Buffer 4 (5M guanidine hydrochloride, pH 1.5) eluate
Lane 7: 0.1M NaOH eluate
Lane 8: Molecular weight standards

6.3.5 Synthesis of Perloza-S-(Ac)-Cys-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 (Resin 2)

6.3.5.1 Reactions of α-bromoacetamido Perloza

These experiments were carried out to determine the viability of anchoring a cysteine-containing peptide-ligand to α-bromoacetamido functionalised Perloza via a stable thioether bond. One of the concerns in this study was whether a cysteine-containing peptide would react quantitatively with α-bromoacetamido Perloza. The presence of unreacted bromine was of concern in that it might be available for reaction with cysteine-containing proteins derived from solutions to be affinity purified. If other proteins coupled to the resin they could potentially add another binding component to the affinity matrix. Therefore a study was carried out to test reagents that could be used to block unreacted α-bromoacetamido groups.

α-Bromoacetamido Perloza 500 Medium resin was made using the procedure reported in Section 6.2.10.1. The bromine substitution was 0.50 mmole/g by elemental analysis (0.52 mmole/g theory). A ninhydrin assay of the α-bromoacetamido resin indicated that the reaction of α-bromoacetic anhydride with aminopropyl Perloza had gone in 99.8% yield. 2-Mercapto-ethanol, ethanolamine, NaOH (0.1M), and Na_2CO_3 (0.1M), solutions were tested for reaction with α-bromoacetamido Perloza. The results of the reactions of α-bromoacetamido Perloza with βME, NaOH, and Na_2CO_3 are given in Tables 6.4, 6.5, and 6.6 respectively. The reaction of ethanolamine is discussed below.

Table 6.4 Reaction of α-bromoacetamido Perloza 500 Medium with βME

Time (min)	Initial	15	60
Br substitution (mmole/g)	0.50	0.08	0.06
Sulphur substitution (mmole/g)*	0	0.51	0.45

* Theory for quantitative reaction: 0.52 mmole/g

Table 6.5 Reaction of α-bromoacetamido Perloza 500 Medium with 0.1M NaOH solution

Time (min)	Initial	5	15	30	60
Br substitution (mmole/g)	0.50	0.45	0.43	0.45	0.38

Table 6.6 Reaction of α-bromoacetamido Perloza 500 Medium with 0.1M Na_2CO_3 solution

Time	Initial	5	15	30	60
Br substitution (mmole/g)	0.50	0.51	0.53	0.52	0.57

The results given in Tables 6.4-6.6 showed that only βME and 0.1M NaOH reacted to a significant extent with α-bromoacetamido Perloza over 60 minutes. From the result of the analysis for bromine, βME (Table 6.4) appeared to have given about 84% reaction after 15 minutes, and 88% reaction after one hour. The 0.1M NaOH solution (Table 6.5) gave slow cleavage of the bromine from the resin. The 0.1M Na_2CO_3 solution (Table 6.6) appeared to give an increase of bromine substitution, but the apparent increase with time was probably due to experimental variation in the elemental analysis result. Ethanolamine reacted with α-bromoacetamido Perloza to form a resin-bound secondary amine (Scheme 6.1), which could be titrated with picric acid.

Scheme 6.1 Reaction of α-bromoacetamido Perloza with ethanolamine

$$\text{Perloza-CH}_2\text{-Br} + \text{HO-CH}_2\text{CH}_2\text{-NH}_2 \rightarrow \text{Perloza-CH}_2\text{-NH}_2^{+}\text{-CH}_2\text{-CH}_2\text{-OH} + \text{Br}^{-}$$

Titration of the resin after one hour gave an amine substitution of 0.06 mmole/g. Picrate titration of the initial α-bromoacetamido Perloza gave an amine substitution of 0.01 mmole/g. As the initial bromine substitution was 0.52 mmole/g, the result indicated that only 10% of the bromine groups had reacted with ethanolamine after one hour. This result may be compared to the reaction with βME (Table 6.4), which

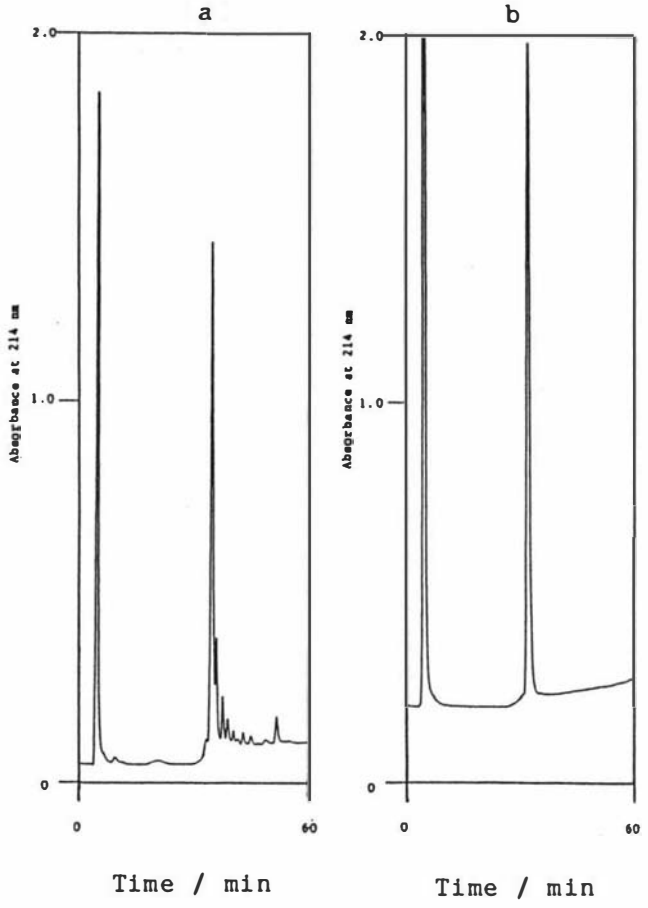
showed approximately 88% reaction after one hour. A peptide intended to be anchored to α-bromoacetamido Perloza would probably contain at least an N-terminal amino group, as well as the thiol intended for reaction with the α-bromoacetamido resin. The significance of the results of the ethanolamine and βME reactions is that they showed that reaction of the α-bromoacetamido group with a peptide-thiol would probably be favoured over reaction with a peptide-amine, at the pH used in this study.

Only 2 equivalents of βME were used in the βME coupling experiment (Table 6.4). The result of the elemental analyses for bromine suggested that not all of the α-bromoacetamido groups had reacted with βME after one hour. Possibly a larger excess of βME would give quantitative blocking of the α-bromoacetamido groups. The results found for the βME experiment suggested that some of the α-bromoacetamido groups may not be available for reaction with thiol-containing ligands, even those of low molecular weight such as βME. If the α-bromoacetamido groups were not available for reaction with low molecular weight thiols due to steric reasons, they would not be expected to be available for reaction with high molecular weight proteins.

6.3.5.2 Synthesis of Ac-Cys-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2

The peptide Ac-Cys-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 was synthesised on an amide-linker Perloza resin using the Fmoc solid phase peptide synthesis protocols reported in Chapter 5, but with Fmoc-amino acid activation via HOBt esters formed in DCM (Section 6.2.8.1). Two cleavages of the peptide were carried out. The first resulted in a cleavage yield of 58%, with a 25% yield of purified peptide, based on the initial amino acid substitution of the resin. Amino acid analysis of the purified peptide gave the expected ratios: Ser 0.82 [1], Pro 1.00 [1], Gly 2.00 [2], Cys 0.77 [1], Leu 0.98 [1], Tyr 1.00 [1], Arg 0.98 [1]. The purified peptide was used for reaction with α-bromoacetamido Perloza in a preliminary experiment to determine whether the peptide would couple to the resin. The excess of peptide over α-bromoacetamido groups was 1.33X. The coupling reaction was carried out in 0.1M $NaHCO_3$ solution for one hour. Picrate titration of the arginine N^G gave an amine substitution of 0.36 mmole/g (theory for quantitative reaction: 0.37

Figure 6.9 HPLC traces of a) crude, b) purified, Ac-Cys-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2



mmole/g). The dried resin remaining from the picrate titration was used for elemental and amino acid analyses. Elemental analysis showed the resin had a bromine substitution of 0.07 mmole/g. The sulphur substitution was 0.35 mmole/g (theory 0.37 mmole/g). The titration and sulphur elemental analysis results suggested that the coupling reaction had gone in greater than 94% yield, however the presence of bromine suggested otherwise. The amino acid analysis result showed a peptide substitution of 0.37 mmole/g (theory 0.37 mmole/g) based on glycine. The results of three analyses to determine the peptide substitution level (picrate titration, elemental analysis for sulphur, amino acid analysis) indicated that the coupling reaction had gone in near quantitative yield. The presence of bromine was therefore not expected. A more rigorous investigation into the reasons for non-quantitative displacement of bromine by thiol-containing ligands will have to be carried out.

Regardless of some doubt concerning the coupling yield, this experiment showed that a cysteine-containing peptide could be coupled to α-bromoacetamido Perloza in very high yield using only a slight excess of the peptide over resin bound α-bromoacetamido groups.

A second cleavage of Ac-Cys-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 peptide was carried out. The cleavage yield was 66%, the yield of purified peptide was 30% based on the initial amino acid substitution of the resin. Amino acid analysis of the purified peptide gave the expected ratios: Ser 0.69 [1], Pro 0.93 [1], Gly 2.00 [2], Leu 0.94 [1], Tyr 0.88 [1], Arg 0.92 [1], Cys 0.78 [1]. HPLC traces of the crude and purified peptide are given in Figure 6.9. The purified peptide (1.5 eq) was coupled to α-bromoacetamido Perloza for two hours in the presence of tri-n-butyl phosphine. Tri-n-butyl phosphine was added to maintain the peptide thiol moiety in a reduced state during the course of the coupling reaction (Ruegg and Rudinger, 1977; Kolodny and Robey, 1990). After 2 hours βME was added to block unreacted α-bromoacetamido groups, and reaction was continued for another hour. Elemental analysis of the peptide-resin gave a bromine substitution of 0.05 mmole/g. The sulphur substitution was 0.41 mmole/g (theory 0.37 mmole/g). The peptide substitution as determined by amino acid analysis was 0.36 mmole/g (theory 0.37 mmole/g). The sulphur elemental analysis result, and amino

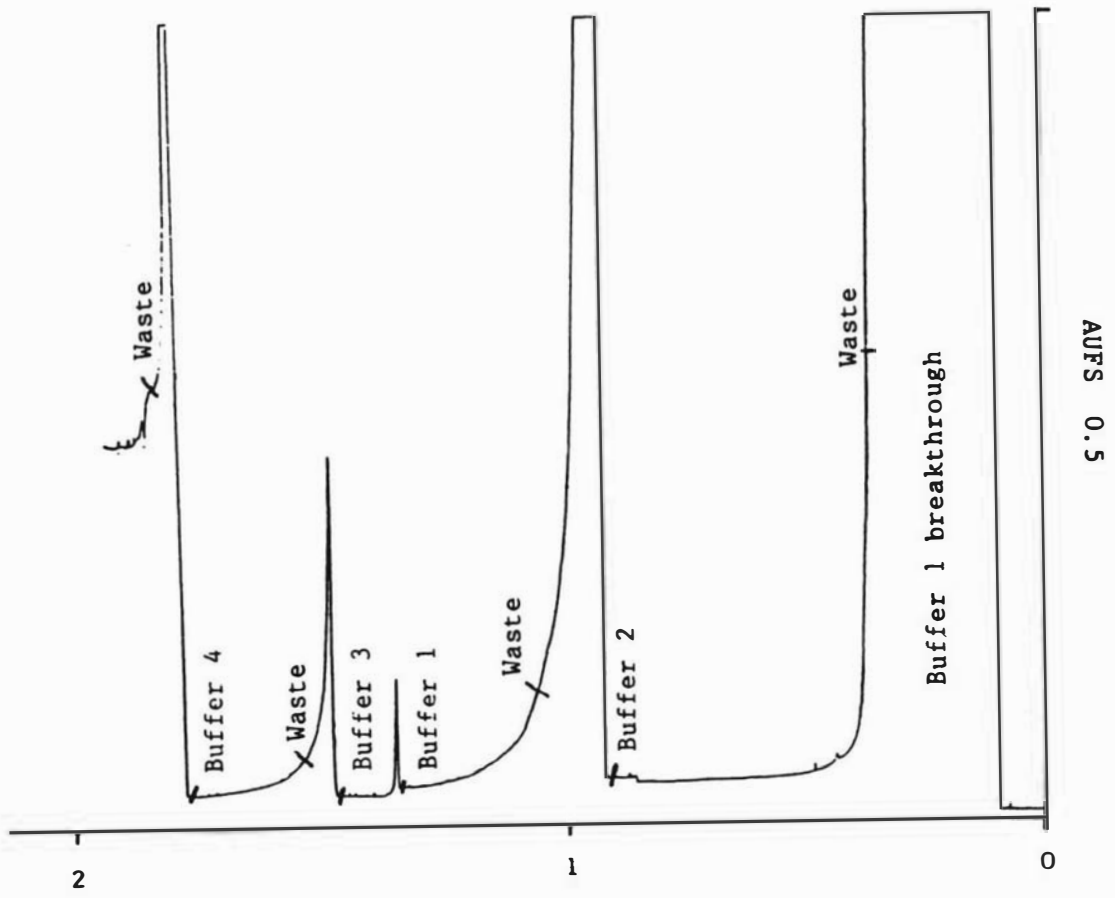
acid analysis result, suggested that the peptide coupling reaction had gone in high yield.

In this peptide coupling reaction steps were taken to maximise the coupling yield by addition of tri-n-butyl phosphine, and to block unreacted α-bromoacetamido groups with βME. Even though βME was added after 2 hours to block unreacted α-bromoacetamido groups, bromine was still found by elemental analysis (0.05 mmole/g). For steric reasons, it was considered unlikely that any residual α-bromoacetamido groups would be available for reaction with thiol-containing proteins.

6.3.6 Use of Resin 2 for binding antibodies to LHRH

Serum containing antibodies to LHRH was subjected to ammonium sulphate precipitation as a first step to isolate immunoglobulins from other serum components. The precipitate containing the serum immunoglobulins was dissolved in pH 7.5 10mM Tris buffer, and then dialysed against the same buffer. The dialysed solution was then subjected to affinity chromatography using Resin 2 as the affinity matrix (Section 6.2.11). A copy of the A_{280} trace of the antibody affinity purification is given as Figure 6.10. Samples from this run were analysed by SDS-PAGE, a photograph of the gel is given as Figure 6.11. Lanes 5 and 6 of Figure 6.11 are of the buffer 1 and buffer 2 washes respectively. Both of these fractions show the presence of heavy and light immunoglobulin chains. Lane 7 was the fraction from buffer 3, and lane 8 was the fraction from a wash of the column with buffer 4, the elution buffer. Fractions represented by lanes 7 and 8 contained a band, heavier than the immunoglobulin heavy chain, that was of the same weight as albumin (run in lane 2). RIA of the fractions run in lanes 5-8 indicated that antibodies to LHRH were present in all of the fractions. However, the fraction represented by lane 8 in Figure 6.11 (lane 8: buffer 4 {5M guanidine.HCl, pH 1.5}) appeared to contain mainly light and heavy immunoglobulin chains, with some heavier contaminant protein, which had a similar weight to albumin. The results of this preliminary experiment (not optimised) showed that it was possible to use thioether chemistry to immobilise a peptide-ligand onto Perloza for the affinity isolation of antibodies specific to the ligand.

Figure 6.10 A_{280} **trace of purification of antibodies to LHRH using Resin 2**



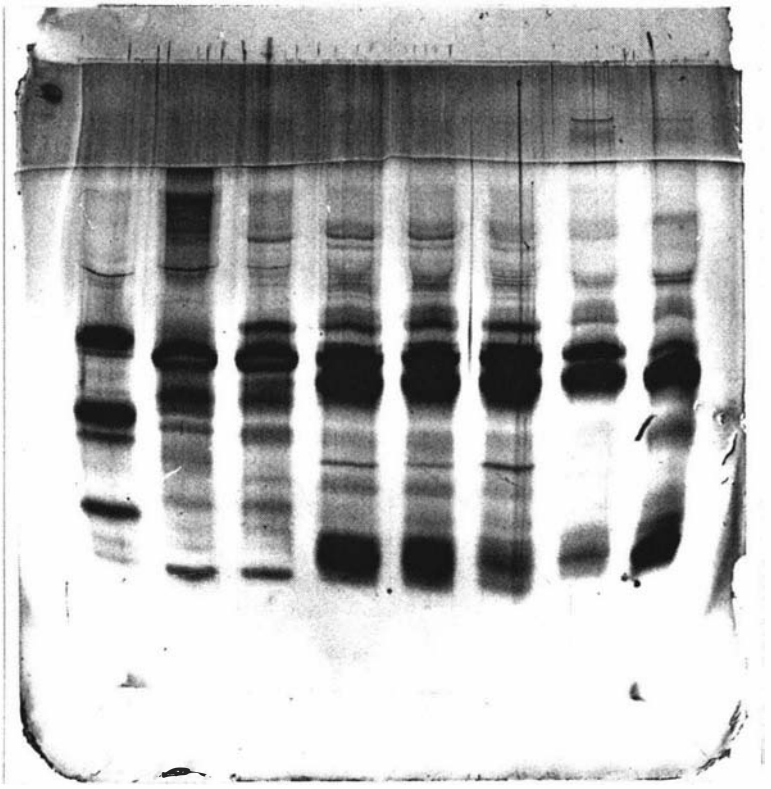
Buffer 1: 10mM Tris, pH 7.5

Buffer 2: 10mM Tris, pH 7.5, 500mM NaCl

Buffer 3: 10mM citrate, pH 3.0

Buffer 4: 5M guanidine hydrochloride, pH 1.5

Figure 6.11 SDS-PAGE gel of fractions from affinity purification of antibodies to LHRH using Resin 2

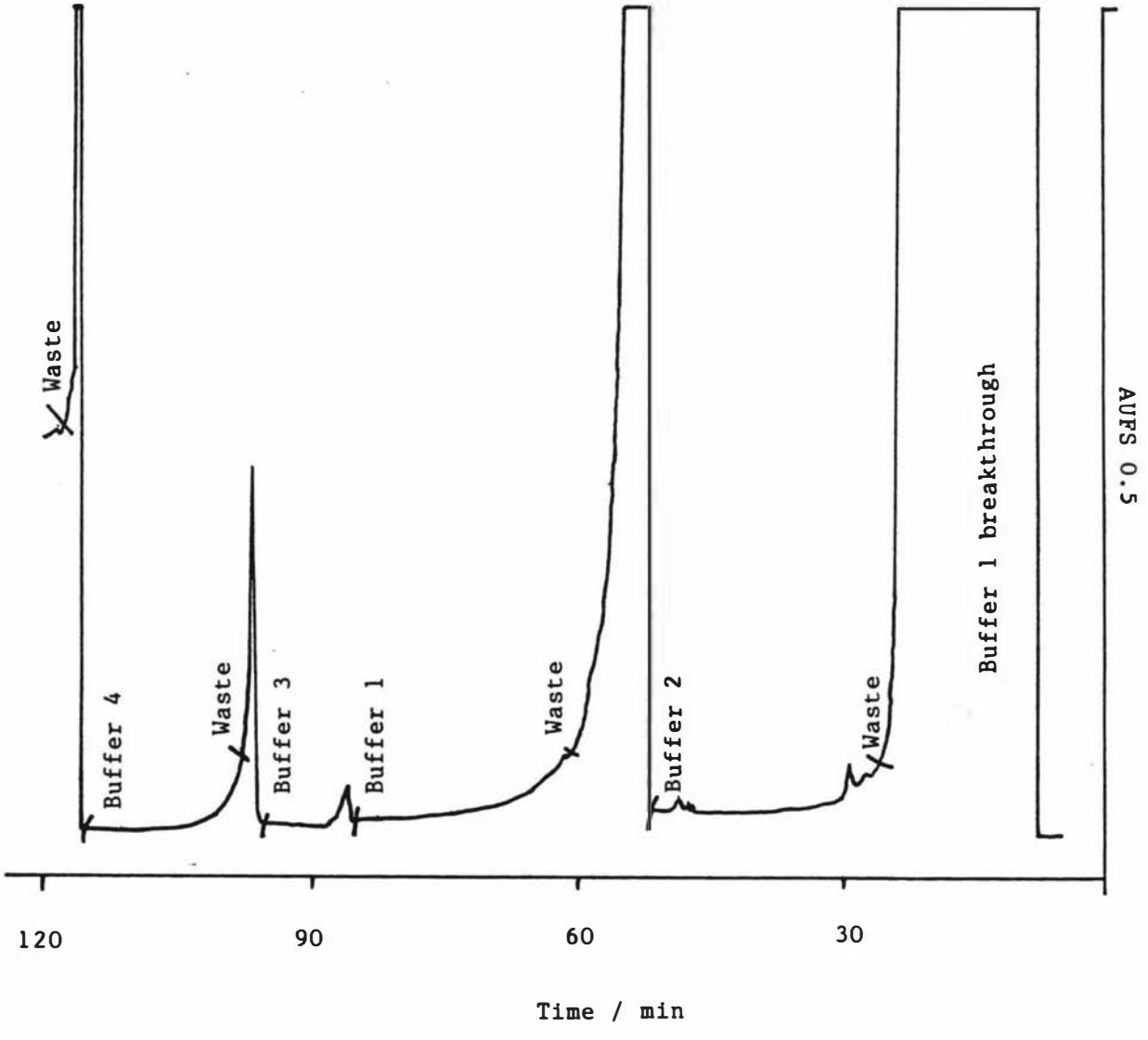


Lane 1: Molecular weight standards
Lane 2: ICP bovine albumin
Lane 3: Supernatant from ammonium sulphate fractionation of serum
Lane 4: IgG serum fraction loaded onto column
Lane 5: Buffer 1 (10mM Tris, pH 7.5) breakthrough fraction
Lane 6: Buffer 2 (10mM Tris, pH 7.5, 500mM NaCl) eluate
Lane 7: Buffer 3 (10mM citrate) eluate
Lane 8: Buffer 4 (5M guanidine hydrochloride, pH 1.5) eluate

6.3.6.1 Binding of serum components to Resin 2

The purpose of this experiment was to determine whether serum components would bind non-specifically to the peptide-resin. The lamb serum used in this study was not subjected to ammonium sulphate precipitation in order to give a "worst case" indication of non-specific binding. Lamb serum was diluted and run through Resin 2 using the same set of washes employed for the antibody isolation run. A copy of the A_{280} trace is given as Figure 6.12. Samples of the fractions were analysed by SDS-PAGE, a photograph of the gel is given as Figure 6.13. Lanes 5, 6 and 7 of Figure 6.13 represent the buffer 3, elution buffer (buffer 4) and 0.1M NaOH washes of the peptide-resin. Figure 6.13 shows that significant amounts of non-specifically bound protein were only eluted using relatively harsh conditions (lane 7: 0.1M NaOH). The major protein present appeared to have the same weight as albumin (run in lane 1). Very little protein appeared to be eluted by the wash with buffer 4 (the elution buffer, 5M guanidine.HCl, pH 1.5). From the results given in the previous Section (see Figure 6.10, lane 8), it appeared that albumin was also eluted when the column, with antibodies to LHRH bound (as shown by RIA), was washed with buffer 4. From the results given in these two Sections (6.3.6, 6.3.6.1), it appeared that there was some non-specific binding of a serum component (possibly albumin) to the peptide-resin, as well as binding by the antibodies to LHRH. However, it appeared that only low levels of the non-specifically bound protein were eluted on washing the column with the antibody elution buffer (buffer 4: 5M guanidine hydrochloride, pH 1.5). In addition, lane 8 of Figure 6.11 (the LHRH antibody purification using Resin 2) showed that heavy and light chains of immunoglobulin appeared to be the major component eluted from the column on washing with buffer 4.

Figure 6.12 A_{280} **trace of experiment to determine binding of serum components to Resin 2**



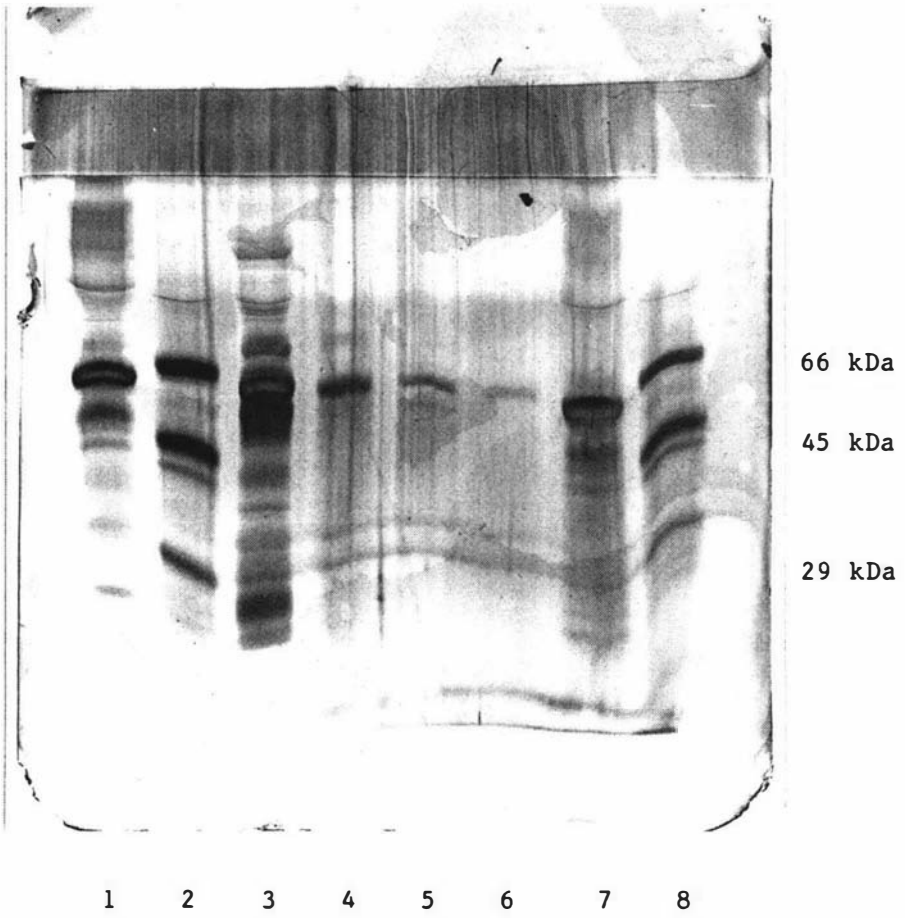
Buffer 1: 10mM Tris, pH 7.5

Buffer 2: 10mM Tris, pH 7.5, 500mM NaCl

Buffer 3: 10mM citrate, pH 3.0

Buffer 4: 5M guanidine hydrochloride, pH 1.5

Figure 6.13 SDS-PAGE gel of serum components bound to Resin 2



Lane 1: ICP bovine albumin

Lane 2: Molecular weight standards

Lane 3: Lamb serum loaded onto column

Lane 4: Buffer 2 (10mM Tris, pH 7.5, 500mM NaCl) eluate

Lane 5: Buffer 3 (10mM citrate, pH 3.0) eluate

Lane 6: Buffer 4 (5M guanidine hydrochloride, pH 1.5) eluate

Lane 7: 0.1M NaOH eluate

Lane 8: Molecular weight standards

6.3.7 Binding and elution of antibodies to LHRH: a summary

Both Resin 1 and Resin 2 were able to bind antibodies, even though the two peptide ligands presented to the solution were the opposite ends of the LHRH molecule. It is interesting to speculate as to whether the antibodies bound by the two resins represented two populations, each specific to a different portion of the LHRH molecule. For example, Copeland et al (1979) tested binding of a number of different antisera to a series of LHRH analogues and found wide variation in the binding specificity of the different antisera. It may be expected that a variety of polyclonal antibodies would be generated in a sheep, with each population able to recognise different portions of the LHRH molecule. It was not the aim of this study to study the LHRH-Ab ↔ Ag interaction as such, but rather to demonstrate binding and elution of antibodies with retention of biological activity. The results of this study suggested that this objective had been accomplished.

6.4 CONCLUSIONS

1. A peptide-ligand could be synthesised directly onto aminopropyl Perloza for affinity chromatographic purposes using Boc methodology if no side chain protected amino acids were present. Further study will be needed, especially on deprotection of trifunctional amino acids, before this method becomes as generally applicable as that found in the Fmoc study.

2. A peptide-ligand could be synthesised directly onto aminopropyl Perloza for affinity chromatographic purposes using Fmoc methodology. Side chains could be deprotected using Reagent 4 (DCM, 80%; TFA, 16%; TMSBR, 1%; thioanisole, 1%; EDT, 1%; m-cresol, 1%) without significant cleavage of peptide from the resin, and without degradation of the resin. The peptide-resin could then be used for affinity chromatographic purposes.

3. A cysteine containing peptide could be coupled to α-bromoacetamido Perloza in high yield. The peptide-resin could then be used for affinity chromatographic purposes.

4. Perloza was again shown to be a suitable matrix for affinity chromatographic applications.

CHAPTER 7

PERLOZA BEADED CELLULOSE AS A SUPPORT FOR SPPS: A SUMMARY AND ASSESSMENT OF POSSIBLE FUTURE DIRECTIONS

CHAPTER 7 PERLOZA BEADED CELLULOSE AS A SUPPORT FOR SPPS: A SUMMARY AND ASSESSMENT OF POSSIBLE FUTURE DIRECTIONS

The results of the studies reported in this thesis demonstrated the utility of Perloza beaded cellulose as a solid support for a number of applications. The key step in these studies was synthesis of aminopropyl Perloza by reduction of cyanoethyl Perloza with diborane. It was possible to obtain cyanoethyl Perloza with controllable nitrogen substitution for subsequent reduction to aminopropyl Perloza. This study appeared to be one of only two (see Daly and Munir, 1984) dealing with diborane reduction of cyanoethyl cellulose.

Peptides were synthesised using Boc SPPS methodology with Perloza as the support, and a novel cleavage reagent, BF_3/dioxane, for Boc cleavage. Peptides were also synthesised on Perloza using essentially standard Fmoc SPPS methodology, but with peptide cleavage workup protocols modified to cater for the differences between Perloza and the standard supports used for Fmoc SPPS.

It was found that Perloza was usable for SPPS in both a low pressure pumped continuous flow peptide synthesiser (LKB Biolynx 4175), and in a discontinuous batchwise peptide synthesiser (ABI 430A). This may possibly be a unique feature of Perloza as an SPPS support, because the supports commonly used for SPPS can usually only be used in one of the two types of peptide synthesiser, but not both.

Peptide-ligands were synthesised onto aminopropyl Perloza for subsequent use in affinity purification of biological macromolecules. Both chymosin and antibodies to LHRH were isolated using peptide-ligands synthesised directly onto Perloza. A mild procedure was developed for cleavage of amino acid side chain protecting groups of Perloza-bound peptides synthesised using the Fmoc SPPS methodology.

In addition, a means for achieving directed orientation of support-bound peptide-ligands was conceived and developed. A cysteine-containing analogue of LHRH was reacted with α-bromoacetamido Perloza to immobilise the peptide to the support via a stable thioether bond. The peptide-Perloza conjugate was used for the affinity isolation of antibodies to LHRH.

However, a number of aspects of the work reported in this thesis may bear further investigation. These are discussed below.

It was found in this study that cyanoethyl Perloza could be reduced both at room temperature over 20 hours, or under reflux for 3 hours. The reflux reduction was eventually chosen for routine use because of the superior flow properties of aminopropyl Perloza made using this method. However, it may be possible to achieve similar quality products by reduction at room temperature by using swirling instead of magnetic stirring as the means of agitating the resin. One aspect of the reflux reduction which could be worth considering is the variability in the reduction yields. In some case quantitative reduction was achieved, while in others the yield was as low as 52%. It may be that all that is required is greater time spent refluxing the cyanoethyl Perloza.

As an alternative to reduction of cyanoethyl Perloza, simpler, cheaper methods of functionalising Perloza to give an amine group linked to the support via a spacer arm and stable ether bond may be worth investigating. One possible means would be to functionalise the resin with epichlorohydrin, followed by treatment with anhydrous ammonia solution to open the epoxide ring and generate a primary amine group at the end of the spacer arm. A second means of providing Perloza with amine groups may be to reinvestigate carboxymethylation of Perloza followed by reaction with a diamine in the presence of a condensing agent such as EDC or DIC. This method was initially investigated (see Chapter 2), but was shelved because reaction yields were only about 50%, and more success was being achieved in the studies of reduction of cyanoethyl Perloza.

The ninhydrin assay sometimes gave resin-bound amine substitution results significantly lower than those obtained by picrate titration. The ninhydrin assay is more convenient and faster to carry out than the picrate titration. Therefore, it may be worthwhile to investigate the reasons for the disagreement between the results of the two methods, and to develop a ninhydrin assay suitable for use with Perloza.

Two peptides, LAGV and Leu-enkephalin, were synthesised on Perloza using Boc SPPS methodology and the base-labile glycolamide linker. However,

four attempts to synthesise the decapeptide ACP 65-74 (VQAAIDYING) using the same methodology met with failure. However, it was possible to synthesise this peptide on Perloza using Fmoc SPPS methodology. A more rigorous investigation of SPPS on Perloza using the Boc SPPS methodology may be worth undertaking. One approach which could be tried is to use the PAM linker to anchor Boc-amino acids to Perloza for subsequent SPPS. Cleavage of peptides would have to be using either liquid HF (which dissolves Perloza) or a strong acid such as TFMSA in TFA.

A potential means of synthesising short protected peptides using Boc chemistry, BF_3/dioxane for Boc cleavage, and the acid labile HMPA linker (see Chapter 5) arose from the observation that valine-HMPA-Perloza (initial amine substitution: 0.53 mmole/g) treated with 1M BF_3/dioxane for one hour showed minimal loss of valine as determined by picrate titration (final amine substitution: 0.51 mmole/g, a loss of 4% over one hour). One hour's exposure to 1M BF_3/dioxane is equivalent to 6X 10 minute Boc cleavage cycles. Therefore, it may be possible to synthesise short peptides on Boc-amino acyl-HMPA-Perloza using BF_3/dioxane for Boc cleavage, and to cleave the peptides using TFA. The side chain protecting groups used for the Boc SPPS methodology are stable to TFA, and therefore the peptide that resulted from such a synthesis and cleavage would be side chain protected. A side chain protected peptide could then be used for fragment couplings in solution.

To test the feasibility of using a combination of Boc chemistry, BF_3/dioxane for Boc cleavage, and the acid labile HMPA linker, the tripeptide Val-Val-Arg was synthesised using Fmoc-Arg(Mtr)-HMPA-Perloza as the starting resin (Arg substitution after Fmoc cleavage: 0.35 mmole/g). The Fmoc group was cleaved using standard piperidine treatment. Boc-Val was then coupled, and the Boc group was cleaved using 1M BF_3/dioxane. Boc-Val was then coupled to the free amino terminus, and the Boc group again cleaved using 1M BF_3/dioxane. The amine substitution of the peptide-resin at the end of the synthesis was found to be 0.33 mmole/g (theory: 0.33 mmole/g), which indicated that significant cleavage of the peptide-HMPA linkage by 1M BF_3/dioxane had not occurred. The peptide was cleaved from the support using Reagent B (Chapter 5), and was purified by HPLC. Sequencing of the purified peptide gave the expected amino acids.

Use of the Fmoc SPPS methodology with Perloza gave peptides of acceptable quality in reasonable yields. One of the procedures used in the Fmoc study was anchoring of Fmoc-amino acids to Perloza by reaction of preformed Fmoc-amino acyl-4-oxy-methylphenoxyacetic acid 2,4-dichlorophenyl esters with aminopropyl Perloza. In some cases the coupling yields obtained using this procedure, while adequate for SPPS, were lower than desirable. An investigation to optimise this reaction may be worthwhile. Another aspect of the use of Fmoc SPPS methodology with Perloza would be to investigate SPPS of peptides longer than the maximum of 21 amino acid residues reported in this thesis. Another investigation which may be worth pursuing is optimisation of ABI 430A SPPS protocols for use with Perloza. The modified protocols used in this study were satisfactory, but were wasteful of solvent. It was thought that they could be further optimised to reduce solvent consumption. Finally, it was noted in this thesis that, in the synthesis of KMP4 (Cys-tBu) (Chapter 5, Section 5.3.3.9), there was a low coupling yield for one of the two arginine residues. Low yields in coupling Fmoc-Arg(Mtr) are not confined to Perloza, as they have been reported with other supports (Harrison et al, 1988). Optimisation of Fmoc-Arg(Mtr) couplings would be beneficial.

Because it is possible to synthesise aminopropyl Perloza with amine substitution levels of at least 3 mmole/g, Perloza may find some use as a support for high load peptide synthesis. Perloza may also be useful as a support for synthesis of hydrophobic peptides. Lebl et al (1991) used cellulose cotton as the support for the synthesis of a very hydrophobic decaalanine sequence. They noted that

> *...possible hydrogen bonding of the growing peptide chain may prevent self-association effects which complicate the synthesis on the classical carriers in several cases. This was shown for example in the synthesis of decaalanine sequence, where after the fifth or sixth step the synthesis performed on a polystyrene or polyamide resin usually collapsed. We were able to proceed with this synthesis up to the tenth step without observable slowing of the coupling rate. The only trouble was the cleavage of the synthesised peptide Ala^{10}-Val-Gly from the carrier.*

Because Perloza is also a cellulose support, it would be expected that it would be possible to achieve similar results in the synthesis of a hydrophobic peptide such as that made by Lebl et al. Indeed, Wade (1992) has recently reported some success in a synthesis of decaalanine using aminopropyl Perloza supplied by the author.

The results of this study indicated that synthesis of peptide-ligands directly onto aminopropyl Perloza was a viable means of generating peptide-ligand affinity supports. Both Boc and Fmoc SPPS chemistry were used in this study, however Boc chemistry was not used for synthesis of a peptide-ligand with protected side chains because of the strong acid conditions required for their cleavage (liquid HF or TFMSA in TFA). However, it may be possible to use Reagent 4 (80% DCM, 16% TFA, 1% TMSBR, 1% thioanisole, 1% EDT, 1% m-cresol; Chapter 6, Section 6.2.8.4) to cleave side chain protecting groups from Perloza-bound peptides synthesised using the Boc SPPS chemistry. For example, Nomizu et al (1991) used 1M TMSBR / 1M thioanisole in TFA (plus 1% EDT, 1% m-cresol) to cleave side chain protecting groups from the peptide Boc-Cys(p-MeOBzl)-Ser(Bzl)-Cys(p-MeOBzl)-Ser(Bzl)-Ser(Bzl)-Leu-Met(O)-Asp(OBzl)-Lys(ClZ)-Glu(OBzl)-Cys(p-MeOBzl)-His(Tos)-Leu-Asp(OBzl)-Ile-Ile-Trp(CHO)-Val-Asn-Thr(Bzl)-Pro-Glu(OBzl)-His(Tos)-Val-Val-Pro-Tyr-(BrZ)-Gly-Leu-Gly-Ser(Bzl)-Pro-Arg(Mts)-Ser(Bzl)-PAM-resin over one hour at 4^{o}C. The deprotected peptide was then cleaved from the polystyrene support using HF / 1% m-cresol / 0.5% EDT at 4^{o}C for one hour. The side chain protection reagent used by Nomizu et al had similar components to Reagent 4 (see above). Therefore, if it were desired that Boc chemistry also be used for SPPS of peptide-ligands onto aminopropyl Perloza, Reagent 4 may be a useful starting point for development of a viable means of cleaving side chain protecting groups of peptides synthesised on Perloza using the Boc SPPS methodology.

Direct verification (for example by HPLC) of the amount of a desired peptide-ligand synthesised onto Perloza was not possible using the methodology employed in the study reported in Chapter 6. One means of cleaving the peptide-ligand for HPLC determination of the relative amounts of peptide present on the resin would be to insert a cleavable linker between the peptide and the support. In the study of Nomizu et al (see previous paragraph) the PAM linker was used to anchor the

peptide. The peptide synthesised in that study was side chain deprotected while the peptide was left bound to the resin via the PAM linkage. Because the deprotection reagent used by Nozimu et al was similar to Reagent 4 employed in Chapter 6, it may be possible to synthesise a peptide-ligand onto Perloza using the Fmoc SPPS methodology, and cleave the side chain protecting groups using reagent 4 while leaving the peptide bound via the PAM linker. It may then be possible to cleave a small portion of the peptide using liquid HF, and to analyse the product by HPLC to verify the peptide composition. One of the potential problems with this approach is that the PAM linker may introduce another binding component to the resin. However, it may be possible to determine whether the PAM linker binds non-target proteins in any proposed affinity purification by synthesising a PAM linker-Perloza resin, and investigating whether any proteins bind to PAM as a ligand.

The major problem encountered with the thioether approach to peptide immobilisation was the presence of bromine at the end of the reaction period. The bromine was not reactive towards small thiol-containing ligands such as βME, and was therefore not expected to be available for reaction with thiol-containing proteins for steric reasons. A full investigation into means for blocking unreacted Perloza-bound α-bromoacetamido groups should be carried out if this approach to peptide immobilisation is to become generally used.

One possible application of peptide immobilisation via thioether bond formation, as developed in this thesis, relates to antibody generation. It may be possible to react the N^{ε}-amine groups of a carrier protein, such as BSA, with α-bromoacetic anhydride to give a bromine functionalised protein (see Hansen et al, 1991, for a discussion of the use of BSA as a solid support for SPPS). The protein-bound α-bromoacetamido groups could then be reacted with thiol-containing peptides, **in the absence of tri-n-butyl phosphine**, to result in immobilisation of the peptides to the protein for antibody generation. One of the potential advantages of this approach over that of Kolodny and Robey (1990) (see Chapter 6) is that the length of the peptides to be immobilised would not be limited by synthetic difficulties encountered in the synthesis of bromine-containing peptides by the Boc SPPS methodology (Robey et al, 1992).

During the investigation of peptide-resins which bind antibodies to LHRH, it was found that peptide-ligands with either the C or the N terminus directed into the aqueous phase bound antibodies to LHRH. It is known that antibodies to LHRH may recognise different portions of the molecule (Pique et al, 1978; Copeland et al, 1979), and it would be interesting to determine the binding specificity of the antibodies bound by the 2 peptide-resins employed in the study reported in Chapter 6.

Another investigation would involve immobilisation of LHRH (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2) to α-bromoacetamido via the middle of the peptide, thus leaving both the C and N-termini of the peptide directed into the aqueous phase. One possible means of achieving this would be to synthesise an analogue containing [Cys^6] in place of [Gly^6], and to immobilise the analogue to Perloza using the thioether strategy. The peptide-Perloza could then be tested for its ability to bind antibodies to LHRH. The reason for such a study is that one possible population of antibodies to LHRH recognises the full 3-dimensional structure of the LHRH molecule (Ellinwood et al, 1985), and it would be interesting to determine whether a peptide-ligand could be immobilised with the correct orientation to bind these antibodies.

The investigations outlined in the last two paragraphs could be used to determine whether the peptide immobilisation strategies developed in Chapter 6 could result in peptide-ligand-Perloza resins able to **specifically** bind three different types of antibodies to LHRH:

i) antibodies that recognise the C-terminus of LHRH.
ii) antibodies that recognise the N-terminus of LHRH.
iii) antibodies that recognise the entire three dimensional structure of LHRH.

Another application of the methodology reported in this thesis would be to use other carbohydrate supports, for example cellulose paper, for affinity purifications. One of the reasons for using a planar support such as paper is that some biological entities (for example whole cells) would be unable to enter the pores of Perloza to interact with Perloza-bound peptide-ligands. However, cell surface peptide-receptors may be able to interact with peptide-ligands bound to a planar surface. It may

therefore be possible to apply the methodology developed in this thesis to supports for specifically binding populations of cells which possess receptors for a specific peptide-ligand. A first step would be to functionalise paper using the cyanoethylation / diborane reduction methodology. In an early segment of this investigation, cyanoethyl paper (Whatman 540) was synthesised (CN substitution: 0.72 mmole/g), and then reduced for 3.5 hours with diborane in THF under reflux. The amine substitution of the aminopropyl paper was 0.55 mmole/g, which indicated a 76% reduction yield.

In summary, Perloza was found to be a viable support for SPPS by both the Boc and Fmoc SPPS methodologies. However, to date the greatest success has been with the Fmoc methodology. Peptide-ligands could be synthesised directly onto aminopropyl Perloza using either Boc or Fmoc methodology, although the Fmoc methodology was the only viable means for synthesis of peptide-Perloza resins where the peptide-ligand contained protected side chains. The SPPS methodology employed resulted in direction of the N-terminus of the peptide into the aqueous phase. Peptide-resin conjugates synthesised using this methodology were successfully used for affinity purifications of an enzyme (chymosin) and antibodies to a short peptide (LHRH). A methodology for achieving directed immobilisation of a peptide-ligand, via thioether chemistry, was developed and successfully employed for affinity purification of antibodies to LHRH. Finally, the methodology developed in this thesis may be applicable to a number of other hydroxyl-containing supports. Both natural (for example, carbohydrate) and synthetic (for example, polyvinyl alcohol) hydrophilic solid phases may prove useful in the union of solid phase peptide synthesis and affinity chromatography.

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