ANTIGENIC PROPERTIES OF SNAKE VENOM CARDIOTOXINS

by

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M.Sc. PU for CHE

A Dissertation submitted in partial fulfillment
of the requirements for the degree of

DOCTOR OF SCIENCE

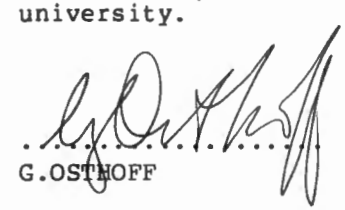
in the

Faculty of Science
(Department of Biochemistry)
Potchefstroom University
for
Christian Higher Education
Potchefstroom

May 1989

DECLARATION

I hereby declare that this dissertation is the result of my own work and is not being submitted to any other university.



G.OSTHOFF

ACKNOWLEDGEMENTS

I would like to express my thanks to the following:

Professor C.J.Reinecke, supervisor of this project, for helpful advice and encouragement.

Dr.F.J.Joubert, co-supervisor.

Members of the Molecular Biochemistry Division of the old NCRL for sharing their expertise with me.

Alida Nel for sharing with me the expertise she learned during her stay in Italy.

Drs. R.C.Clark and F.H.H.Carlsson for their advice in the preparation of this dissertation.

Therese, fuer ihre Geduld.

Meine Eltern, dass sie mir die vorhergehenden Studien als Anlauf zu diesem Studium ermoeglicht haben.

The DPCMT (and old NCRL), CSIR for allowing me to use work done under their auspices for this dissertation.

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ABSTRACT

In this dissertation an innitial investigation was made to locate antigenic determinants of cardiotoxin $V^{II}1$ from N.nivea with a future view of applying the knowledge obtained in the design of synthetic vaccines. The conformation and surface properties of this toxin differs from that of proteins of which the immunological properties are known, so that this information cannot be directly applied.Parallel to the investigation of the cardiotoxin, a similar study was carried out on bovine phospholipase A_2, which was used as control in the experimental work. Peptides derived from the sequences of N.nivea $V^{II}1$ and bovine phospholipase A_2, were synthesized chemically. The conformation of these peptides in various solvents, was determined by circular dichroism spectroscopy, for relating immunological properties to epitope structure. Antiserum was raised against the two proteins, and the reactivity thereof towards the synthetic peptides determined.

It was found that the peptides derived from both N.nivea $V^{II}1$ and phospholipase A_2, were immunologically inactive, either because of the absence of continuous antigenic sites in the proteins, or because of the absence of conformation of the synthetic peptides in the aqueous solvent in which the immunological tests were carried out.

Three peptides, sequence 15-25 derived from N.nivea $V^{II}1$ and sequences 1-12 and 90-109 from bovine phospholipase A_2 , displayed an α-helical conformation when dissolved in trifluoroethanol. Analysis of the α-helical conformations showed that peptide sequences 15-25 derived from N.nivea

$V^{II}1$ and 90-109 from bovine phospholipase A_2 are amphipathic, and could be potential T-cell epitopes of their respective mother proteins.

The present investigation was preceeded by a project for a dissertation for a M.Sc. degree by the present author, the results of which were published in the following journals:

1. Osthoff,G. , Louw,A.I. and Visser,L. 1987. Reversed-phase and hydrophobic-interaction High performance liquid chromatography of Elapid cardiotoxins. Analytical Biochemistry, 164:315-320.

2. Osthoff,G., Louw,A.I. and Reinecke,C.J. 1988. Correlation between the surface hydrophobicities and elution orders of Elapid neurotoxins and cardiotoxins on hydrophobic-interaction high-performance liquid chromatography. Toxicon, 26:475-483.

3. Osthoff,G. 1988. Differing stabilities of snake venom cardiotoxins in acidic aqueous acetonitrile. International Journal of Biochemistry, In Press.

The results of the present dissertation are presented in the following Journals:

1. Osthoff,G. and Nel,A.J. 1988. Far-UV CD-spectroscopy, IR-spectroscopy and immunological properties of synthetic peptides including an α-helical peptide from bovine phospholipase A_2. Biopolymers, In Press.

2. Osthoff,G. 1988. Far-UV CD-spectroscopy and immunological properties of synthetic sequential peptides derived from cardiotoxin $V^{II}1$ of Naja nivea venom; an amphipathic α-helix formed by sequence 15-25 of a β-protein. International Journal of Biochemistry, In Press.

OPSOMMING

In hierdie skripsie is 'n aanvanklike ondersoek uitgevoer om antigeniese determinante te lokaliseer vir kardiotoksien $V^{II}1$ van Naja nivea, met die doel om die betrokke inligting toekomstig aan te wend in die ontwerp van sinteties vaksiene. Die konformasie en oppervlak eienskappe van hierdie toksien verskil van die van proteiene waarvan die immunologiese eienskappe bekend is, sodat hierdie inligting nie geredelik toegepas kan word nie. Parallel met die ondersoek op die kardiotoksien, is 'n soortgelyke studie op bees fosfolipase A_2 uitgevoer, wat as kontrole gebruik is in die eksperimentele werk.

Peptiede wat afgelei is van die aminosuurvolgorde van N.nivea $V^{II}1$ en bees fosfolipase A_2 is chemies gesintetiseer. Die konformasie van hierdie peptiede in 'n verskeidenheid oplosmiddels is bepaal deur sirkulere dikroisme spektroskopie, om die immunologiese eienskappe met epitoopstruktuur in verband te bring. Antiserum is teen die twee proteiene berei, en die interaksie daarvan met die peptiede bepaal.

Dit is bevind dat die peptiede, afgelei van N.nivea $V^{II}1$ en fosfolipase A_2 immunologies onaktief is, eendersyds weens die afwesigheid van liniere antigeniese determinante in die proteiene, of andersyds weens die afwesigheid van konformasie van die peptiede in die waterige medium waarin die immunologiese toetse uitgevoer is.

Drie peptiede, volgorde 15-25 van N.nivea $V^{II}1$ en volgordes 1-12 en 90-109 van bees fosfolipase het 'n α-heliese konformasie vertoon in oplossing in trifluoroetanol. Analise van hierdie α-heliese konformasies het aangetoon dat peptiedvolgorde 15-

25 van N.nivea $V^{II}1$ en 90-109 van bees fosfolipase A_2 amfipaties is, en dus potensiele T-sel epitope van hul onderskeie moederproteiene kan wees.

Hierdie studie is voorafgegaan deur 'n projek vir 'n M.Sc.-skripsie van die outeur, waarvan die resultate in die volgende joernale gepubliseer is:

1. Osthoff,G., Louw,A.I. and Visser,L. 1987. Reversed-phase and hydrophobic-interaction High performance liquid chromatography of Elapid cardiotoxins. Analytical Biochemistry, 164:315-320.

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IMMUNOLOGICAL PROPERTIES OF A SNAKE VENOM CARDIOTOXIN

GENERAL INTRODUCTION

Injection of a toxic protein into an animal induces the production of specific antibodies which are generally neutralizing the function of the toxin [1]. This observation has led to the extensive use of toxic proteins, viruses and bacteria as vaccines against a large variety of diseases. Antisera raised by vaccination of a sub-lethal dose of toxin, are used to protect individuals from the toxic effects after encountering this toxin at a later stage [2].

Both the uses of substances for protection against diseases and infections, or for the production of antisera, face several major problems, for example the lack of sufficient material from natural sources or safety considerations of some of these substances [3]. These problems could be overcome by the use of synthetic vaccines, the design of which will be explained below.

The observation that neutralizing antibodies against a toxic substance can be produced, implies that one or more of these immunoglobulins present in the antisera bind to critical epitopes that are responsible for the toxic action. Identification of such epitopes constitutes an important step in the understanding of the molecular mechanisms that are associated with the inactivation of a toxin by its specific immunoglobulins. Mimicing such an epitope by a synthetic substance, e.g. a synthetic peptide, can also lead to eliciting antibodies able to neutralize the native mother substance.

It was originally assumed that proteins contain a limited number of such epitopes, also called antigenic determinants [4-6]. The synthetic peptide to be used in the form of a synthetic vaccine thus had to mimic the antigenic site in all aspects, i.e. sequence, as well as conformation [7,8]. It was later demonstrated that a prior knowledge of the antigenic determinants in the native protein was unnecessary, and that the apparent lack of correspondence between peptide sequence and conformation was not necessarily a limiting factor [9]. However, this does not seem to be true for all proteins [10].

Snake venom toxins are also used in raising antisera for treating victims of snake bite [2]. The same problems, i.e. lack of sufficient material from natural sources and safety considerations, are encountered here. An additional problem experienced by patients treated for snake bite, is serum sickness [11], where the patient's immune system reacts to the serum proteins from the host animal (e.g. horse) in which the antiserum was raised. This situation is occasionally fatal. The use of only the relevant toxic venom proteins for immunization, could reduce the variety of unnecessary antibodies in such an antiserum that would be raised to irrelevant venom proteins of low or no toxicity. One can speculate on synthetic peptide vaccines, mimicing the antigenic determinants of the toxic proteins, and even more futuristic, the use of monoclonal antibodies from such synthetic immunogens [12]. Cardiotoxin $V^{II}1$ of Naja nivea (Cape cobra)[13], a member of a family of toxic proteins found in Elapid venom (cobras and rinkhals), was used in this study with a view to investigate the immunological properties of these proteins. The results obtained could be of future

value for designing synthetic peptide vaccines to raise polyclonal antisera, or possibly monoclonal antibodies, for treating victims of snake bite.

CHAPTER 1 LITERATURE REVIEW

1 Immunological Properties of Proteins

1.1.1 Introduction

Immune protection in vertebrates is provided by a dual system that maintains two basic defence systems against foreign substances, called antigens, such as proteins [11]. Each system responds in a specific way to most foreign substances. The duality of the immune system results from two populations of morphologically distinguishable lymphocytes. One class of lymphocytes, the B-cells, mediate extracellular phases of infections by production and secretion of antibodies. The other class of lymphocytes, the T-cells, mediate cellular immune responses against cells that have taken up or have been penetrated by a foreign substance. A region of a protein antigen that is recognized by either B-cell produced antibodies or T-cells in the immune response to the whole protein, is referred to as a protein antigenic site, antigenic determinant or epitope (three terms which are used interchangeably)[14,15]. The structural and functional properties of these antigenic sites are of special significance for this investigation, and will be discussed subsequently.

1.1.2. Protein antigenic sites

The description of an antigenic site was given in the previous paragraph. Short peptide sequences may also elicit antibodies reactive with determinants on the native protein from which the sequence was derived. Such a determinant itself may not be part of an antigenic site and should not be confused with one. The present study

involves the investigation of antigenic sites of a native protein, which will be discussed in detail, and not of peptide sequences able to elicit immunological responses.

Protein antigenic sites have been found to belong to two architectural classes [14,15]. They can occupy a surface region of the molecule which constitutes a continuous part of the peptide sequence, and are thus termed continuous antigenic sites (also called sequential or linear sites). Alternatively, a protein antigenic site may comprise surface regions that come into close proximity by virtue of the folding of the peptide chain, but which are generally not directly linked by peptide bonds. These are termed conformational (also discontinuous) antigenic sites. Regions outside these antigenic sites may also be antigenic, but to a lesser extent [4,14,16].

Not all researchers agree with the classification of antigenic sites into the two architectural classes mentioned above [14], but rather state that the whole surface of a protein is antigenic, and that all antigenic sites are conformational. Continuous antigenic sites only constitute part of conformational epitopes. These contradictory opinions will be reviewed and discussed below.

It was found that B-cell produced antibodies and T-cells may recognize the same antigenic site in some proteins, e.g. myoglobin [5] and lysozyme [6], whereas different antigenic sites for these two immunological systems have also been reported. The nature of B- and T-cells will be discussed separately.

1.1.2.1. B-cell epitopes

1.1.2.1.1 Introduction

The first proteins for which complete antigenic structures were determined were sperm whale myoglobin [4], hen egg lysozyme [16], bovine serum albumin [17-20] and human hemoglobin [21-23]. Because of the complexity of protein structure, protein antigenic sites cannot be determined by the exclusive application of a single approach. Atassi and co-workers were involved in immunological studies of all the proteins mentioned above, and have developed a systematic approach for the delineation of antigenic sites which has been adopted and used by other researchers involved in similar studies of other proteins. The strategy used by Atassi and co-workers relies on the following five approaches [4]:

a) to study the effect of conformational changes on the immunochemistry of the protein

b) to study the immunochemistry and conformation of specific chemical derivatives of the protein

c) to isolate and study a large number of overlapping peptide fragments to identify immunochemically reactive fragments that can quantitatively account for the total anti-protein antibodies

d) to study the immunochemistry and conformation of specific chemical derivatives of immunochemically reactive peptides

e) synthesis of the reactive regions after having narrowed them down by approaches a-d to the smallest size.

None of these approaches is likely to produce the correct antigenic structure on its own. The findings of the one approach has to be confirmed by the findings from the others, as will be discussed in the following paragraphs.

The delineation of region 145-151 of myoglobin[34] (Figure 1.1) will be used as example in the explanation and discussion of the application of the approaches. This antigenic site was selected as an example because, not only were the typical procedures of delineation of antigenic sites observed, but also problems were encountered which had to be dealt with in different ways. One such a problem encountered was the low reactivity of this peptide with antiserum, which normally indicates that the peptide is not part of an antigenic site. However, chemical modification of amino acid side chains in this peptide abolished the reactivity completely, indicating that this peptide should definitely be part of an antigenic site.

1.1.2.1.2. Delineation of B-cell epitopes

a) Effects of conformational changes.

The ideal situation for studying the effect of conformational changes of a protein on its immunochemistry, is to have derivatives of the protein in which conformational changes are intentionally imposed, but where the modified location is not part of an antigenic reactive site. In myoglobin, for example, the heme group is not part of an antigenic site and thus an ideal candidate for modification [24]. In immunochemical studies on myoglobin, the heme group was removed, and replaced by copper and zinc metalloporphyrins [25] as well as heme derivatives prepared by nitration [26] and esterification of the vinyl

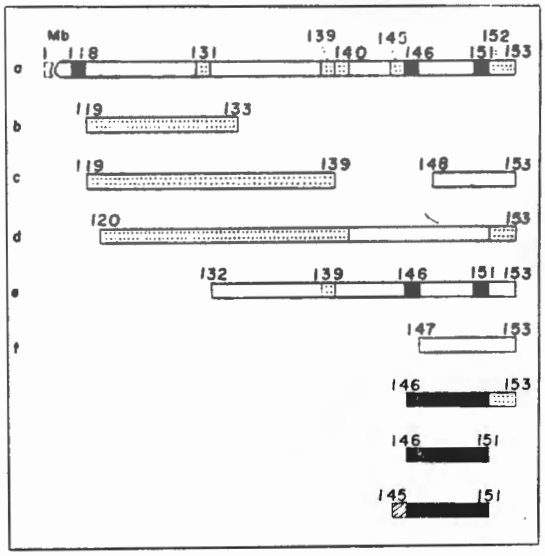


Figure 1.1. Schematic diagram of step-by-step delineation of antigenic site 145-151 of sperm whale myoglobin [ ].
Solid blocks represent segments and residues that are part of a reactive region. Dotted parts represent segments and residues that have been shown to be outside a reactive region. Accurate delineation was derived from the following findings:
a) Removal of the C-terminal dipeptide from the intact protein did not influence its reactivity. Chemical modification of Met 131 in myoglobin by periodate oxidation or carboxylation, Arg 139 by cyclohexanedione, or Lys 140 and Lys 145 by acylation, did not influence the antigenic reactivity of the respective myoglobin derivatives. Modification of Tyr 146 and Tyr 151 by nitration and Arg 118, showed them to be located within a reactive region.
b) Peptide 119-133 was non-reactive with antisera to myoglobin.
c) Peptide 119-139 was almost entirely non-reactive. It can therefore be concluded from a-c that sequence 113-139 is outside the reactive region. Since residues 140 and 145 are also not part of the reactive region, the non-reactive region can be extended to regions 113-145. Peptide 148-153 exhibited a small inhibitory activity towards reaction of apomyoglobin (and not myoglobin) with antisera to myoglobin.
d) Reactions of peptides 120-153 and 132-153 were Quantitatively identical, confirming that sequence 120-131 was not part of a reactive region.
e) Modification of Arg 139 in peptide 132-153 did not influence the antigenic reactivity of the peptide, while nitration of Tyr 146 and Tyr 151 abolished its activity entirely.
f) Synthetic peptides 145-151, 146-151 and 146-153 had comparable inhibitory activities towards reaction of myoglobin with two antisera. With one antiserum only, peptide 145-151 had significantly higher activity than peptides 146-151 and 146-153. Peptide 147-153 did not inhibit myoglobin immune reaction. The reactive region thus occupies sequence 146-151 entirely, and Lys 145 may be included into this region, since it plays an active role with some antisera.

side chains with pyrid-4-ylpropanol [27]. Circular dichroism (CD) spectroscopic data showed that native and copper myoglobins possessed identical conformations, while the zinc myoglobin showed an appreciable degree of unfolding [25]. Myoglobin containing heme modified at the vinyl side chains showed a small conformational change [25]. With the exception of myoglobin prepared with copper metalloporphyrin, which was conformationally identical to native myoglobin, all derivatives showed a decrease in antigenic reactivity.

Extensive changes in immunological reactivity may not be observed for all conformationally altered proteins. Lysozyme, in which Tyr-20 and Tyr-23 were chemically modified to 3-aminotyrosine, showed an altered conformation by CD-spectroscopy [28]. Although the enzymatic activity of this modified derivative was decreased by 50%, the antigenic reactivity was not extensively affected [29].

These results, found for myoglobin [25-27] and lysozyme [28,29], suggest that the interpretation of data obtained in immunological studies of one protein cannot necessarily be extrapolated and applied to other proteins. This implies that with regard to immunological properties, each protein has to be investigated individually. It might afterwards be possible to make some generalizations on the immunological properties of proteins, provided that a sufficient number of proteins have been studied in detail.

b) Immunochemistry of specific chemical derivatives.

Immunochemical studies of protein derivatives where specific amino acid side chains are modified and no conformational changes induced, yield information for the incorporation of specific amino acid residues into antigenic reactive sites [30,31]. In the delineation of region 145-151 of myoglobin [4], nitration of Tyr-146 and Tyr-151 of myoglobin resulted in impaired antigenic reactivity, suggesting that these two Tyr-residues form part of an antigenic site. Acylation of Lys-140 and Lys-145 with 3,3-tetramethylene- glutaric anhydride, however, showed that they were not involved in an antigenic site. When no change in activity is observed after modification of such an amino acid residue, the nature of the chemical modification has to be considered. It was mentioned in approach a) that the modified lysozyme containing 3-aminotyrosine, although conformationally altered, had the same immunological properties as native lysozyme. When the same Tyr-residues were nitrated, a loss in antigenic properties were observed. This indicates that the extent of change in size and charge of a side chain determines its influence on biological activity [28,29]. It might thus be necessary to modify the amino acid side chain in question in more than one way, if possible [29,30].

c) Immunochemistry of overlapping peptides.

If an immunochemically reactive region is removed from an intact molecule, it might still interact with antibodies to the whole molecule. Overlapping peptides are then selected. In the delineation of epitope 145-151 of myoglobin [32], for example, peptides 119-133, 119-139, 120-153, 132-153 and

148-153 were prepared by enzymatic cleavage (Figure 1.1). From the reactivity of peptides 132-153, 120-153 and 14-153, and non-reactivity of peptides 119-133 and 119-139, it was concluded that sequence 119-139 was outside the reactive region. Inspection of the tertiary structure of the region of myoglobin in which regions 119-139 and epitope 145-151 are located, shows that a large part of the former region is located in an α-helix, whereas sequence 145-151 is not involved in any specific hydrogen bonded secondary structure [4]. A low content of helical conformation was observed by CD-spectroscopy for peptide 120-153 [33]. Non-reactivity of peptide 119-139, which forms part of sequence 120-153, could thus be the cause of structural impairment.

An isolated peptide may not always adopt the conformation it had in the parent protein [33,34], and then prove to be non-reactive. A non-reactive peptide however, may still be part of a reactive region in the intact protein. Peptide 1-7 of myoglobin, for example, was shown to be inactive, but improved the reactivity of peptide 8-55 when incorporated to form peptide 1-55 [33]. This emphasizes the importance of information from a variety of methods in the delineation of antigenic sites.

d) Modified immunochemically reactive peptides.

Further delineation of antigenic sites, after having narrowed a region down to a certain peptide fragment as discussed in c above is achieved by specific chemical modification of selected amino acid residues in the reactive peptide [35]. From the results obtained, specific amino acid residues

in the peptide fragment can then either be included in or excluded from the antigenic site.

By this technique the status of peptide fragments with low or no reactivity as a result of structural impairments, discussed in c above, can also be studied, e.g. in the delineation of site 145-151 in myoglobin [34]. Peptide 148-153 only showed a low reactivity with antiserum to myoglobin, but nitration of Tyr-146 and Tyr-151 in this peptide abolished the reactivity completely [33], indicating that this peptide sequence, especially the side chains of either Tyr-146 or Tyr-151 or both, is part of an antigenic site.

e) Accurate delineation with synthetic peptides.

Having narrowed down the reactive regions on a protein molecule, by the foregoing approaches, to conveniently small sizes, ultimate delineation is achieved by the chemical synthesis and immunological studies of various parts of each region which cannot be obtained by other methods e.g. enzymatic cleavage. For the accurate delineation of epitope 145-151, the possible site was narrowed down to segment 139-153. Synthetic peptides 145-151, 146-151 and 146-153 had comparable inhibitory activities towards reaction of myoglobin with antiserum [36]. Peptide 147-153 did not inhibit the myoglobin immune reaction. The smallest peptide necessary for the reactive antigenic site was thus derived as being located at residues 145-151 [36].

1.1.2.1.3. Structure of B-cell epitopes

As already mentioned, not all researchers agree on the definitions of linear or continuous antigenic sites. In fact, not all agree on the locations of epitopes in various proteins. Contradictory results for myoglobin, lysozyme and serum albumin have been found, and are listed in Table 1.1. At least some agreement was found for the antigenic structure of myoglobin. Three sites coincide to a large degree in the peptide regions 15-29, 56-59 and 145-153. Whereas Atassi found additional sites 94-99 and 113-119 [4], Crumpton and Wilkinson [42] reported additional regions at 70-76 and 139-146. Evidence for conformational epitopes in myoglobin was also presented [43]. This site includes the continuous site 15-22 [4]. Cross-reactivities of different myoglobins with the fraction of antibodies to beef myoglobin specific for peptide region 1-55, showed that these antibodies bound with markedly different affinities to beef, sheep and pig myoglobins, respectively, in spite of their identity in the sequence 15-22.

Regions outside antigenic sites have been found not to be totally non-immunogenic. Atassi reported that less than 2% of the antisera raised "to myoglobin [4] and lysozyme [16] reacted with regions outside the antigenic sites. In contrast, Hurrell et al [44] and East et al [43] found that 30-40% of antibodies raised in either goat, sheep or rabbit did not bind to any of the CNBr-peptide fragments of myoglobin, implying that they are directed to sites other than those reported by Atassi [4].

From the above it can be concluded that any part of the surface of a protein may be antigenic, but that "immunodominant" sites exist, containing

Table 1.1. Contradictory data on the localization of B-cell epitopes of proteins.

Myoglobin	
Peptide sequences of continuous epitopes found by:	
Atassi[4]	Crumpton and Wilkinson[42]
15-22	15-29
56-62	56-69
	70-76
94-99	
113-119	
	139-146
145-151	147-153
Lysozyme	
Residues in conformational epitopes found by:	
Atassi[16]	Others[35-41]
Site I: 5,7,13,14,125	Multideterminant
Site II: 33,34,113,114,116	Almost whole
Site III: 62,87,89,93,96,97	protein surface
Albumin	
Results obtained by:	
Atassi[17-20]	Benjamin and Teale[41]
Two determinants on carboxy terminal	Almost whole surface

certain sequences or amino acid residues to which most of an immune response is directed.

More detailed information of the specific interaction of antibodies and antigenic sites was achieved by Geysen et al [45] with the use of monoclonal antibodies. A monoclonal antibody is an antibody produced by a single clone of B-lymphocyte [12]: all the antibodies have the same amino acid sequence and hence the same binding properties. They are produced in culture by B-cells, of an immunized animal, hybridized with myeloma cells. One cell line producing monoclonal antibodies with a high affinity for a specific site on the protein immunogen is then selected. In the work by Geysen et al [45], monoclonal antibodies were raised against foot-and-mouth disease virus and tested for the specific interaction with a large number of peptides. The results showed that only a few amino acid side chains at specific positions played a key-role in the interaction with an antibody. Whether continuous or conformational, only a few amino acid side chains within an antigenic site thus take part in the interaction with an antibody.

Monoclonal antibodies, raised to a native protein cannot be used in delineating immunodominant antigenic sites, since this man-designed random selection of an antibody could be to a site outside an immunodominant site, and thus not reflect the antigenic picture of a protein as selected by nature. It has already been mentioned that polyvalent antiserum raised to a native protein will be used in the present study.

1.1.2.1.4. Species specificity of B-cells

A further disputable character of antigenic sites is whether they are recognized to a similar degree by antisera raised in different hosts. According to Atassi, the same antigenic sites of myoglobin [4,46], lysozyme [16], albumin [18] and human hemoglobin [22,23] are recognized by antisera raised in various hosts, e.g. rabbit, goat and mouse. Contradictory results [45] however show that;

a) different species responding to a common continuous epitope may produce antibodies with different fine specificities,

b) different species may not recognize any common sequential epitope, and

c) even within the same species, individuals do not necessarily respond to the same small number of epitopes.

Antigenic sites on a protein antigen thus comprise regions which differ in sequence from their counterparts in the immunized host, e.g. myoglobin. Therefore they are not peculiar to the protein, but are defined with reference to the host species in which antibodies are raised [47,48].

1.1.2.1.5. Prediction of antigenic sites

The proteins for which antigenic structures have been defined as discussed so far, are all of known tertiary structure. Since antigenic sites are surface features, and with a known three dimensional structure, regions with a high potential of being antigenic sites may be predicted for a protein simply by locating exposed regions. For most proteins, however, only the primary structure is available, and such

predictions are thus not possible. Because the elucidation of the tertiary structure and the antigenic structure of a protein is difficult, time consuming and expensive, ways were sought to predict structural features, surface regions and antigenic properties with only the sequence of a protein available.

To achieve this goal, the physico-chemical properties of proteins with known tertiary structure, concerning the positions of secondary structures (α-helices, β-pleated sheets and turns) [49,50], properties of surface regions [51], hydrophilic and hydrophobic regions [52], as well as the properties of known antigenic sites, were thus investigated, and the results then applied to proteins for which these properties are not known.

From the analyses of antigenic sites, it was learned that they are surface features of proteins, frequently associated with regions that have a high degree of exposure to solvent [4;47], consist of charged, hydrophilic amino acid side chains, and that most antigenic sites are between 5 and 8 [4,16,51,53], but seldom as long as 13 [54,55] amino acid residues in length. The peptide regions carrying antigenic sites have also been shown to be highly mobile or flexible [56,57]. Experimentally or statistically derived numerical values for hydrophilicity [52] or flexibility [42] were determined and then assigned to each amino acid residue of a protein sequence. These were then repetitively averaged for certain intervals over the length of the polypeptide chain. Stretches of sequence that obtained high values of hydrophilicity or flexibility, were then predicted to be possible antigenic sites.

Although prediction methods are frequently used [59,60], they are not very accurate, because not all highly exposed or hydrophilic regions are antigenic [61], not all proteins have their hydrophobic amino acid side chains buried in the interior with only hydrophilic ones exposed [62,63] and hydrophobic amino acid side chains and proline are frequently found as part of antigenic sites [14].

1.1.2.1.6. Discussion on B-cell epitopes

The differing opinions, mainly between Atassi's group, and other researchers, on the structure and location of B-cell epitopes are summarized in Table 1.2. It can be concluded that an understanding of the immunochemistry of proteins, concerning B-cell epitopes, is far from complete. A view on which most of the authors mentioned seem to agree, is that the surface of a protein antigen consists of a complex array of continuous overlapping antigenic determinants [15]. So-called "immunodominant" regions do occur, which depend upon the conformational integrity of the native molecule. Those regions to which an individual host animal responds, are dictated by host regulatory mechanisms, and not necessarily by an inherent property of the protein molecule reflecting restricted antigenicity or limited antigenic sites.

Irrespective of the different opinions on antigenic sites of proteins, the strategy outlined by Atassi [4](see 1.1.2.1.1) is widely used. Approach c, immunological properties of overlapping peptides with antisera, is a key approach and the most convenient method to obtain an initial idea of which parts of a protein molecule could be involved in immunological

Table 1.2. Differences in opinions on the properties of B-cell epitopes.

Atassi et al	Other researchers
a)Continuous and conformational [4,16]	a)Only conformational "Continuous" sites are part of conformational sites [37,41]
b)Discrete boundaries [4,16]	b)Whole surface with immunodominant regions [28,32]
c)Same discrete boundaries irrespective the host of antiserum [4,16,18,22,23,46]	c)Regions differ for antisera from different host species, as well as from different individuals of same specie [45,47,48]
d)Charged and hydrophilic amino acids [4,16,51,53]	d)Not only hydrophilic and charged residues; hydrophobic residues and Proline included [61]
e)Sites of lower reactivity outside epitopes [4,16]	e) less reactive regions found [43,44]

interactions. Other examples, apart from Atassi's group, where this approach was applied in the delineation of antigenic sites, are histone proteins [64], tobacco mosaic virus proteins [53], myohemerythrin [65] and horse muscle acylphosphatase [61].

1.1.2.2. T-cell epitopes

Less is known about the T-cell reactive properties of proteins than about the B-cell epitopes. The only known difference, is that proteins have to be taken up by cells, e.g. macrophages, and that parts of the antigenic protein have to be presented on the cell surface of the macrophage for interaction with the T-cell lymphocyte [11]. Most of the research involving T-cell epitopes was carried out on the same proteins for which B-cell epitopes have been delineated, i.e. myoglobin [5] and lysozyme [6].

1.1.2.2.1. Delineation and structure of T-cell epitopes

The same approach as was used for the delineation of B-cell epitopes (1.1.2.1.1) was used to delineate T-cell epitopes of myoglobin [7] and lysozyme [6]. Atassi showed that the same continuous B-cell epitopes found for myoglobin [4] and conformational epitopes in lysozyme [16] are also recognized by T-cells. An additional T-cell epitope was found for lysozyme [6], hence B-cell epitopes are also recognized by T-cells, but T-cell epitopes are not always recognized by antibodies.

Again there is disagreement on the nature of T-cell epitopes. Contradictory results were reported for lysozyme, suggesting that antibodies and T-

cell lymphocytes recognize totally different sites [66,67].

1.1.2.2.2. Presentation of T-cell epitopes

As already mentioned, an antigenic protein, or part thereof, ingested by a macrophage, has to be presented on the cell surface for interaction with T-cells. Berzofsky [in ref. 68] found that myoglobin only has to be unfolded, and Unanue and co-workers [68,69] found that lysozyme has to be processed by both unfolding and proteolytic cleavage, and then presented for recognition by T-cells. It was also shown that, in contrast with studies with antibodies, T-cells elicited by immunization with a native protein, react equally well when challenged with either the native or denatured forms of the antigen, or its peptide fragments [70,71], thus supporting the above. In contrast, Bixler and Atassi reported intact lysozyme [72] and myoglobin [73] to stimulate T-cells.

1.1.2.2.3. Prediction of T-cell epitopes

In order to be able to predict T-cell epitopes of proteins, the physico-chemical properties of known T-cell epitopes of proteins were investigated and the proposed method of prediction, discussed below, tested for accuracy.

The finding that T-cell epitopes do not distinguish between native and denatured structures of a protein, since proteins have to be processed and presented for recognition, suggested that these epitopes reflect lower-order structural properties of their sequences [68,69]. Further, T-cells recognize antigen in association with a presenting cell, suggesting that such lower-order

structural properties might be induced and stabilized by interactions with hydrophobic surface structures of the presenting cell. De Lisi and Berzofsky [74] thus suggested that for interaction with a cell membrane, the induced ordered secondary structure should be amphipathic, i.e. with one face consisting of relatively hydrophobic residues, and the opposite face of polar residues. Analysis of residues 132-145 of the known T-cell epitope of myoglobin [5] show that they are encompassed within an amphipathic α-helical segment of the native structure. The same appears to be true for cytochrome c [75] and epitope 87-92 of lysozyme [76]. Although one of the other sites of lysozyme is not helical in the native structure, and another only partially helical [77], and furthermore none of the T-cell sites of influenza hemaglutinin are helical [78], there still is a correlation of helical periodicity with amphipathic properties for these segments. It was thus concluded that it is not the native secondary structure in the intact protein, but the ability of a peptide segment to form an α-helical structure, induced and stabilized by the amphipathic environment at the surface of an antigen-presenting cell, which is a characteristic of a T-cell epitope.

This method is not 100% accurate, and when evaluated was applicable to only 10 out of 12 epitopes investigated. De Lisi and Berzofsky also warn that this analysis does not necessarily indicate the presence of a T-cell epitope; since for several amphipathic helical peptide sequences predicted for lysozyme, only one coincided with such an epitope [74]. The inaccuracy was also indicated by a T-cell epitope found for the α-chain of an acetylcholine receptor, which did not fulfill these criteria [79]. Since only limited

data on T-cell antigenic sites of proteins is available, this method is frequently utilized to predict possible sites before extensive experimental work is undertaken to locate them [80].

1.1.2.2.4. Discussion on T-cell epitopes

The different opinions on the nature of T-cell epitopes are summarized in Table 1.3, which shows that knowledge about these epitopes is vague. Researchers are however tending to support the view that antigenic proteins have to be processed, either by unfolding or by proteolytic cleavage, in order to present their T-cell epitopes [68-71].

1.1.3. General discussion on Protein Antigenic Sites

More information is available for B-cell than for T-cell antigenic sites. Authors often disagree on the characteristics as well as the localization of the latter. The general conclusion that can be drawn is that the whole surface of a protein consists of overlapping antigenic determinants, some of which may be "immunodominant" [19-41], and which differ among host species into which the antigenic protein is injected [45,41,48]. T-cell antigenic sites may often have the same location as B-cell sites, the sequences of which were frequently found to have the potential of forming amphipathic α-helical structures [74-78]. T-cell epitopes usually have to be processed before being presented on the surface of the host macrophage cell.

Since characteristics of antigenic sites can differ among different types of protein, e.g. myoglobin [4,24-27] and lysozyme [16,28,29], the

Table 1.3. Differences in opinions on the properties of T-cell epitopes.

a)T-cell epitopes same as B-cell epitopes. Additional T-cell sites found [5,66]	a)T-and B-cell epitopes at different sites [66,67]
b)Antigenic protein not processed for presentation [72,73]	b)Processing of antigenic protein by either unfolding or proteolytic cleavage [68-71]
c)Not necessarily potential of forming amphipathic α-helical structures [79]	c)Potential of forming α-helical structures [74-78,80]

results found for one protein cannot be readily applied to another. Each protein has to be investigated individually. The proteins for which immunological properties have been investigated and which have been discussed so far, are all of globular structure containing α-helical hydrogen bonded structures and having hydrophilic surfaces. Information about the immunological properties of fibrous proteins, proteins having a high content of β-pleated sheet [81,82-87] and proteins with hydrophilic surfaces [82-86,88-93] is lacking.

In the present study, the immunological properties of a snake venom cardiotoxin is to be studied. In the M.Sc. study of the present author [94], the surface properties of this family of proteins were found to be highly hydrophobic [63,94]. In the same study an attempt was made to predict the possible location of antigenic sites of a cardiotoxin [94]. The structural properties of cardiotoxins are known and were shown to have a high content of β-pleated sheet [62,95]. The cardiotoxins thus differ considerably in structural properties from the proteins for which immunological properties are known. This contrast makes application of known immunological properties of other proteins to these toxins even more impossible. They are thus ideal candidates for studying immunological properties.

In the approach of the present study a control protein was used. This is to prevent artifactual results which may occur. The peptides to be synthesized are hydrophobic and could interact non-specifically with antibodies through hydrophobic interaction, giving false positive results. This is a possible problem which could be observed for these peptides, but not for the hydrophilic peptides discussed in 1.1.2.1.2. The

control protein should have structural properties in common with both cardiotoxin and proteins with well-characterized immunological properties. The protein chosen was bovine phospholipase A_2 (PhA), which has a globular structure containing α-helical hydrogen bonded structures, but with a hydrophobic surface [96]. Peptides derived from this protein would thus also be hydrophobic, and would also interact non-specifically with an antibody raised to cardiotoxin, indicating such an interaction with a cardiotoxin derived peptide as non-specific.

The bovine enzyme was also chosen as control protein because it is non-toxic. PhAs also occur in snake venoms, some of which are toxic [97,98]. The PhA found in cobra venom was also shown to function synergistically with cardiotoxins [99]. The results obtained for the bovine enzyme in this study, should be useful for repeating and comparing with the snake venom enzyme.

Since the structural properties of PhA have been studied [100] and it is a hydrophobic protein of practically unknown immunological properties, it is also an ideal candidate for immunological studies.

The properties of cardiotoxins and PhA are reviewed in the following paragraphs.

1.2. Cardiotoxins

Cardiotoxins, also known as cytotoxins or membrane active polypeptides, have been found only in the venoms of the genera Naja and Hemachatus (Cobras and Rinkhals)[101] which belong to the family Elapidae [102]. Extensive research has been undertaken on the structure of snake venom

cardiotoxins, while various functional aspects of these proteins have been reported. Fewer reports deal with the immunological properties of cardiotoxins. The structural, functional and immunological properties will be discussed separately.

1.2.1. Structural aspects of cardiotoxins

Cardiotoxins constitute a family of homologous single-chain small globular proteins (Mr 6800), over 50 sequences of which have been reported [103]. They contain 60 or 61 amino acid residues and four intrachain disulfide bonds. According to their primary structures, cardiotoxins were subdivided into three different types; the Naja -type, DLF-type (direct lytic factor) and cardiotoxin homologues (Figure 1.2)[101].

With three known exceptions, 36 positions of the Naja -type cardiotoxins are invariant; 3, 6, 8, 12-15, 26, 33-39, 41-44, 46, 48, 50, 51, 53, 54, 56, 59 and 60. Seven positions i.e. 7, 10, 26, 32, 47-49 are invariant with respect to a hydrophobic amino acid. Positions 1 and 32 also show this property with a single exception each. Position 58 contains either Lys or Arg as basic amino acid, and position 25 usually contains an aromatic residue.

The DLF-type cardiotoxins from H.haemachates have an extra residue after position 4 (4a). They differ from the Naja-type toxins in positions 6, 18, 33, 34-35, 39, 41, 44, 46, 50, 51 and 56, and have an additional Trp in position 61 in two of the six DLF-type toxins [101].

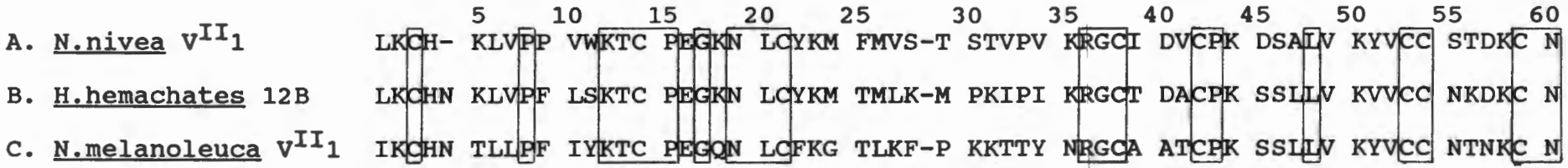


Figure 1.2. Primary structures of cardiotoxins.
A.Naja-type cardiotoxin
B.DLF-type cardiotoxin
C. Cardiotoxin homologue
Letters in boxes indicate invariant residues between the three cardiotoxin groups.

The cardiotoxin homologues differ from the DLF-type toxins in positions 22 and 24. Positions 43, 46 and 50, are the same as in the Naja-type cardiotoxins.

The tertiary structure of a cardiotoxin, N.m.mossambica $V^{II}4$, as determined by X-ray diffraction [62], only became known recently. According to these results, the peptide chain is folded in such a way that the intrachain disulfide bonds give a triple-loop structure (Figure 1.3). Two main secondary structures are apparent; a large three-stranded antiparallel β-sheet formed by residues 20-27, 34-39 and 49-54, and a smaller one formed by residues 2-5 and 10-13. A β-turn is formed by residues 46-49. Comparison with earlier studies by circular dichroism (CD)[95], n.m.r. [104,105] and laser Raman spectroscopy [106] as well as with the X-ray structure of the homologous short neurotoxins [107,108] show that all cardiotoxins have this triple-loop structure and high content of β-pleated sheet.

Small differences in the structures of cardiotoxins do exist, as was indicated by CD-spectroscopy [81,109]. On the basis of the distinctive far-ultraviolet CD-spectra, they were classified as belonging to class I, class II or neither of these two classes. It was then found that the class I cardiotoxins possess invariant residues Pro-9, Pro-10, Phe-11, Trp-12 and Met-54. It was furthermore shown in a study preceding this investigation [94,109] that the class I and non-classified cardiotoxins were more stable to structural disruption by acidic aqueous acetonitrile, often used for purification of proteins by high performance lliquid chromatography (HPLC)[94], than the class II toxins. The structures of the class II

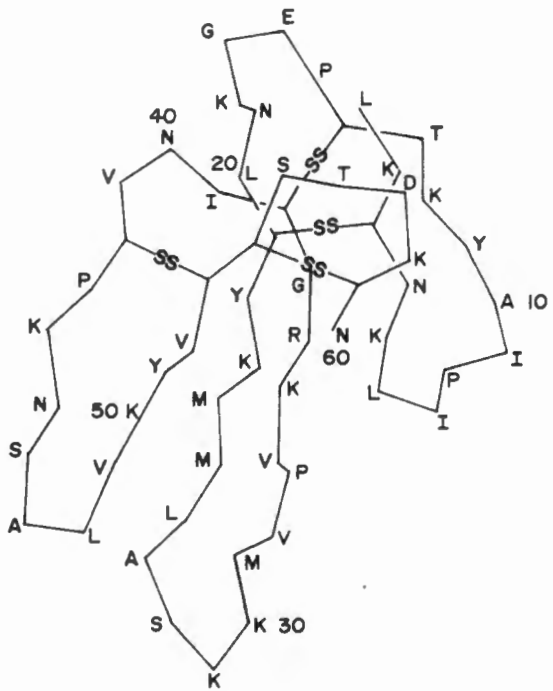


Figure 1.3. The X-ray structure of cardiotoxin V^{II}_1 of N.m.mossambica [79].
(Disulfide bonds are indicated by -SS-)

cardiotoxins were partially disrupted by 10% acetonitrile and totally disrupted by 30-50% acetonitrile. For class I and non-classified cardiotoxins, structural disruption commenced at 20% acetonitrile and was complete at concentrations above 50% acetonitrile.

A number of studies have been directed at discovering the surface accessibility and functional roles of amino acid side chains of cardiotoxins. Region 6-12 has been shown to interact with membrane lipids [110,111] and is therefore accessible at the surface. Other surface accessible amino acid side chains have been identified by the binding of monoclonal antibodies to residues 1, 11, 12, 23, 27-31, 45, 47, 49, 51, 52, 57 and 58 [82,87]. Photochemically induced nuclear depolarization (photo-CIDNP) has also shown that Tyr-11, 22, 25 and 51 in these toxins are accessible [112], and chemical modification experiments have provided supplementary evidence for Tyr-22 and Tyr-25 [83] as well as the accessibility of Met-24 and Met-26 [85,86] and Arg-36 [84]. From X-ray crystallographic data [62] the side chains of Asp-40, Val-41 and Lys-44 could be added to this list. The combined results of these studies indicate that the side chains of at least amino acid residues 1, 6-12, 22-31, 36, 40, 41, 44-49, 51, 52, 57 and 58, i.e. 52%, are surface accessible. Correlation between the surface hydrophobicities and elution orders of Elapid neuro- and cardiotoxins on hydrophobic-interaction HPLC showed that the surfaces of the extending loops of cardiotoxins are very hydrophobic [63].

1.2.2. Functional aspects of cardiotoxins

To date the specific mechanism of action of cardiotoxins has not been conclusively determined, although various pharmacological effects have emerged. These toxins interact with various types of cells, and can impair both structure and function of cell membranes which leads inter alia to muscle paralysis and systolic arrest of the heart [113]. The LD_{50} values for mice is 0,75-3,0μg administered intravenously [114-118].

Binding of cardiotoxins to cell membranes causes lysis of various types of cells e.g. leucocytes, lymphocytes, bone marrow cells and erythrocytes [119] which is caused by changes in permeability of membranes, i.e. inhibition of transport of small anions, amino acids and calcium [120,121]. It also affects membrane enzymes; activation of glyceraldehyde-3-phosphate dehydrogenase, adenylate cyclase, 3-phosphoglycerate kinase and aldolase [122] and inhibition of sodium and potassium dependent ATPase [120], calcium dependent ATPase [123], cholinesterase [124] and phospholipid-sensitive, calcium dependent protein kinase [125].

Synergism with phospholipase A_2 (PhA)[99] plays an important role in the action of cardiotoxins. Where highly purified cardiotoxin has moderate haemolytic activity on erythrocytes, the presence of as little as 0,0007% (w/w) PhA enhances the rate of cardiotoxin induced haemolysis up to 30 times [99]. It was proposed that cardiotoxin makes the phospholipids of erythrocyte membranes, which are normally resistant to PhA, available for cleavage [126].

Two molecular mechanisms were suggested for interaction of cardiotoxins with membrane bilayers. It was found that residues 6-12 of cardiotoxin $V^{II}3$ of Naja mossambica mossambica , when interacted with liposomes containing phosphatidylserine, were unavailable for proteolytic degradation [111]. This suggested that interaction between the cardiotoxin and the membrane takes place between the first loop of the cardiotoxin and the membrane bilayer only.

In the second hypothesis it was proposed that interaction with bilayer membranes involves all three loops of the cardiotoxin [127], because the distribution of hydrophobic and charged amino acids within the three loops is such that interaction with a membrane is possible. Figure 1.4 presents a schematic view of the proposed mechanism [127], adapted to fit the reported structure of cardiotoxin [62]. The four hydrophobic fragments in the three hairpin loops of the cardiotoxin are located in the hydrophobic interior of the membrane, and the central loop penetrates the lipid bilayer. In a membrane containing negatively charged lipids, the positively charged ϵ-amino groups of the eight Lys-side chains can form salt bridges in the polar head group regions of the inside layers (Lys-29 and 30) and the outside layers (Lys-5, 12, 23, 35, 44 and 50) of the membrane. The major portion of the protein globule, which contains all the disulphide bridges, extends into the aqueous phase. This model was however, only proposed, and no experimental evidence was provided.

The importance of loop II for the biological activity of cardiotoxins was demonstrated by chemical modifications of amino acid side chains

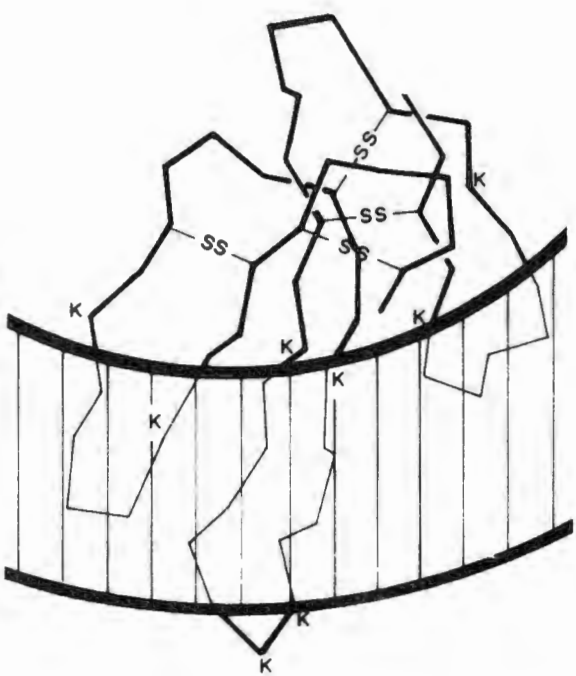


Figure 1.4. Schematic presentation of the structure proposed for the cardiotoxin-membrane complex [107] of N.m.mossambica $V^{II}1$ as derived from the X-ray structure [79]. (Disulfide bonds are indicated by -SS-)

located in this loop. Oxidation of Met-24 and Met-26 of cardiotoxin $V^{II}1$ of N.melanoleuca [85] led to a loss in toxicity and hemolytic potency. Nitration of Tyr-22 and Tyr-51 (loop III) in N.melanoleuca $V^{II}1$ and N.h.annulifera $V^{II}1$ [83] and also modification of Arg-36 with 1,2-cyclohexanedione of the latter toxin had minor effects on the toxicity.

The importance of all three loops for biological activity was demonstrated by the neutralization of toxin gamma of N.nigricollis with two monoclonal antibodies which interacted with amino acid residue 11 in loop I, and residues 25, 27, 29-31 in loop II and 45, 49 and 52 in loop III [82,87].

1.2.3. Immunological aspects of cardiotoxins

Cardiotoxins fall within one serological group, differing from the homologous short and long neurotoxins and show partial identity as a consequence of small differences in primary structure [128]. The two structural groups of cardiotoxins [81,109] mentioned in 1.2.1 were also found to differ in immunological properties [81]. Antibodies raised to N.nigricollis toxin gamma, a toxin belonging to structural class I toxins, cross reacted with all the other toxins of the same group, but with weaker affinity than those of class II. As mentioned in 1.2.1, the class I toxins have invariant residues Pro-9, Pro-10, Phe-11 and Trp-12 in loop I, whereas the toxins of class II have various amino acid residues at these positions. Since it was shown that altering of certain key-residues in a peptide also alters the affinity of antibodies to this peptide [45], it would suggest that these amino acid side chains of

loop I do play a role in the antigenic properties of cardiotoxins.

Although the chemical modifications of amino acid residues 22, 24, 26, 36 and 51, discussed in 1.2.2 altered the functional aspects of cardiotoxins, no major changes in immunological properties were found [83-86]. It has however to be taken into account that the technique of immunodiffusion was used, which is not sensitive enough to detect small changes or differences in affinity to antiserum, rendering this information not very useful.

The possibility that the three loops of toxin gamma of N.nigricollis could be antigenic, was shown by the interaction of neutralizing monoclonal antibodies with sites incorporating amino acid residues within these loops [82-87]. One monoclonal antibody was found to bind to a site involving Trp-11 in loop I, and the other to residues 25, 27, 28-31 of loop II and 45, 47, 49, and 52 of loop III. Since monoclonal antibodies are selected individually, they could be of high affinity to a less reactive region of a protein [4] and therefore not reflect an "immunodominant" site (see 1.1.2.1.2.). However, polyclonal antisera also neutralize the activity of cardiotoxins [2], but sites of interaction are unknown. Since the monoclonal antibodies showed the same neutralizing abilities as polyvalent antiserum, it is possible that they were raised to immunodominant antigenic sites. These studies with monoclonal antibodies thus show the importance of all three loops in the immunological properties of cardiotoxins. Non-neutralizing monoclonal antibodies were also reported, without defining any epitope [129].

From the above it can be concluded that not much is known about the antigenic structure of snake venom cardiotoxins. From experimental evidence provided, all three loops seem to be involved in the antigenic structure, possibly including residues 9-12 in loop I [81], residues 25-31 in loop II and 45-47, 49 and 52 in loop III [82,87].

1.2.4. Discussion on Cardiotoxins

From the above it is clear that the structural properties of this homologous family of proteins is well defined. Many structure- function aspects, including the toxic function as well as immunological properties, still need to be solved. Since the structural properties are known, cardiotoxins are interesting candidates for studying immunological properties. Unlike the proteins mentioned, on which immunological studies have been performed, which are globular, contain α -helical secondary structures and hydrophilic surfaces, cardiotoxins have a high content of β - pleated sheet [62,95] and hydrophobic surfaces [63]. This could add further important information to the knowledge of immunochemical properties of proteins.

1.3. Phospholipase A_2

Phospholipases A_2 (PhA)(EC 3.1.1.4) are present in most cells. This enzyme is widely found in venoms of snakes, bees, scorpions, as well as animal tissue, e.g. pancreas [101,130,131]. One of the richest sources of PhA is the venom of snakes of the families Viperidae (vipers and adders), Crotalidae (rattle snakes), Elapidae (cobras and rinkhals) and Hydrophiidae (sea snakes). The venom enzyme has also been designated phospholipase A, phosphatidase A, lecithinase A or hemolysin,

because of its ability to indirectly hemolyse red blood cells. The tertiary structures of a few PhA's and the enzymatic function of this enzyme family is well characterized, but the toxic activities and immunological properties still lack information. The structural, functional and immunological properties of this enzyme family will be discussed separately.

1.3.1. Structural aspects of Phospholipase A_2

A high degree of homology occurs in the primary structures as well as the tertiary structures of this enzyme family [96,100,101,130,132]. They contain between 108 and 140 amino acid residues, six or seven disulfide bonds and a molecular weight in the range of 11000-31000. This large difference in molecular weight is a result of the method of isolation; a dynamic equilibrium between dimer and monomer seems to exist, which can shift towards either of the forms, depending on conditions of isolation, e.g. ionic strength and calcium ions in the medium.

Of the over 50 known toxic and non-toxic PhA's, the sequences of only a few have been reported [101,103,130]. There is a high degree of similarity (33%), especially in regard to the positions of the 14 Cys-residues and the 7 disulfide bonds (Figure 1.5).

The tertiary structures of the porcine [96] and bovine [100] pancreatic and Crotalus atrox venom [132] enzymes have been determined, which show a high content (50%) α -helical structure, and a high degree of homology [94,132].

The bovine enzyme used in this study has a molecular weight of 14000, consists of 123 amino

```
                          10         20         30         40         50
Bovine pancreatic    ALWQFNGMIK CKIPSSQPLL DFNNYGCYCG LGGSGTPVDD LDRCCQTHDN

Porcine pancreatic   ALWQFRSMIK CAIPGSHPLM DFNNYGCYCG LGGSGTPVDE LDRCCETHDN

Crotalus atrox       SLVQFETLIM KIAG-RSGLL WYSAYGCTCG WGGHGLPQDA TDRCCFVHDC

         60         70         80          90         100        110        1201        130
 CYKQAKKLDS CKVLVDNPYT NNYSYSCSNN  EITCSSENNA CGAFICNCDR  NAAICSFKV- P-YNKEHKNL  DK-KNC

 CYRDAKNLDS CKFKVDNPYT ESYSYSCSNT  EITCNSKNNA CEAFICNCDR  NAAICFSKA- P-YNKEHKNL  DTKKYC

 CYGKATD-   C-----NPKT VSYTYSEEWG  EIICGG-DDP CGTQICECDK  AAAICFRDNI PSYDNKYWLF  PP-KDCREEP  EPC
```

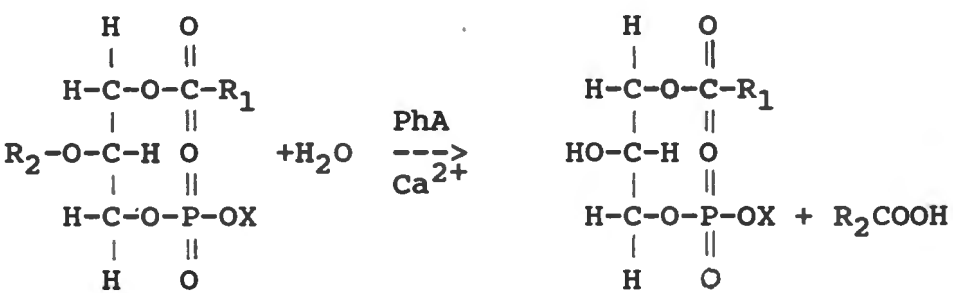
Figure 1.5. Primary structures of three phospholipases A_2 with homology alignment of the sequences.

acid residues and has 7 disulfide bonds [100]. The molecule consists of two antiparallel α -helices, amino acids 40-58 and 90-108, each having 5 full turns, which are connected by two disulfide bonds (Figure 1.6). Sequences 1-12 was also found to be an α -helix, while 74-84 is an antiparallel β -sheet. Residues 19-22 form a single helical turn [100,132] and 59-68 a "paperclip"-loop [133].

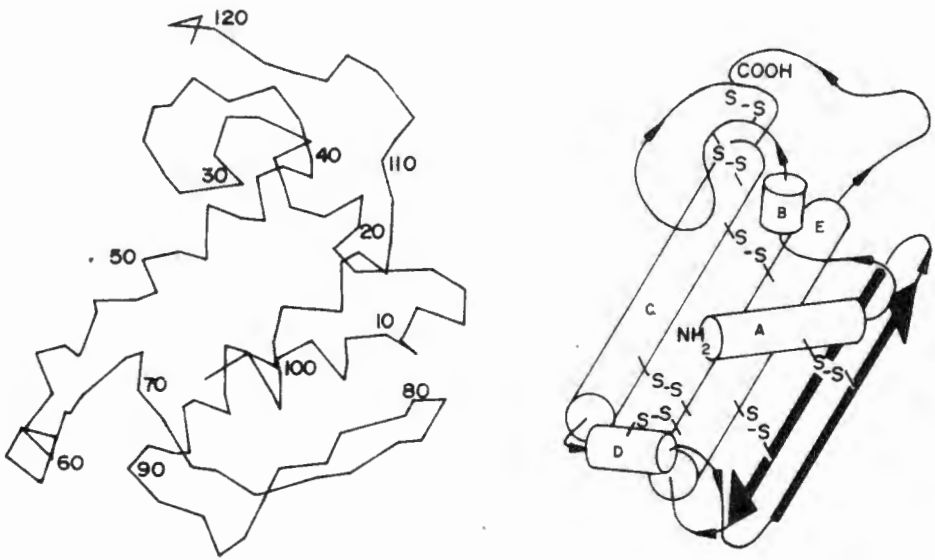
The molecular surface of PhA is very hydrophobic, as can be deduced from chromatographic studies to obtain PhA-free snake venom cardiotoxins by hydrophobic interaction chromatography [134]. The phospholipase could only be eluted from the phenyl Sepharose by denaturation with 6M urea.

1.3.2. Functional aspects of Phospholipase A_2

Despite the structural homology, PhA's induce a variety of pharmacological effects, some of them more than one. Venom PhA's induce myotoxic [97], cardiotoxic [98], hemolytic [135], anticoagulant [129] and pre-synaptic neurotoxic [101] effects. Yet all the PhA's catalyze specifically the hydrolysis of the ester bond at the C2 position of 3-sn-phosphoglycerides [131]:



These enzymes require Ca^{2+} for catalytic activity and are active in the alkaline pH-region, pH7,5-



'igure 1.6. A.The X-ray structure and B. schematic drawing of bovine phospholipase A_2

8,5 [67]. The active site of PhA as inferred from the bovine enzyme, is made up of several invariant residues; His-48, Asp-99, Phe-5, Ile-9, Ala-103, Phe-106 and the disulfide bond between Cys-45 and Cys-99 [137]. The essential Ca^{2+}-ion is also in this region.

The specific sites responsible for toxic action have not yet been solved. Attempts at predicting such sites were reported [138,139]. It was found that the presynaptically acting PhA's have a distinct hydrophobic region around the residues 80-110, which might possibly be involved in the interaction with presynaptic membranes [138].

Presynaptically neurotoxic PhA's which are also myotoxic , were shown to have a distinct charge distribution pattern, with a characteristic cationic site around residues 79-87 [139].

1.3.3. Immunological aspects of Phospholipase A_2

Limited information is available on the immunochemistry of PhA's. In comparative studies of the immunological properties of PhA's from N.melanoleuca and N.nigricollis, it was found that these enzymes contain common antigenic sites, this being demonstrated by gel immunodiffusion [128]. Nair et al [88] observed that specific antisera to PhA from N.naja and N.nigricollis inhibited PhA activities of the venoms of only closely related species in vitro . Similar cross-reactivities between PhA's of closely related species have been reported for rat pancreatic and rat microsomal PhA [89],and rat liver mitochondrial and rat liver platelet PhA [90]. Contradictory results have also been reported; no detectable cross-reactivity between human seminal and human pancreatic PhA [90].

Although it was found that PhA from unrelated species do not cross-react, as was found between pancreatic and venom enzymes [92], and rat liver mitochondrial and porcine pancreatic or Crotalus atrox enzymes, contradictory results were also reported. It was for instance shown that both snake venom and porcine pancreatic PhA's were inhibited by monoclonal antibodies to human plasmin lecitin-cholesterol acyltransferase, which also acts as a PhA, and polyclonal antiserum to N. naja PhA cross-reacted with different mammalian enzymes [93].

No information on either the precise or even the possible location of antigenic sites have been reported.

1.3.4. Discussion on Phospholipase A2

The review above indicates that the structural properties of this family of enzymes is clarified, with X-ray structures for three of these enzymes [94,100,132]. The enzymatic mechanism of these proteins is also understood, but the pharmacological properties of the toxic members of this enzyme family are vague. The immunochemistry of these enzymes also need to be solved. Since the structural properties are solved, these proteins are also interesting candidates to study immunological properties. PhA's are also globular proteins containing α-helical structures, but unlike the reviewed proteins for which immunological properties have been reported, these enzymes have a hydrophobic surface. Immunochemical data for PhA could thus also provide additional results for the immunological properties of proteins in general.

1.4. Aim of this study and proposed experimental design

Snake venom toxins are used in raising antisera for treating victims of snake bite [2]. Problems encountered in the production and use of these antisera, are the lack of sufficient material from some of the natural sources and safety considerations in the use of some of these substances. Patients treated for snake bite usually develop serum sickness [11], a situation where the patient's immune system reacts to the serum proteins from the host animal (e.g. horse) in which the antiserum was raised. This situation is occasionally fatal. The use of only the relevant toxic venom proteins for immunization , could reduce the variety of unnecessary antibodies in such an antiserum that would be raised to irrelevant venom proteins of low or no toxicity. One can speculate on synthetic peptide vaccines, mimicing the antigenic determinants of the toxic proteins, and even more futuristic, the use of monoclonal antibodies [12].

Synthetic vaccines would involve the use of synthetic peptides, derived from a protein, to raise antibodies, which are able to neutralize the function of the mother protein. The design of such synthetic vaccines usually needs a proper knowledge of the immunological properties of the mother protein [7,8].

From the review presented, it is indicated that extensive research has been undertaken on the structure of snake venom cardiotoxins [62,81,95,101-109], as well as various aspects of the functional properties [83,99,111]. Only a few reports deal with the immunological properties of

cardiotoxins [2,81-87,128]. Antigenic sites have not yet been identified.

Although data on the immunological properties of some proteins are available [4,16-45], it has been pointed out that such data cannot be readily applied to other proteins [27-29] and that every protein has to be studied separately. It was also pointed out that the structural properties of the proteins of which immunological properties have been studied, differ from the cardiotoxin used in this study, making application of known data to these toxins even more impossible. The immunological properties of cardiotoxins thus have to be studied seperately.

For an initial study of the immunological properties, it was chosen to adopt one of the approaches, discussed in 1.1.2.1.1.c), where the reactivity of antiserum raised to an intact protein, towards a large number of peptides of overlapping sequence, derived from the protein, is determined. This approach has been used successfully in the delineation of antigenic sites of a number of proteins, e.g. tobacco mosaic virus protein [53], histone proteins [64], myohemerythrin [65] and horse muscle acylphosphatase [61]. From the results of this initial approach the investigation can then be extended to other approaches, discussed in 1.1.2.1.2 for a more accurate delineation of antigenic sites. This approach was chosen because successes of other researchers using it, have been reported [53,61,64,65].

Briefly the approach was as follows:

1. Polyvalent antiserum against cardiotoxin V^{II}_1 of Naja nivea was raised in guinea pigs. The reasons for the specific use of polyvalent antiserum raised to a native protein have been discussed. Polyvalent antiserum was also raised against the control protein, bovine phospholipase A_2.

2. The antisera were tested for their specificity for the proteins they were raised against by immunodiffusion as well as enzyme linked immunosorbent assay (ELISA).

3. Peptides derived from the sequences of the cardiotoxin and PhA were synthesized chemically by a solid phase procedure.

4. The antisera were tested for their reactivity towards the peptides by ELISA. This is where the role of the antiserum raised against the control protein and peptides derived therefrom, is important. The proteins of which immunological properties have been studied to date are hydrophilic. The proteins in the present study are hydrophobic, as are the peptides derived from them. Possible non-specific hydrophobic interactions between antibodies and these peptides could give artifactual results. This possibility could be avoided by using antiserum to a hydrophobic protein with different structural properties, as a control, rather than solely depending on control sera of unimmmunised animals.

5. For relating the immunological properties observed with respect to the conformation of the peptides, the conformation of each peptide in various solvents, was determined by CD-spectroscopy. Here the use of peptides derived from the control protein is also important. Where the cardiotoxin has a high content of β-pleated sheet, the PhA contains α-helical structures. The ability of the peptides, derived from the

respective mother proteins, to adopt specific conformations, should thus differ.

From the structural analysis of the synthetic peptides, together with information concerning known T-cell epitopes [74], identification of possible T-cell epitopes in both N. nivea $V^{II}1$ and bovine phospholipase A_2 was possible.

CHAPTER 2

PEPTIDE SYNTHESIS

2.1. Introduction

For peptide mapping of antigenic sites of proteins [4], peptides can be obtained either by cleavage of the native protein by proteases and chemical methods[53,61,140], or by chemical synthesis [64,141]. Enzymatic cleavage requires large amounts of protein material, and extensive methods of purification and characterization of peptides by chromatography and electrophoresis. Since cleavage by enzymes can only be achieved at certain amino acid residues, peptides of a specific sequence cannot be obtained. When peptides are synthesized chemically, any required sequence can be obtained, and extensive purification and characterization of peptides are unnecessary. It was thus chosen to synthesize the required peptides chemically by a solid phase procedure [142].

For an initial investigation in mapping of antigenic sites of *N.nivea* $V^{II}1$, by investigation of the reactivity of antiserum to peptides, the procedures for which were discussed in 1.1.2.1.1 and 1.1.2.1.5, peptides derived from the sequence of the cardiotoxin were synthesized to mutually overlap with six amino acid residues (see Table 2.1).

The peptide sequences derived from bovine phospholipase A_2 were chosen from regions adopting hydrogen bonded secondary structures within the tertiary structure of the enzyme, as was discussed in 1.3.1. The sequences of these peptides are listed in Table 2.2.

Table 2.1. Sequences of synthetic peptides derived from N.nivea $V^{II}1$

Residue Position	Sequence*
1-10	LKCHKLVPPVG
5-15	KLVPPVWKTCPG
10-20	VWKTCPEGKNLG
15-25	PEGKNLCYKMFG
20-30	LCYKMFMVSTSG
25-35	FMVSTSTVPVKG
30-40	STVPVKRGCIDG
35-45	KRGCIDVCPKDG
40-50	DVCPKDSALVKG
45-55	DSALVKYVCCSG
50-60	KYVCCSTDKCNG

* A Gly-residue was used as a spacer in each peptide between the C-terminus and the solid support.

Table 2.2. Sequences of synthetic peptides derived from Bovine Phospholipase A_2

Residue Position	Sequence*
1-12	ALWQFNGMIKCKG
13-30	IPSSEPLLDFNNYGCYCGG
31-42	LGGSGTPVDDLDG
43-54	RCCQTHDNCYKQG
55-74	AKKLDSCKVLVDNPYTNNYSG
75-85	YSCSNNEITCSG
90-109	ACEAFICNCDRNAAICSFKVG
113-125	YNKEHKNLDKKNCG

* A Gly-residue was used as a spacer in each peptide between the C-terminus and the solid support.

In this chapter the methods and results of peptide synthesis will be presented, while the conformational properties of the peptides as determined by CD-spectroscopy, will be dealt with in Chapter 3.

2.2. Materials

The 9-Fluorenylmethyloxycarbonyl (F-moc) amino acids (L-configuration), tert-butyloxycarbonyl (t-Boc) Arginine, dicyclohexylcarbodiimide (DCC) and 1-hydroxybenzotriazole (HoBt) were purchased from Fluka AG, Switzerland, and fluorescamine from Pierce Chemical Company, USA. Piperidine was distilled from sodium hydroxide pellets under nitrogen, and redistilled under nitrogen [143]. Dimethylformamide (DMF) was distilled from ninhydrin and calcium oxide under nitrogen *in vacuo* and stored over type 4Å molecular sieve (BDH). Dichloromethane (DCM) was distilled from anhydrous sodium carbonate and stored over type 4Å molecular sieve [143], Dioxane was washed with hydrochloric acid, dried over potassium hydroxide, boiled under reflux and nitrogen over sodium metal until the sodium remained unoxidized, and then redistilled over sodium metal [143]. Both DCM and dioxane were passed through a column of aluminium oxide 90 (Merck) prior to use [143]. Isopropanol, and trifluoroacetic acid were obtained from Merck, Darmstadt, and acetonitrile from Millipore.

2.3. Methods

2.3.1. Solid phase peptide synthesis

The sequences of the synthetic peptides derived from *N.nivea* $V^{II}1$ [13] and phospholipase A_2 (PhA)[100] are listed in Tables 2.1 and 2.2

respectively. The peptides derived from N.nivea $V^{II}1$ will be referred to as Niv -X-Y, where X is the first and Y the last residue of the peptide, corresponding to the peptide sequence derived from the protein. Similarly, peptides derived from PhA will be refered to as PhA-X-Y.

The peptides were synthesized by a solid phase procedure, using amino acids of which the N-terminals were protected by 9-fluorenylmethyl (F-moc) groups [142,144]. The α-amino group of Arg was protected by the tertiary-butyl-oxycarbonyl (t-Boc) group [145]. Two different deprotection procedures [145] are required to split these protecting groups from the α-amino groups of the N-terminal amino acid residues, as will be discussed below.

One Gly residue was included in every peptide as spacer between the solid support and the C-terminal amino acid residue. This Gly-spacer was reported not to affect the immunological properties of peptides [146].

Synthesis was carried out manually in a custom built glass cell [142](volume of 4ml), and cleavage of peptides from the resin with anhydrous hydrogen fluoride (HF) in a custom built small-scale open circuit line.

In a previous study, the M.Sc.-thesis of the present author, the procedures of Chang et al [147] were followed for the chemical synthesis of peptides [94]. In those procedures a symmetric anhydride of the amino acids were formed as the activated form of the amino acid. It was found that those procedures gave rise to a extensive racemization of amino acid residues. Consequently it was suggested in the thesis that the synthesis

according to procedures described by König and Geiger [148] should be applied in future peptide synthesis. In this method 1-hydroxybenzotriazole (HoBt) is used to form an activated ester of the amino acid, and was reported to reduce the process of racemization [148]. The latter procedures were used in the present study.

2.3.1.1. Preparation of Boc-Glycine (Cesium salt)

Boc-Gly (4mmol;700mg) was dissolved in a mixture of 4ml ethanol and 1,5ml distilled water, and then titrated to pH7,0 with cesium carbonate (4mmol;1,3 g) dissolved in 4ml water. This was evaporated to dryness, washed four times with 10ml benzene, and then dried in vacuo over phosphorus pentoxide.

2.3.1.2. Preparation of Glycyl-Resin [145,149]

The sequence of the synthesis procedures is given in Figure 2.1, and will be referred to in the text. The resin, chloromethylpolystyrene 1% divinylbenzene (0,7 meq/g) (1,7mmol;2,5g), and Boc-Gly (cesium salt) (1,7mmol;530mg) were suspended in 20ml anhydrous DMF, and stirred with a magnetic stirrer for 24 hours at 50°C in a water bath (Step A, Figure 2.1). The resin was then washed with DMF:water (1:1, v/v), methanol and dichloromethane (DCM). Substitution of chlorine with Boc-Gly was assayed by determining the remaining chlorine content, by oxygen flash combustion and titrimetic determination with mercury (II) nitrate [150] (done by Mr H.H. Lachman, DPCMT, CSIR). Substitution of chloride by Boc-Gly was found to be 97% complete.

Boc-Gly-Resin (300 mg) was treated with 60% trifluoroacetic acid (TFA) in DCM containing 5% 1,2 ethanedithiol, to remove the Boc-protection

group [143] (Step B, Figure 2.1). Deprotection of the Boc-Arg-residues during synthesis was carried out by the same method. After this deprotection procedure, the resin was washed with two cycles of DCM, methanol and DMF.

2.3.1.3. Preparation of Activated Esters

A ratio of 2:1 of an Fmoc-amino acid (usually 130 μmol) to the free amino groups available on the resin, and 1-hydroxy- benzotriazole (HoBt)(150 μmol) were reacted in DMF with dicyclohexyl-carbodiimide (DCC)(150 μmol) at 4°C for 1 hour, and then at room temperature (22°C) for 1 hour to form an activated ester (Step C, Figure 2.1). The reaction mixture was then filtered through Millex SR (0,5μm) filters (Millipore) to remove the byproduct, dicyclohexylurea, which was formed during the reaction.

2.3.1.4. Elongation of the Peptide Chain

Coupling of the next amino acid residue was performed by reacting the activated ester, described in 2.2.1.3, with the Gly-Resin (see 2.2.1.2). Coupling was carried out in DMF for 1 hour at room temperature (Step D, Figure 2.1). This coupling reaction was then repeated to ensure complete coupling. For Fmoc-Asn and Fmoc-Tyr, the initial reaction time was 5 hours, and the second 18 hours [147]. The resin was then washed with two cycles of DMF and DCM.

Completeness of coupling was monitored after each coupling step by subjecting a few resin particles to a ninhydrin test [151]. This is a relatively simple procedure and gives reliable results. A

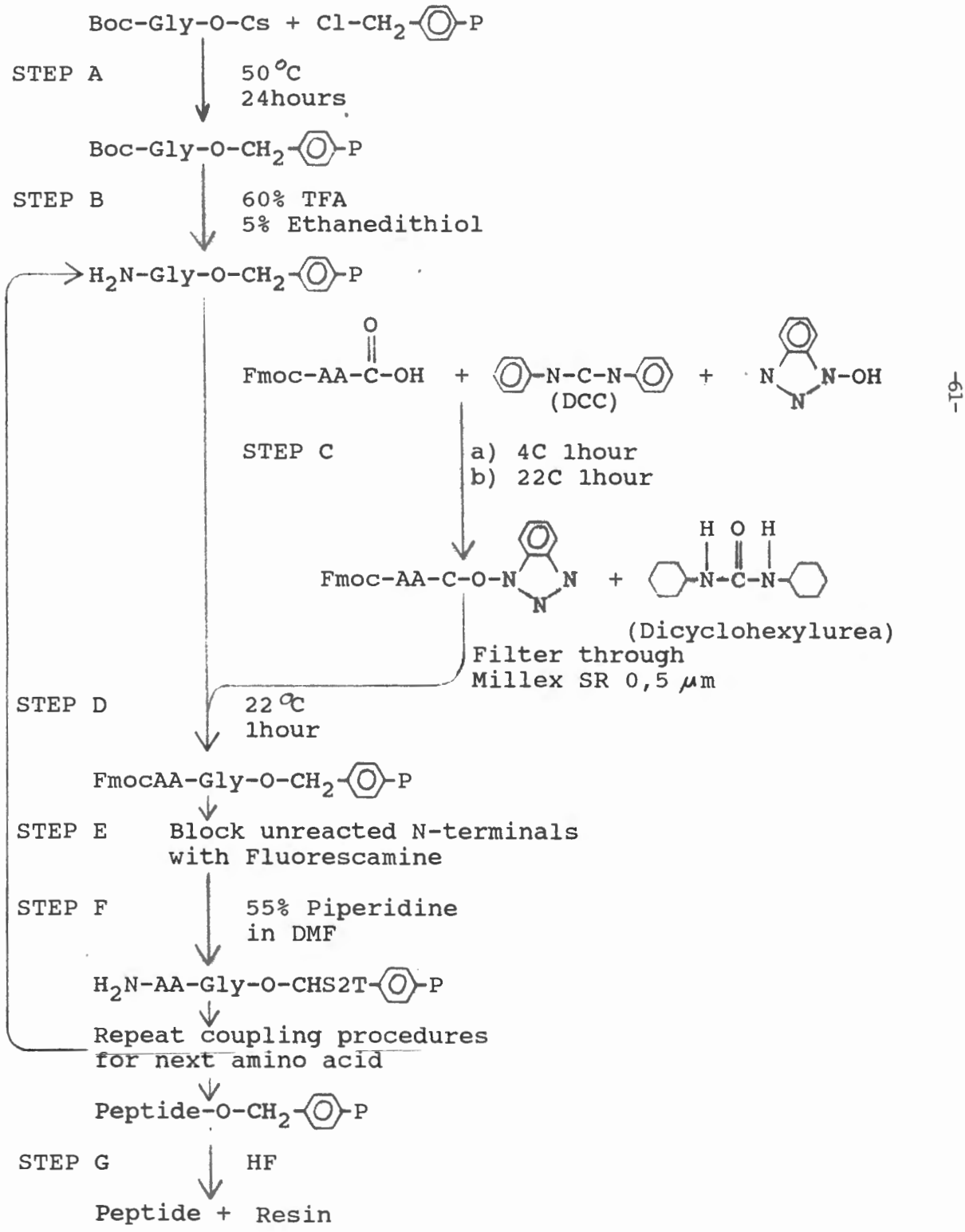


Figure 2.1. Schematic presentation of solid phase peptide synthesis.

color change to blue indicated the presence of free amino groups and thus an incomplete coupling step, which had to be repeated.

After complete coupling, possible unreacted N-terminals were blocked by addition of 10 mg fluorescamine in 100µl DCM to the resin, and a reaction time of 15 minutes [152] (Step E, Figure 2.1). The resin was then washed with two cycles of isopropanol, DCM and DMF.

Deprotection of the coupled Fmoc-amino acid was carried out by treatment with 55% piperidine in DMF (Step F, Figure 2.1). After this deprotection step the resin was washed with two cycles of DMF, dioxane:water (2:1), DMF, DCM and DMF. The next amino acid residue was then added to the growing peptide chain.

2.3.1.5. Cleavage of Peptides from the Resin [41]

Since a commercial hydrogen fluoride (HF)-line was not available for use, a model working on different principles was designed and built. Parallel to the design of the model discussed here, a model operating on the same principle, but able to handle 25 samples simultaneously, was reported by other researchers [153]. Both these open circuit HF-lines were as efficient in cleaving peptides from resin supports as a commercial vacuum system [145]. The small-scale open circuit HF-line, described here, used dried nitrogen as a carrier and purging gas, and was constructed as shown in Figure 2.2. All components exposed to HF, either gas or liquid (Components B-G in Figure 2.2),were of polytetrafluoroethylene (PTFE, Teflon) construction. Published cleavage conditions [145,154] were followed (Step G, Figure 2.1). The resin-bound peptide (300mg) and

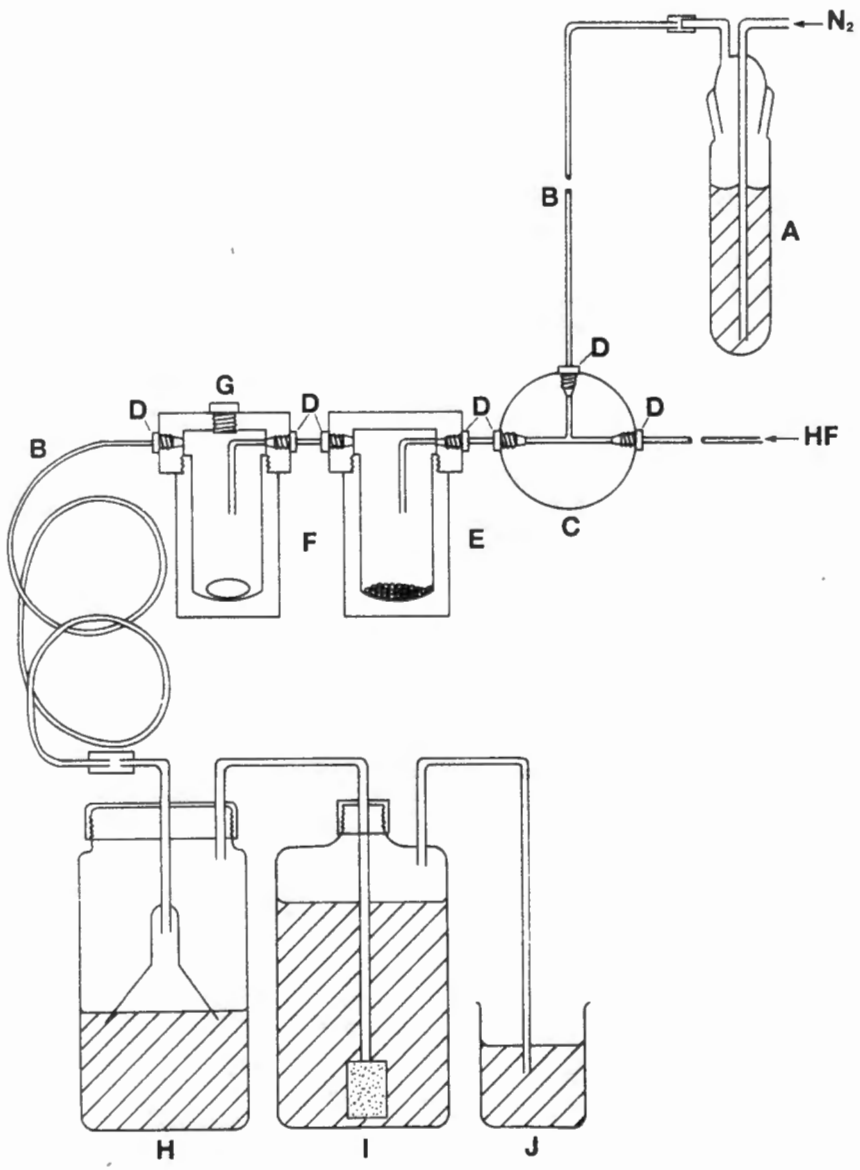


Figure 2.2. The small-scale open circuit hydrogen fluoride line.
A: Bubbler. B: PTFE-tubing. C: T-piece. D: Ferrules. E: Vial containing CoF_3. F: Reaction vial with PTFE-coated magnetic stirring bar. G: Stopper. H: Polyethylene bottle and funnel containing 400ml 5N NaOH with 0,1% methyl orange. I: Polyethylene bottle containing a sintered bubbler (PTFE) and 400ml 5N NaOH with 0,1% methyl orange. J: Methyl orange (0,1%) in water to detect unreacted HF.

anhydrous anisole (400µl) were placed in vial F with a PTFE-coated magnetic stirring bar, and anhydrous cobalt fluoride (1g) in vial E. The whole system was then assembled. The system was flushed with high purity nitrogen gas at ca 150 ml/min for 1-2 minutes, and the flow then reduced to ca 6 bubbles/min at bubbler A for carrying HF gas. Vial E was immersed in solid CO_2/acetone. The HF was condensed in vial E. By backlighting of vial E , the level of accumulating HF liquid was observed, and 5ml collected. This was transferred to vial F by cooling vial F and warming vial E with water (40°C), again observing the rise of HF liquid level. After liquid transfer was complete, vial F was kept at 0°C in ice/water for 1 hour while stirring magnetically. Vial F was thereafter warmed with water (40°C) and the nitrogen flow raised to 280ml/min until all the liquid therein had transferred to trap H, containing 5M NaOH. The peptide was washed from the resin alternately by 80% acetonitrile in water containing 0,01% TFA, and water. Recovery of peptide was determined by amino acid analysis. Peptides were freeze-dried and purified by reversed phase high performance liquid chromatography (RP-HPLC).

2.3.2. Analysis of peptides

2.3.2.1. Purification of peptides

Peptides were purified by RP-HPLC on a C18 µBondapak column (Waters Ass) using linear gradients of 0,01% TFA as initial and 80% acetonitrile in water containing 0,01% TFA as limiting solvent. The flow speed was 1ml/min [155] and elution was monitored at 229 nm.

2.3.2.2. Amino acid analysis [142]

Approximately 3µg of each HPLC-fraction of each peptide preparation was hydrolyzed with 500µl 6N constant boiling hydrochloric acid in evacuated tubes at 110°C for 24 hours. To protect the Tyr-residues against chlorination, 10µl phenol was added. The hydrolyzed material was dried *in vacuo*, dissolved in citrate buffer (pH2,2) with 200nmol/ml norleucine as internal standard, and analyzed with a Waters HPLC-system under standard conditions [156].

A fraction of the remaining resin was also subjected to amino acid analysis to determine completeness of cleavage and recovery of each peptide. Resin-bound peptides were hydrolyzed at 130°C with a mixture of 6N hydrochloric acid/propionic acid (1:1, v/v) for 3 hours in sealed evacuated tubes [142].

2.4. Results

The recoveries of peptides from the resin after HF-cleavage, as determined by amino acid analysis for Niv-15-25 and 40-50, and PhA-1-12, as examples, are shown in Table 2.3. In general the recoveries were 84-96%. The results obtained for the above three peptides are shown and discussed below as typical examples.

Chromatograms of peptides Niv-15-25 and 40-50, and PhA-1-12, shown in Figure 2.3, indicate the presence of more than one fraction. The peptide content of each fraction obtained for every crude synthetic peptide preparation, is listed in Table 2.4. The main fraction of each peptide was rechromatographed to purity, under the same

Table 2.3. Recoveries of peptides from resin as determined by amino acid analysis.

Peptide	moles Peptide Recovered		
	Free	Resin	% of Total
Niv 15-25	73,0	2,4	96
Niv 40-50	54,2	8,6	86
PhA 1-12	55,5	9,4	85

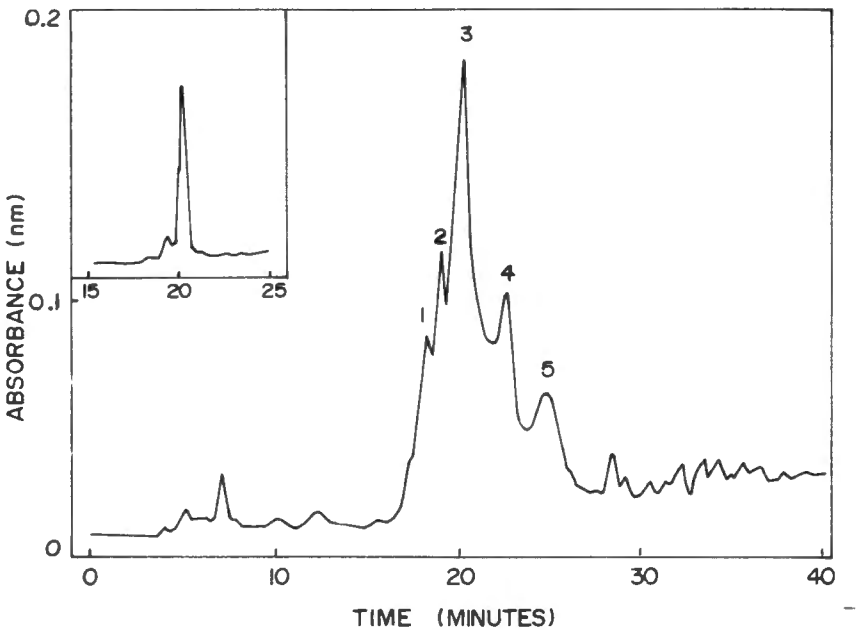


Figure 2.3.1. HPLC chromatogram of peptide 15-25 derived from N.nivea $V^{II}1$ cardiotoxin.
Insert; rechromatography of fraction 3.
Chromatography was carried out on a C18 μBondapak column and a 20 minutes linear gradient of 20% Acetonitrile/0,01% TFA to 60% Acetonitrile/0,01% TFA.

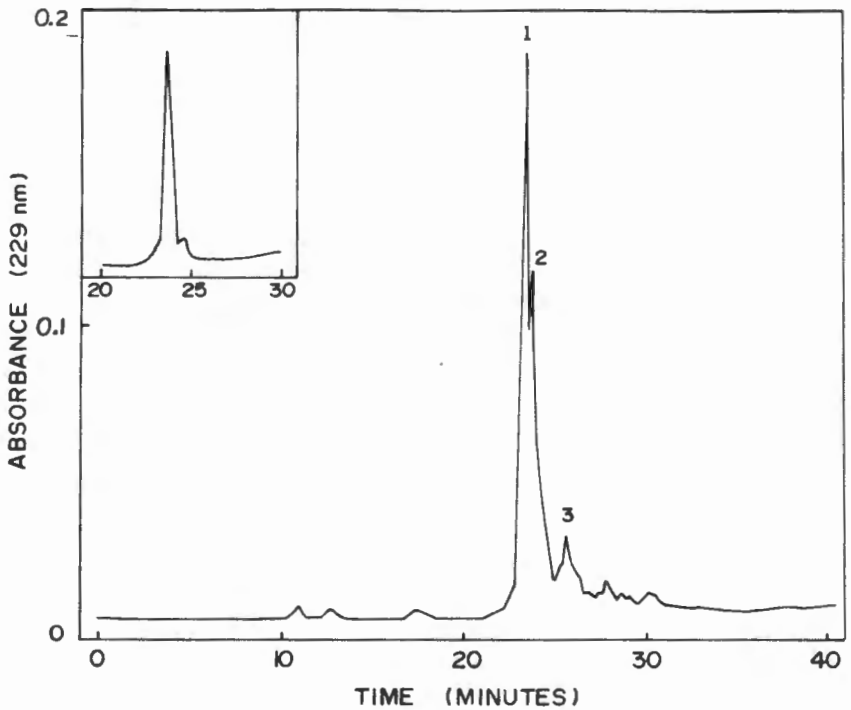


Figure 2.3.2. HPLC chromatogram of peptide 40-50 derived from N.nivea $V^{II}1$ cardiotoxin. Insert; rechromatography of fraction 1. Chromatography was carried out with a linear gradient of 5% Acetonitrile/0,01% TFA to 50% Acetonitrile/0,01% TFA.

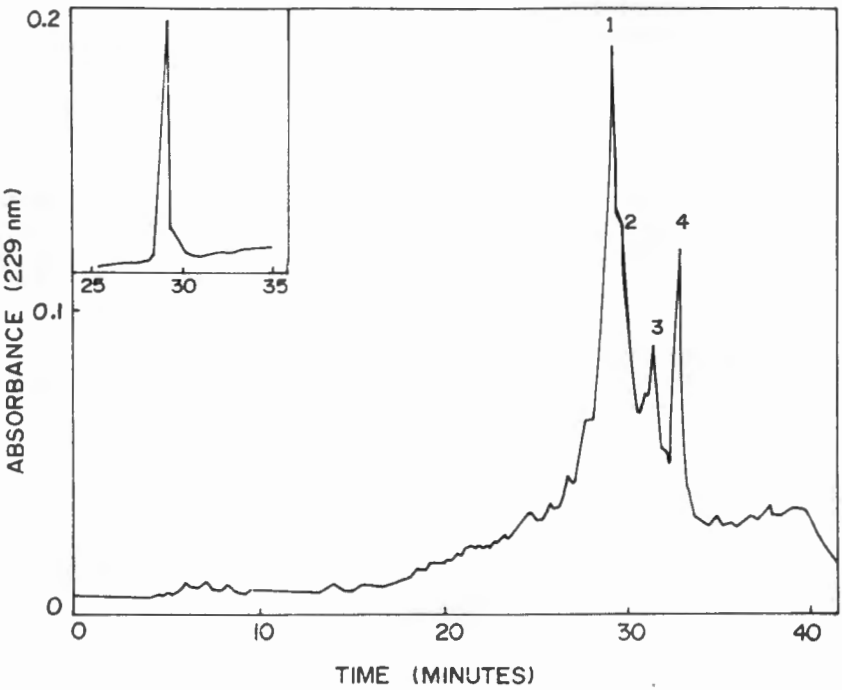


Figure 2.3.3. HPLC chromatogram of peptide 1-12 derived from phospholipase A_2.
Insert; rechromatography of fraction 1.
Chromatography was carried out with a linear gradient of 20% Acetonitrile/0,01% TFA to 60% Acetonitrile/0,01% TFA.

Table 2.4. Recovery of fractions of crude synthetic peptides which were chromatographed on reversed-phase HPLC. Main fractions are underlined.

Peptide	Recovery of HPLC-fractions, w/w (%)				
	1	2	3	4	5
M.nivea V[II]1					
1-10	76,9	28,9	4,2	0,0	-
5-15	0,0	63,2	30,5	6,3	-
10-20	7,2	36,0	41,4	12,7	2,7
15-25	18,0	21,5	46,3	14,2	0,0
20-30	12,8	63,7	23,5	-	-
25-35	15,3	60,2	24,5	0,0	-
30-40	21,5	73,2	6,3	0,0	-
35-45	65,3	25,7	9,0	0,0	-
40-50	72,9	24,8	2,3	0,0	-
45-55	7,3	21,3	69,4	2,0	-
50-60	0,0	59,8	22,9	17,3	0,0
Bovine phospholipase A_2					
1-12	58,6	32,5	8,9	0,0	-
13-30	15,2	22,7	58,4	3,7	0,0
31-42	54,7	30,1	13,1	2,1	0,0
43-54	3,7	69,0	27,0	0,3	-
55-74	6,2	28,1	59,7	6,0	0,0
75-85	0,0	31,9	41,2	15,9	11,4
90-109	0,9	10,3	49,3	31,5	8,00
113 125	0,0	16,5	53,7	22,3	7,5

chromatographic conditions as the first chromatogram of the corresponding crude peptide preparation.

Each peptide fraction obtained by HPLC, as well as the pure peptide were subjected to amino acid analysis. The results of the three examples are given in Table 2.5.

Chromatography was carried out with a linear gradient of 20% Acetonitrile/0,01% TFA to 60% Acetonitrile/0,01% TFA.

2.5. Discussion

The amino acid analyses of the individual HPLC-fractions of peptide preparations (see Table 2.5), as well as the purified peptides, show that each fraction had an amino acid content corresponding to the theoretical content of the peptide. This indicated that no truncated fragments were formed due to either incomplete synthesis or hydrolysis during the HF-cleavage step. In that case the peptide fragments would have shown amino acid contents different from those of the theoretical contents of each peptide. This observation, together with the high recoveries of peptide after HF-cleavage, both indicated that the custom-built HF-line functioned with an effectivity and reliability similar to that of a commercial one [145] and the open circuit system described by others [153].

Multiple fractions of synthetic peptides as observed here, were also observed by other researchers [157-159], but no analysis or explanation was provided. A possible explanation is the presence of amino acid enantiomers, which

Table 2.5.1. Amino acid composition of peptide 15-25 derived from N.nivea $V^{II}1$.

Amino acid	Residues per mole					
	Theoretical	HPLC-fraction				
		1	2	3	4	Pure
Asp	1	1,12	1,15	1,11	1,08	1,11
Thr	0	0,00	0,00	0,00	0,00	0,00
Ser	0	0,10	0,00	0,21	0,08	0,15
Glu	1	0,63	0,57	0,84	0,74	0,85
Pro	1	0,95	0,94	0,91	0,96	0,92
Gly	2	2,34	2,22	2,38	2.40	2,28
Ala	0	0,00	0,00	0,08	0,00	0,00
Cys	1	1,15	1,23	1,25	1,40	1,20
Val	0	0,00	0,00	0,05	0,00	0,00
Met	1	1,03	1,10	1,08	0,09	1,03
Ile	0	0,03	0,05	0,02	0,06	0,03
Leu	1	0,86	0,90	0,83	0,91	0,87
Tyr	1	1,48	1,22	1,03	1,11	1,03
Phe	1	1,07	1,00	1,11	1,34	1,10
His	0	0,15	0,27	0,18	0,11	0,19
Trp	0	0,29	0,24	0,11	0,35	0,11
Lys	2	2,26	2,06	2,19	2,18	2,09
Arg	0	0,12	0,29	0,19	0,09	0,11
Recovery, w/w (%)		18,0	21,5	46,3	14,2	

Table 2.5.2. Amino acid composition peptide 40-50 derived from N.nivea $V^{II}1$

Amino acid	Residues per mole				
	Theoretical	HPLC-fraction			
		1	2	3	Pure
Asp	2	2,23	2,29	2,16	2,22
Thr	0	0,00	0,02	0,01	0,00
Ser	1	0,96	0,93	0,85	0,95
Glu	0	0,00	0,01	0,05	0,00
Pro	1	0,90	0,93	0,91	0,91
Gly	1	1,15	1,15	1,17	1,15
Ala	1	1,07	1,12	1,16	1,08
Cys	1	1,10	1,13	1,25	1,13
Val	2	1,96	2,12	1,96	1,95
Met	0	0,00	0,00	0,00	0,00
Ile	0	0,00	0,00	0,03	0,00
Leu	1	0,82	0,80	0,91	0,84
Tyr	0	0,00	0,00	0,00	0,00
Phe	0	0,00	0,04	0,00	0,00
His	0	0,03	0,03	0,01	0,01
Trp	0	0,00	0,02	0,02	0,00
Lys	2	2,30	2,16	2,13	2,23
Arg	0	0,04	0,05	0,15	0,03
Recovery, w/w (%)		72,9	24,8	2,3	

Table 2.5.3. Amino acid composition of peptide 1-12 derived from Bovine phospholipase A_2

Amino acid	Residues per mole					
	Theoretical	HPLC-fraction				
		1	2	3	4	Pure
Asp	1	1,18	1,20	1,47	0,00	1,22
Thr	0	0,00	0,00	0,00	0,00	0,00
Ser	0	0,00	0,00	0,00	0,00	0,00
Glu	1	0,80	0,80	0,71	0,00	0.80
Pro	0	0,00	0,00	0,00	0,00	0,00
Gly	2	2,30	2,28	2,50	0,21	2,21
Ala	1	1,06	1,01	1,10	0,08	1,08
Cys	1	1,22	1,18	1,02	1,00	1,08
Val	0	0,01	0,02	0,02	0,06	0,00
Met	1	1,15	1,11	1,21	0,10	1,12
Ile	1	0,89	0,83	0,87	0,03	0,83
Leu	1	0,92	0,95	0,89	0,05	0,95
Tyr	0	0,00	0,00	0,00	0,00	0,00
Phe	1	1,10	1,18	1,19	0,06	1,12
His	0	0.02	0,19	0,09	0,00	0,00
Trp	1	1,30	1,36	1,42	0.19	1,27
Lys	2	2,00	1,97	1,89	0,00	1,96
Arg	0	0,00	0,00	0,00	0,00	0,00
Recovery, w/w (%)		58,6	32,5	8,9	0,0	

were formed by racemization during synthesis. Steinauer et al [160] have shown that short peptides containing one D-amino acid residue within their sequence, elute from reversed-phase HPLC at times different from those of peptides with the same sequence consisting only of L-amino acids. The peptide fractions obtained here eluted differently and had the same amino acid content, and could thus have contained amino acid enantiomers. The amounts of D-amino acids were not investigated, since the method of amino acid analysis, described above, is unable to detect small amounts of D-amino acids in the presence of large amounts of L-amino acids.

Many additives that reduce racemization, eg. copper(II)chloride [161] and various polyacrylic acid derivatives [162], have been tested and reported. Although racemization of amino acids during peptide synthesis can be reduced by various additives, it cannot be suppressed completely. The use of HoBt [148], employed in this study, is the most frequently used method that reduces the occurrence of racemization [148].

For reasons mentioned above, the purified main fraction of each peptide preparation was not analysed for the presence of D-amino acids. These pure peptides were used for further experimental work. Analysis of the conformation and immunochemical properties will be discussed in the following chapters.

CHAPTER 3 STRUCTURAL ANALYSIS OF SYNTHETIC PEPTIDES

3.1. INTRODUCTION

In the previous chapter the synthesis of peptides derived from the sequences of cardiotoxin $V^{II}1$ of N . nivea and bovine phospholipase A_2 (PhA), was discussed. In the present chapter the conformational properties of these peptides were investigated.

Synthetic peptides are often used in studying the immunological properties of proteins by analyzing their reactivities with polyclonal antibodies raised against native globular proteins [4,141,163,164]. The approaches have been discussed in 1.1.2.1.1. It is also known that such antibodies do not react with isolated peptides derived from native proteins, although they may be involved in an antigenic site [47,165,166]. This failure was ascribed to the peptides not having the same conformation as the segments they were derived from in the native protein [165], but the conformational characteristics of these peptides were not investigated. It was furthermore speculated that the native conformation is one of many conformations an isolated peptide can adopt in solution [167-169], or that the conformation of a peptide is not important for antibody binding, but that the peptides are flexible and can be structured by the antibody specific for this sequence in the mother protein [170], once the peptide-antibody interaction is initiated. Since it was found that antigenic sites are flexible or mobile [56,57](see 1.1.2.1.2. and 1.1.2.1.4.), the many conformations a peptide could adopt in solution, could mimic the various conformations of a flexible antigenic site to which antibodies were

raised, thus promoting interaction between the antibodies and the peptide.

It has been discussed in 1.1.2.2. that isolated peptides derived from T-cell epitopes are often able to adopt stable amphipathic structures, often with the periodicities compatible with α-helical structures [74]. Supportive as well as contradictoryevidence for this finding has been reported [171].

It is evident from the above that researchers do not always investigate the conformational properties in studies relating to immunological properties of peptides. It is important that these properties are studied, since they are required for a better understanding of immunological properties of peptides (Chapter 4).

In the present work, the conformations of the peptides studied, were determined by circular dichroism (CD) spectroscopy, a technique which is often used in investigating peptide and protein structure [172-174], and structure-function aspects of immunological properties of peptides [175,176]. With this technique the presence of hydrogen bonded secondary structures, i.e. α-helices, β-turns or β-sheets, can be detected, but not the exact location of hydrogen bonds.

Nuclear magnetic resonance (n.m.r.) spectroscopy [175,177] was also considered for this purpose. Although this technique is able to reveal the precise secondary structure of a peptide by exact.location and length of hydrogen bonds, such detailed data, in addition to those obtained by CD-spectroscopy, would not necessarily be of more value in analyzing results concerning the

immunological properties of proteins, as done in this study.

3.2. Materials

N-butanol and 2,2,2-trifluoroethanol (TFE) were of spectroscopic grade, and were purchased from Merck (Darmstadt).

3.3 Methods

Deep ultraviolet CD-spectra were recorded on a Jasco Model J 20 spectropolarimeter, using a 0,2mm path length quartz cuvette [63,109]. Peptides (0,5mg/ml) were dissolved in either phosphate buffered saline (pH7,4)(PBS), 2,2,2-trifluoroethanol or n-butanol (Tables 3.1 and 3.2). The reasons for the choice of solvents will be discussed in the next paragraph. The CD-spectra of Niv -15-25 and PhA-1-12 and 90-109 were also recorded in PBS containing various amounts of TFE.

3.4. Results and Discussion

The conformation of each peptide was determined in three different solvents; PBS as aqueous solvent, because the immunological properties (Chapter 4) were studied in the same medium, and two organic solvents, i.e. TFE and n-butanol, which prevent hydrogen bonding between solvent and peptide [178,181,182]. TFE and n-butanol also differ in polarity, resulting in a difference in solvation of the peptides. TFE has a high tendency to solvate the peptide chain, whereas n-butanol promotes hydrophobic interactions between non-polar amino acid side chains of a peptide [178,179]. This in turn can result in promotion of different conformations of a peptide in these solvents [178-183].The use of other organic

solvents were also investigated, but rejected because of poor solubility of the synthetic peptides, and absorbancy of the solvent in the far-UV range [178].

All the peptides dissolved in PBS for CD-spectroscopy, and displayed CD-spectra characteristic of random structure [172] (see Figures 3.1+3.2). The peptides differed in solubility in TFE and n-butanol. Analysis of the amino acid compositions showed that those with a high content of polar and charged amino acids were insoluble in n-butanol, whereas peptides with a high content of hydrophobic amino acids did not dissolve in TFE. All the peptides that dissolved in n-butanol also displayed CD-spectra characteristic of random structure [172] (Figures 3.1+3.2).

It thus appeared that none of the peptides synthesized had the ability to form any of the known ordered conformations for peptides, but retained a random structure when dissolved in n-butanol and PBS.

In contrast, Niv -15-25 and PhA-1-12 and 90-109, when dissolved in TFE, showed ordered structural character. The deep ultraviolet CD-spectra each displayed a large Cotton effect in the region 190-200nm, and two negative bands in the regions of 210 and 220nm, respectively (Figures 3.3-3.5), characteristic of α-helical structure [172]. According to Chou and Fasman [172], the percentage of α-helix that each peptide adopts can be calculated from the CD-spectra, and is given below.

These three peptides, when dissolved in TFE, reacted differently when titrated with PBS. From

Table 3.1. Sequences, solubilities and prediction parameters for secondary structures of synthetic peptides derived from N.nivea $V^{II}1$ in solvents used for CD-spectroscopy.

Residue Position	Sequence	Solvent*			Prediction Parameter**	
		PBS	TFE	n-Butanol	α	β
1-10	LKCHKLVPPVG	+	-	+	0,984	1,025
5-15	KLVPPVWKTCPG	+	-	+	0,899	1,030
10-20	VWKTCPEGKNLG	+	-	+	0,938	0,933
15-25	PEGKNLCYKMFG	+	+	+	0,924	0,949
20-30	LCYKMFMVSTSG	+	-	-	0,948	1,189
25-35	FMVSTSTVPVKG	+	+	+	0,929	1,159
30-40	STVPVKRGCIDG	+	+	+	0,846	1,067
35-45	KRGCIDVCPKDG	+	+	-	0,852	1,006
40-50	DVCPKDSALVKG	+	+	-	0,988	1,002
45-55	DSALVKYVCCSG	+	+	-	0,948	1,098
50-60	KYVCCSTDKCNG	+	-	-	0,840	1,082

* PBS; Phosphate buffered saline, pH 7,4
TFE; 2,2,2-Trifluoroethanol
+; soluble
-; insoluble

** See text for calculation

Table 3.2. Sequences, solubilities and prediction parameters for secondary structures of synthetic peptides derived form Bovine Phospholipase A in solvents used for CD-spectroscopy.

Residue Position	Sequence	Solvent*			Prediction Parameter**	
		PBS	TFE	n-Butanol		
1-12	ALWQFNGMIKCKG	+	+	-	1,009	1,093
13-30	IPSSEPLLDFNNYGCYCGG	+	+	+	0,836	0,946
31-42	LGGSGTPVDDLDG	+	+	+	0,852	0,944
43-54	RCCQTHDNCYKQG	+	+	+	0,879	1,035
55-74	AKKLDSCKVLVDNPYTNNYSG	+	-	-	0,964	0,976
75-85	YSCSNNEITCSG	+	-	-	0,822	0,935
90-109	ACEAFICNCDRNAAICSFKVG	+	+	+	1,020	1,025
113-125	YNKEHKNLDKKNCG	+	+	-	0,962	0,807

* See Table 3.1 for abbreviations of solvents.
** See text for calculation

these titration curves, the lowest percentage of TFE at which each peptide adopted a final stable conformation can be determined. Stages of conformational transition [171], or in the present study conformational disruption, can also be determined.

From Figure 3.3, the percentage α-helix adopted by Niv -15-25 in 100% TFE, was calculated as 42%. Titration of Niv -15-25 showed a stable α-helical conformation at TFE-percentages down to 70% (Figure 3.6). A further decrease in TFE-percentage to 30% gradually decreased the amplitude of the positive band at 190-200 nm, and a sharp decrease in amplitude was observed below 30% TFE. This indicated two transitions in conformational disruption.

The percentage of α-helix formed by PhA 1-12 in TFE (Figure 3.4) was calculated as 31%. Titration of this peptide (Figure 3.7) showed that this maximum of α-helical conformation is only adopted at 100% TFE. A sharp decrease in the 190-200 nm band amplitude was observed from 100% to 90% TFE, and a gradual decrease ranging from 90% to 0% TFE, with two transitional stages within this range.

The percentage of α-helix for PhA 90-109 in 100% TFE (Figure 3.5) was calculated as 52%. A decrease in the percentage of TFE to 70% showed no change in this conformational state (Figure 3.8). A further decrease of TFE-percentage to 0% gradually decreased the amplitude of the positive 190-200 nm band in a linear way, indicating no observable transitional stages in the conformational disruption of this peptide.

According to the model of De Lisi and Berzofsky [74] (see 1.1.2.2.3.), a peptide that could fold

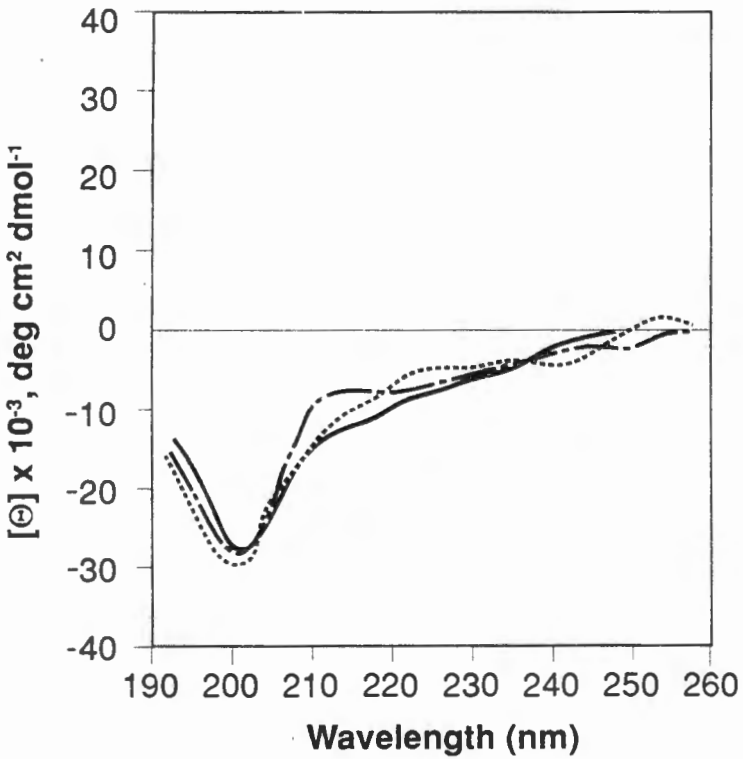


Figure 3.1. Circular dichroism spectra of peptide 25-35 derived from N.nivea $V^{II}1$ cardiotoxin in TFE(———), PBS(•—•—•) and n-Butanol(- - - - -).

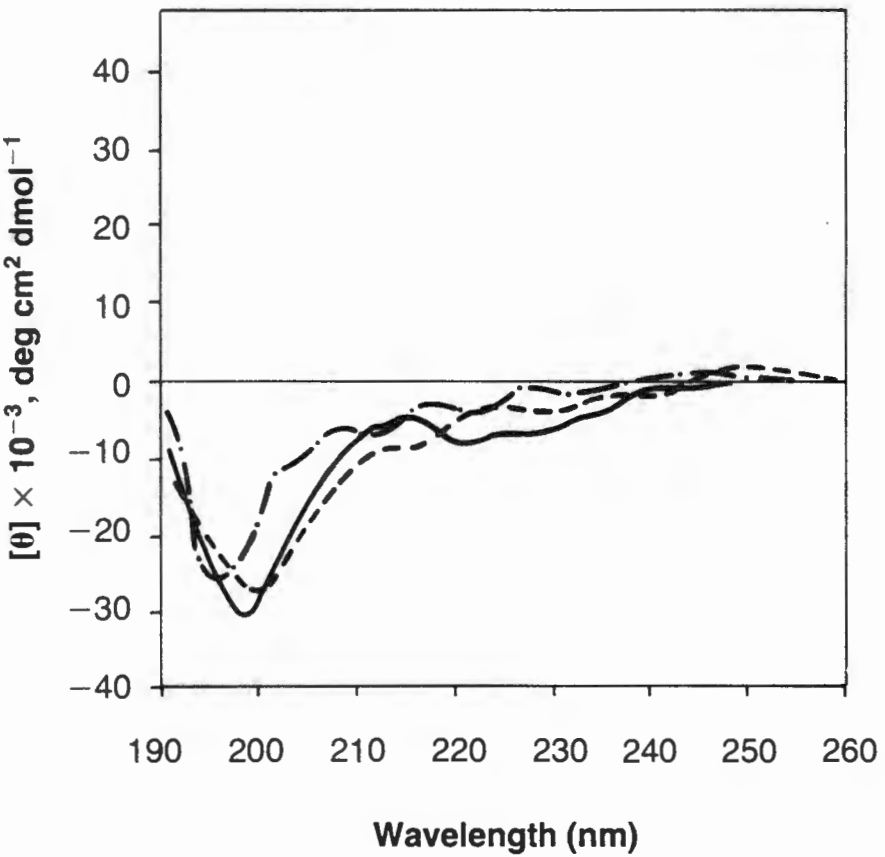


Figure 3.2. Circular dichroism spectra of peptide 31-42 derived from bovine phospholipase A_2 in TFE(———), PBS(—·—) and n-Butanol(- - -).

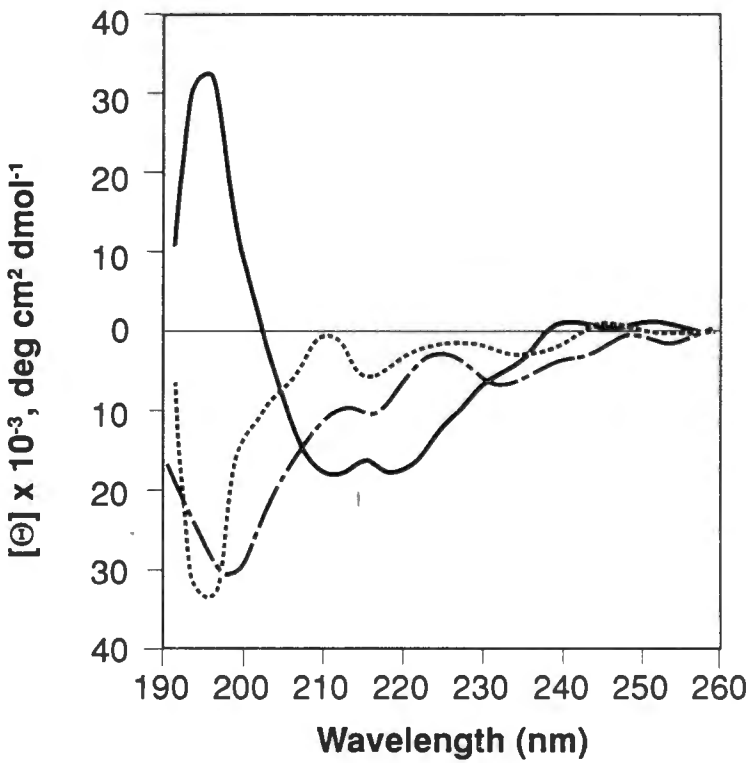


Figure 3.3. Circular dichroism spectra of peptide 15-25 derived from N.nivea $V^{II}1$ cardiotoxin in TFE(———), PBS(-·-·-) and n-Butanol(-----).

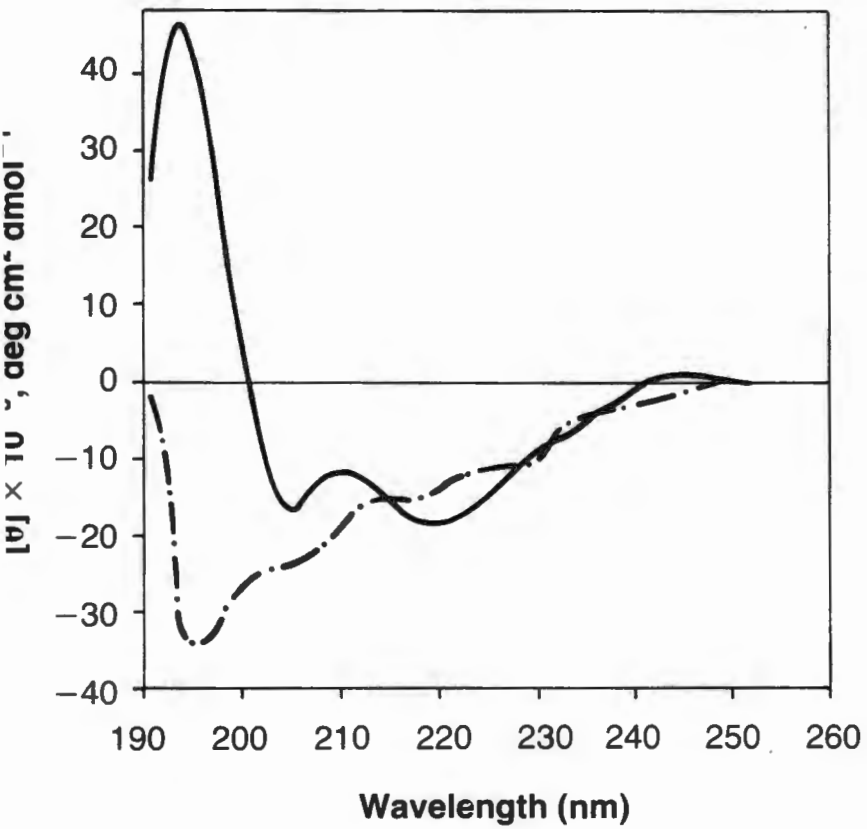


Figure 3.4. Circular dichroism spectra of peptide 1-12 derived from bovine phospholipase A_2 in TFE(———), PBS(·—·—·).

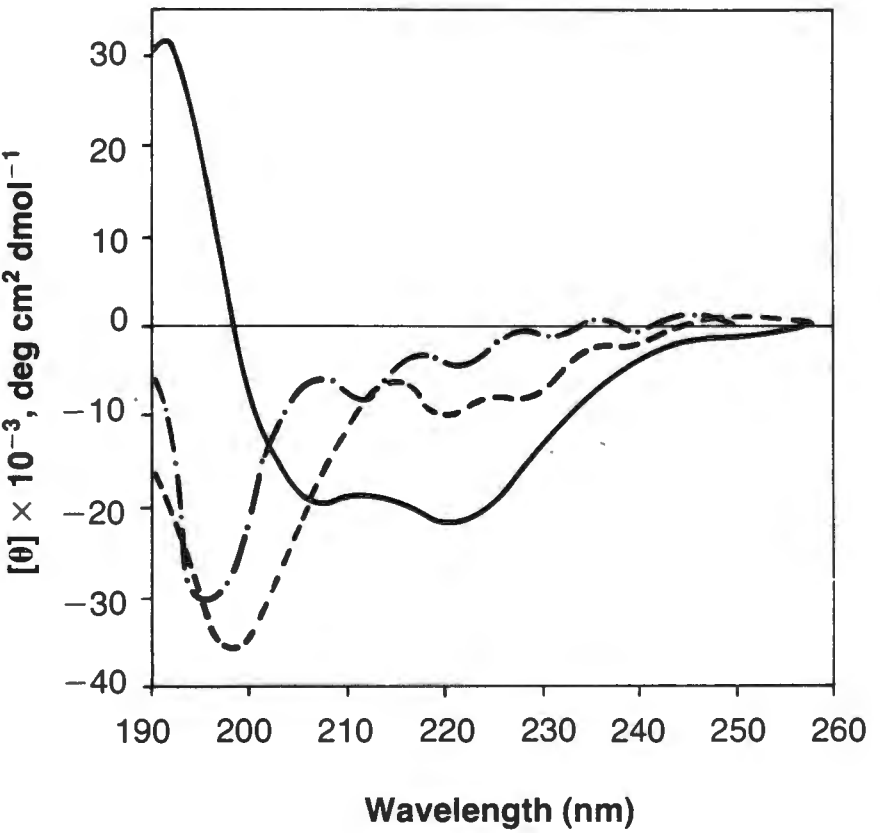


Figure 3.5. Circular dichroism spectra of peptide 90-109 derived from bovine phospholipase A_2 in TFE(———), PBS(·—·—·) and n-Butanol(- - - -).

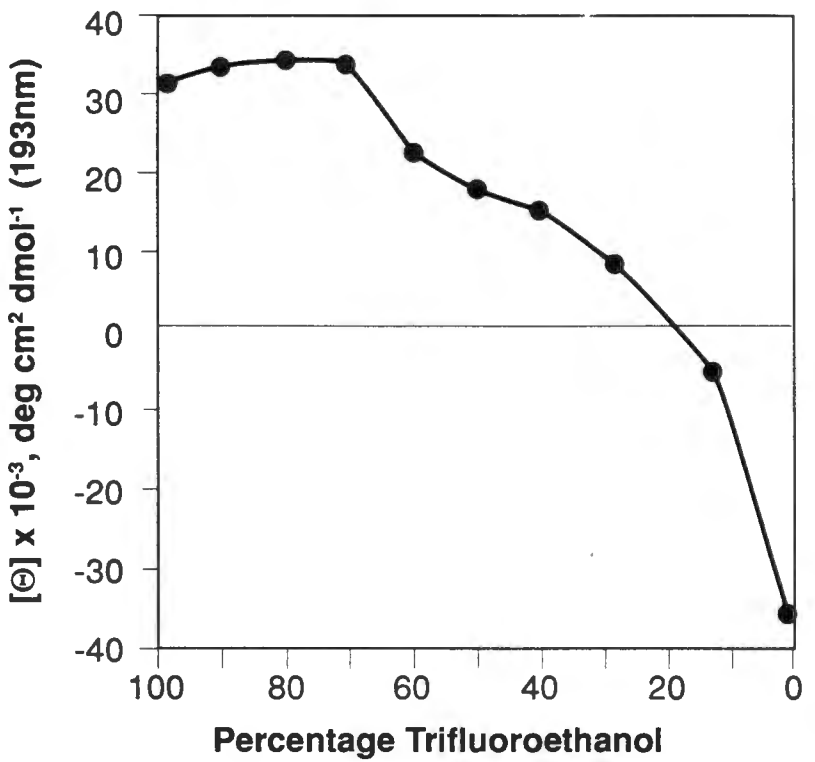


Figure 3.6. The effect of a decrease in TFE-percentage on the mean ellipticity [θ]193nm of peptide 15-25 derived from N.nivea $V^{II}1$ cardiotoxin.

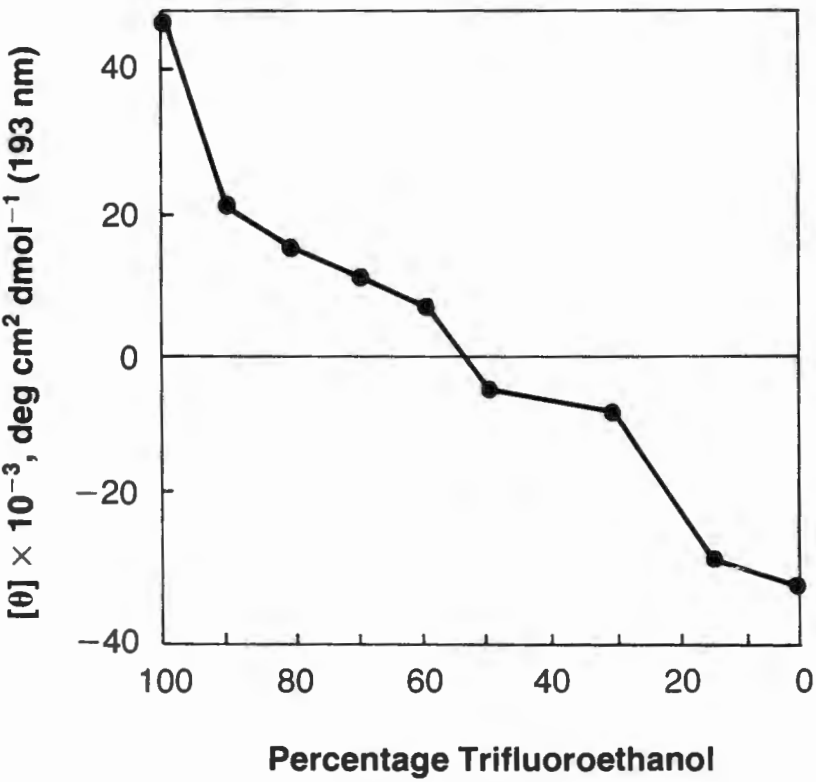


Figure 3.7. The effect of a decrease in TFE-percentage on the mean ellipticity [θ]193nm of peptide 1-12 derived from bovine phospholipase A_2

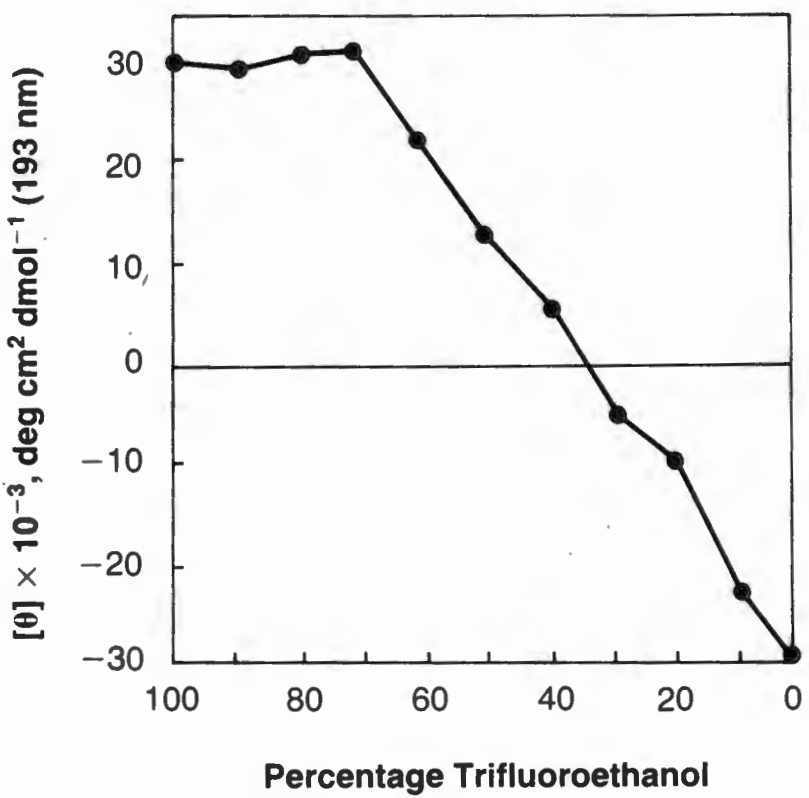


Figure 3.8. The effect of a decrease in TFE-percentage on the mean ellipticity [θ]193nm of peptide 90-109 derived from bovine phospholipase A_2

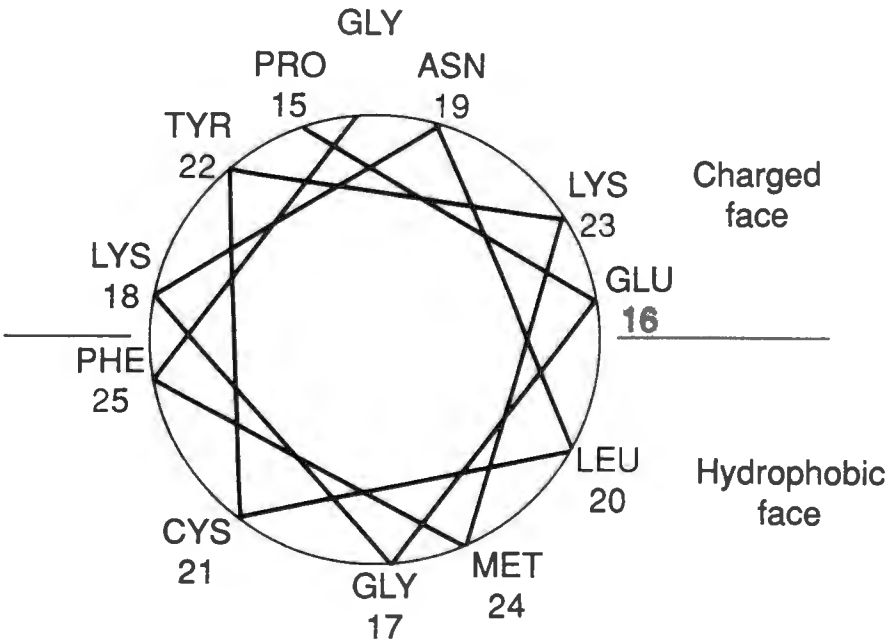


Figure 3.9. Helical wheel representation of the amphipathic helical structure of peptide 15-25 derived from N.nivea II1 cardiotoxin.

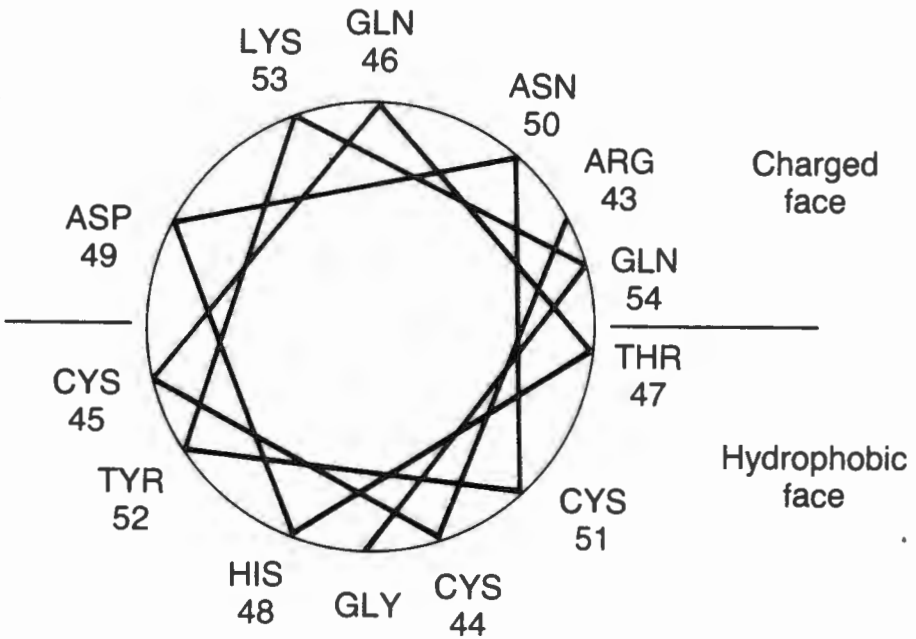


Figure 3.10. Helical wheel representation of the amphipathic helical structure of peptide 43-54 derived from bovine phospholipase A_2

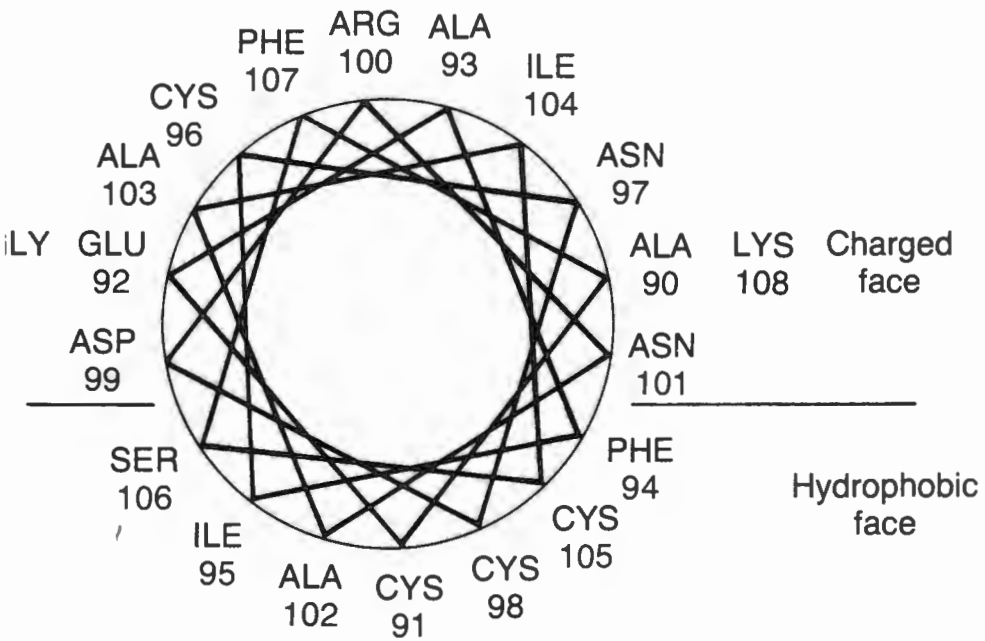


Figure 3.11. Helical wheel representation of the amphipathic helical structure of peptide 90-109 derived from bovine phospholipase A_2

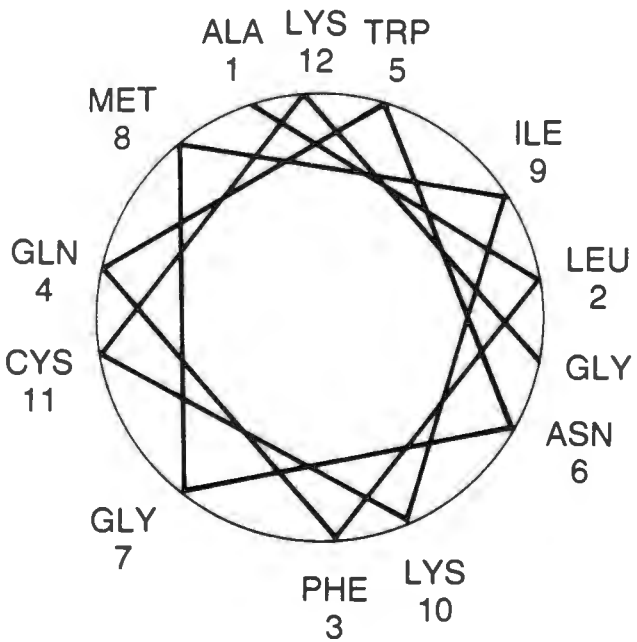


Figure 3.12. Helical wheel representation of the amphipathic helical structure of peptide 1-12 derived from bovine phospholipase A_2

as an amphipathic helix, when presented on the surface of a T-cell after the antigenic parent protein has been processed, could act as a T-cell epitope. Since Niv -15-25, and PhA-1-12 and 90-109 appear to form α-helical structures in TFE, the helical wheel model [184] was applied in order to determine whether these regions could form amphipathic helices. This model was also applied to PhA 43-54, since it has α-helical structure in the intact enzyme [100]. Such analysis showed Niv -15-25 and-PhA 43-54 and 90-109 to be potential amphipathic helices (Figures 3.9-3.11) but not PhA-1-12 (Figure 3.12). Only Niv -15-25, despite being derived from a β-structured protein, and PhA 90-109, are able to form α-helical structures in the free peptide form. Since the helices have distinct charged and hydrophobic faces, they fulfil the criteria of De Lisi and Berzofsky [68] for T-cell epitopes. TFE, a structure-promoting solvent [178,179,183] has been suggested as able to mimic the natural environments of cell membrane receptors [185]. This suggests that peptides Niv -15-25 and PhA 90-109 could also adopt α-helical structures when interacting with cell membranes, supporting further the proposal that these peptide regions can act as T-cell epitopes in their parent proteins.

These results support existing evidence [33,34] that isolated peptides in solution do not always adopt the same hydrogen bonded structures as in the native proteins they were derived from.This finding is important in analyzing results concerning immunological properties of proteins. This especially accounts for peptides in aqueous solutions, in which they often adopt no hydrogen bonded conformations [178,181,182]. Theoretical calculations of the possible conformations the

synthetic peptides could adopt are shown in Tables 3.1 and 3.2. The parameters for α-helix and β-pleated sheet as determined by Chou and Fasman [49] were assigned to each amino acid in the sequence, and the average of all the residues calculated for each peptide.

For the peptides derived from N . nivea $V^{II}1$, only Niv -10-20 and Niv -15-25 showed low average parameter values for β-pleated sheet, suggesting the possibility of preferring α-helical structure. However Niv -10-20 contains a Pro-residue, a helix breaking amino acid, in the center of the sequence. This leaves only Niv -15-25 with the potential to adopt α-helical structure. This peptide was shown to adopt such a conformation in a TFE containing medium as described above. All the other peptides showed higher parameter values for β-pleated sheet than for α-helix, at an average difference of 0,16.

For the peptides derived from PhA, only PhA-1-12 and PhA-90-109 showed high average parameter values for α-helical structure, compared to the other peptides. It was shown above that PhA 1-12 and PhA 90-109 are able to adopt α-helical structures in TFE containing medium. According to the parameter values, PhA-113-125 also shows the possibility of adopting α-helical structure, and PhA-43-54, although involved in an α-helical structure in the native protein, showed a high parameter value for possible β-structure. None of these two peptides adopted any hydrogen bonded structure in solution. All the other peptide sequences showed higher parameter values for β-pleated sheet than for α-helical structure, the average difference being 0,094, which is lower than that observed for the peptides derived from N . nivea $V^{II}1$.

It has to be stressed that these parameter values were designed for structure predictions of peptide sequences within a whole protein and therefore cannot be applied directly to an isolated peptide. These differences in parameters thus have no meaning.

The results obtained by CD-spectroscopy indicate that these predictions of conformation of an isolated peptide in solution is not reliable. The tertiary structure of a protein evidently plays an important role in determining what hydrogen bonded structure a peptide would adopt in the native protein. It does, however not imply that a peptide that adopts no hydrogen bonded secondary structure in solution, would not be immunologically active. As was mentioned in 3.1, researchers speculated that the conformation of a peptide may not be important for interaction with an antibody, but that peptides are flexible, and can be structured by the antibody specific for their particular sequence in the mother protein, once the peptide-antibody interaction is initiated [170]. The immunological properties of all the synthesized peptides will be discussed in the following chapters.

From the above results it can also be seen that the methods of theoretical prediction are not always correct, supporting the warning of De Lisi and Berzofsky [74], discussed in 1.1.2.2.3.

CHAPTER 4 PRODUCTION OF ANTISERA

4.1. INTRODUCTION

A problem is faced that an animal can die when antiserum is to be raised to a toxin such as cardiotoxin $V^{II}1$ from Naja nivea. This is not encountered with the non-toxic bovine phospholipase A_2. The cardiotoxin was therefore detoxified by conjugation to a carrier protein [129]. To improve presentation of the amino acid side chains of the synthetic peptides in an analysis of their reactivity with antisera, these peptides also had to be conjugated to carrier proteins. Antibodies may be raised against the carrier protein as well as the cross linking molecule [186]. Such antibodies would then react with the peptide carrier and thus elicit false positive results. It is thus important that different carrier proteins and conjugation reagents should be used for the cardiotoxin and the peptides respectively. To detoxify N.nivea $V^{II}1$, it was conjugated to poly-L-lysine, and the conjugation agent was a carbodiimide [187].

The interaction of antiserum and synthetic peptides was to be assayed by the ELISA technique. This technique had first to be standardized by determining that interaction took place between the antisera and the proteins they were raised to.

4.2. Materials

Naja nivea VII1 and bovine phospholipase A_2 were kind gifts of Dr. F.J.Joubert, of DPCMT, CSIR. Poly-L-Lysine was purchased from Miles Yeda Ltd, N-ethyl-N'(3-dimethy-laminopropyl)-carbodiimide (EDC) from Merck (Darmstadt), 2,2'-azino-di-(3-ethylbenz-thiazolinsulfate) (ABTS) from Boehringer

Mannheim GmBh, Freund's complete and incomplete adjuvant from Difco Laboratories (Detroit) and casein from Sigma. Rabbit anti-guinea pig IgG was obtained from BioYeda, sheep anti-rabbit Ig from Seravac and rabbit peroxidase anti-peroxidase from Pel Freez.

4.3. Methods

4.3.1. Conjugation of N.nivea $V^{II}1$ to poly-L-lysine

N.nivea $V^{II}1$ (5mg) and poly-L-lysine(HBr)(10mg) with a molecular weight of 56600, were dissolved in 2ml distilled water. The pH was adjusted to 5,0 by addition of 0,2M NaOH. A tenfold molar excess of EDC, dissolved in 600µl water, was added in 3 aliquots at one hour intervals [129,187]. The pH was kept constant at 5,0 by the addition of 0,5N HCl. The mixture was then dialysed twice against 4 litre 0,154M NaCl. The conjugation efficiency was determined by amino acid analysis (see 2.2.2.2 for the procedures).

4.3.2. Antisera

Three guinea pigs were immunized intraperitoneally with 0,11mg N.nivea $V^{II}1$-poly-L-lysine conjugate (containing the equivalent of 50µg cardiotoxin) emulsified in Freund's complete adjuvant in a total volume of 500µl [129]. Immunizations were boosted three times at three-weekly intervals with 0,11mg conjugate in Freund's incomplete adjuvant. By the same procedure, three other guinea pigs were immunized with 25µg PhA.

Five days after the last booster injection, 5ml blood was drawn from each guinea pig by heart puncture. The blood was left to coagulate and then

centrifuged to obtain clear serum. Antisera against each protein were pooled separately.

Control serum was prepared from guinea pigs immunized with EDC-treated poly-L-lysine, as well as an unimmunized animal.

4.3.3. Immunodiffusion

Gel diffusion was carried out in 1% (w/w) agarose gel for 48 hours at room temperature (22°C)[110]. N.nivea $V^{II}1$ and PhA were dissolved in 0.154M NaCl at concentrations of 0,66; 0,33; 0.17; 0,08; 0,04 and 0,02 mg/ml. Gel diffusion was carried out with 10µl toxin or enzyme solution against the undiluted antisera.

4.3.4. Enzyme linked immunosorbant assay (ELISA)

ELISA of cardiotoxin and PhA was carried out in flat bottomed 96-well microtitre plates (NUNC) as follows: After antigen coating of 4µg protein in 200µl of 0,05M sodium carbonate buffer, pH9,6 for 18 hours at room temperature (22°C), each well was subsequently incubated for 1 hour at 37°C with a succession of the following reagents: 0,5% (w/v) casein in phosphate buffered saline (pH7,4)(PBS) [188]; guinea pig antiserum diluted 1:50, 1:100, 1:200, 1:400, 1:800, 1:1600 and 1:2000 in PBS/0,25% casein; rabbit anti guinea pig IgG diluted 1:1000 in PBS/0,25% casein; sheep anti rabbit Ig diluted 1:1000 in PBS/0,25% casein; and rabbit peroxidase anti peroxidase diluted 1:1000 in PBS/0,25% casein. The wells were washed three times with PBS containing 0,05% (v/v) Tween 20 after each of the incubation steps. Substrate, ABTS in 0,1M sodium phosphate/citrate buffer, pH4,3 was then added. The absorbance at 405nm was then read with a Titertek Multiscan photometer

after 10 minutes incubation time at room temperature.

4.4. Results and Discussion

The amino acid analysis of the N.nivea $V^{II}1$-poly-L-lysine is shown in Table 4.1. From this analysis it can be deduced that 9 of the 77 Lys residues were contributed by the cardiotoxin, and thus 68 came from the poly-L-lysine. The coupling efficiency was calculated as 1 nmole cardiotoxin per 68 nmoles lysine. With a molecular weight of 56600, poly-L-lysine consists of approximately 441 moles Lys residues. Therefore:

> 1 nmole cardiotoxin per 0,154 nmoles poly-L-lysine
>
> or: 6,5 nmoles cardiotoxin per 1 nmole poly-L-lysine.

With the molecular weights of N.nivea $V^{II}1$ and poly-L-lysine as 6800 [13] and 56600, respectively, the conjugation efficiency can also be calculated and expressed as 0,78 mg cardiotoxin per 1mg poly-L-lysine.

The immunodiffusions confirmed that antisera had been raised against N.nivea $V^{II}1$ and PhA respectively, since a precipitin band formed between the wells containing proteins and the respective antisera (Figure 4.1). No bands were observed between anti-cardiotoxin and PhA, and anti-PhA and cardiotoxin, respectively, indicating that the antisera were specific for the proteins they were raised to.

Table 4.1. Amino acid composition of *N.nivea* V^{II}1-poly-L-lysine conjugate.

Amino acid	Residues per mole	
	Theoretical for Cardiotoxin	Conjugate
Asp	5	4.9
Thr	4	3,9
Ser	4	3,7
Glu	1	0,8
Pro	5	5,4
Gly	2	2,2
Ala	1	0,9
Cys	8	7,8
Val	8	7,6
Met	2	2,1
Ile	1	1.1
Leu	4	4,2
Tyr	2	1,8
Phe	1	1,2
His	1	1,8
Trp	1	0,6
Lys	9	76,8
Arg	1	0,6
Total	60	127,4

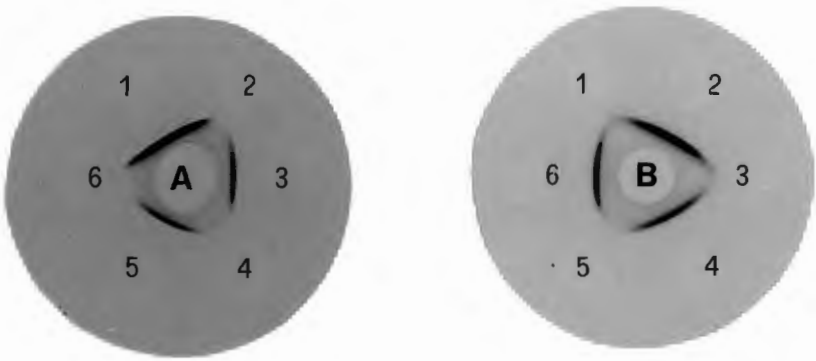


Figure 4.1. Immunodiffusion of antiserum and antigen.
A: Antiserum raised to N.nivea $V^{II}1$
B: Antiserum raised to Bovine phospholipase A_2
Concentrations in mg/ml of N.nivea $V^{II}1$: 0,32(1); 0,16(3); 0,08(5) and bovine phospholipase A_2: 0,08(2); 0,04(4); 0,02(6).

By ELISA, both N.nivea $V^{II}1$ and PhA could be detected by antiserum diluted as much as 2000 times. ELISA also showed that the antisera were specific for the proteins they were raised to (see Table 4.2.). The antisera of the three guinea pigs were pooled to have a mixture of antibodies with maximum activity towards the maximum amount of antigenic sites. This was done because it was shown that within the same species, individual animals do not necessarily respond to the same number of sequential antigenic sites [45,65].

Table 4.2. Reactivity of antisera and control sera as determined by ELISA.

Protein	Antiserum		Control serum			
	Niv	PhA	1*	2*	3*	4**
N.nivea $V^{II}1$	+++	-	-	-	-	-
Phospholipase A_2	-	+++	-	-	-	-

All the sera were diluted 1:200
Scale used for absorbance at 405nm:
+++, above 2,0 O.D.
- , < 0,4 O.D.

* Control sera from guinea pigs immunized with EDC-treated poly-L-lysine.

** Control serum from an unimmunized guinea pig.

CHAPTER 5 MAPPING OF ANTIGENIC SITES

5.1. Introduction

Various reported methods have been employed in studies of immunochemical properties of antiserum, raised against native proteins, to peptides. These methods include affinity chromatography [189], where the peptides are bound to the solid support, inhibition of complement fixation [53], immunoblotting [61,190] and proteolysis of antigen-antibody complexes [191]. The most frequently used meth ods are radio-immuno assays (RIA) [173,192,193] and ELISA [61,173,194]. In the present study ELISA was used, and preferred to RIA because the use of radio-active materials could be avoided.

For coating the ELISA-plates, and to improve the presentation of amino acid side chains for interaction with antibodies, the peptides had to be conjugated to a carrier protein. Coating with peptides in the free form has been reported by some researchers [186,195], and was also done in the present study. As mentioned in Chapter 4, a different carrier protein and conjugation reagent, than was used for the N.nivea $V^{II}1$- poly-L-lysine conjugate, had to be employed.This being to avoid artifactual results from antibodies raised to the carrier protein and conjugation agent [186]. Peptides were conjugated to hen egg phosvitin with glutaraldehyde. Another series of conjugates was prepared with bovine serum albumin as carrier and EDC as conjugation reagent. The use of other large globular proteins as carrier molecules , e.g. myoglobin, lysozyme or casein, was also investigated. However these proteins were found to coagulate upon conjugation. Peptides polymerized

by glutaraldehyde [196] were also used in the assay.

5.2. Materials

Hen egg phosvitin S1 [197] was a gift from Dr.R.C.Clark, DPCMT, CSIR. Bovine serum albumin (BSA) was obtained from Miles Laboratories, glutaraldehyde and L-lysine hydrochloride from Merck (Darmstadt).

5.3. Methods

5.3.2. Conjugation of peptides to carrier proteins

5.3.2.1. Conjugation of synthetic peptides to hen egg phosvitin S1 by glutaraldehyde.

Peptides were conjugated to hen egg phosvitin in a two-step procedure [196]. In the first step phosvitin S1 was activated by dissolving 20mg protein in 0,5ml 0,1M sodium phosphate buffer, pH6,8, containing 1,25% glutaraldehyde (v/v). After 18 hours incubation at room temperature (22°C), The mixture was dialyzed twice against 4l 0,154M NaCl. To the "activated" phosvitin S1, 5mg Niv -peptides, dissolved in 1ml 0,154M NaCl, was added. A buffer, 0,1ml 1M carbonate/bicarbonate, pH9,5, was then added and the mixture left to incubate 24 hours at 4°C The reaction was terminated by the addition of 0,1ml 0,2M L-lysine hydrochloride. The conjugate was dialysed twice against 4l 0,154M NaCl. Aliquots of these conjugate solutions were used for ELISA. The "activated" phosvitin S1 was also blocked with L-lysine hydrochloride as a control. The conjugation efficiency was determined by amino acid analysis (see 2.2.2.2 for the procedures).

5.3.2.2. Conjugation of peptides to bovine serum albumin by carbodiimide.

Conjugation of peptides to BSA was attempted by the above described glutaraldehyde method, but the BSA was found to coagulate upon addition of glutaraldehyde. The same procedure as used for conjugating N.nivea $V^{II}1$ to poly-L-lysine (see 4.2.1) was therefore employed to conjugate Niv- and PhA-peptides to bovine serum albumin, since these conjugates should still be useful in testing the reactivity of anti-phospholipase serum, because no conjugate was used in the preparation. Twenty milligram BSA was reacted with 10mg peptide. The conjugation efficiency was also determined by amino acid analysis (see 2.2.2.2 for the procedures).

5.3.2.3. Polymerization of peptides by glutaraldehyde [98,196].

Synthetic peptides (2mg) were dissolved in 1ml sodium phosphate buffer, pH6,8, containing 1,25% glutaraldehyde (v/v). After stirring for 20 minutes at room temperature, the reaction was terminated by the addition of 0,1ml 0,2M L-lysine hydrochloride.

5.3.3. Enzyme linked immunosorbance assay (ELISA)

ELISA was carried out as described in 4.2.4. Microtiter plates were coated with free synthetic peptides, polymerized peptides and peptides conjugated to phosvitin S1, respectively. The antisera raised against PhA and N.nivea $V^{II}1$ as well as the control sera (see 4.2.2) were used.

5.4. Results and Discussion

The amino acid analyses of Niv-15-25 conjugated to phosvitin S1 and BSA are shown in Tables 5.1 and 5.2 respectively. The analyses of the pure carrier proteins were corrected by calculation so that the amount of the amino acid residues present in these proteins are the same as those for the carrier found in the conjugate. The amino acid residues present in both carrier and peptide were excluded from the calculation. By subtracting the values obtained for the protein from the one obtained for the conjugate, the amount of peptide present in the conjugate could be obtained.

The conjugation efficiency of Niv -15-25-phosvitin conjugate was calculated as:

> 23,6 nmol/ml peptide per 24,8 nmol/ml phosvitin S1, i.e. 0,95 nmol peptide per 1 nmol phosvitin S1.

With the molecular weights of Phosvitin S1 and the peptide as 34000 [197] and 1386, respectively, the conjugation efficiency can be calculated as 0,04 mg Niv-15-25 per 1 mg phosvitin S1. The conjugation efficiency to phosvitin of all the peptides was between 0,05-0,1 mg peptide per 1mg phosvitin.

Similarly the conjugation efficiency of Niv -15-25-BSA was calculated as:

> 40,6 nmol/ml peptide per 9,2 nmol/ ml BSA i.e. 4,4 nmol peptide per 1 nmol BSA.

Table 5.1. Amino acid composition of phosvitin S1 and Niv-15-25-phosvitin conjugate

Amino acid	nmol/ml*			Amino Acid Composition of peptide	
	Conjugate	Phosvitin S1	Peptide	Analysis	Theoretical
Asp	357,3	332,0	25,3	1,07	1
Thr	88,1	85,7	2,4	0,10	-
Ser	2917,6	2914,4	3,2	0,14	-
Glu	275,7	254,3	21,4	0,91	1
Pro	20,9	0,0	20,9	0,89	1
Gly	165,6	111,9	53,7	2,28	2
Ala	154,1	158,1	-4,0	-0,17	-
Cys	31,0	0,0	31,0	1,32	1
Val	64,1	65,8	-1,7	-0,07	-
Met	40,9	18,6	22,3	0,95	1
Ile	44,4	42,1	2,3	0,10	-
Leu	85,7	65,5	20,2	0,86	1
Tyr	30,9	9,1	21,8	0,93	1
Phe	42,5	25,8	16,7	0,69	1
His	235,4	237,4	-2,0	-0,08	-
Trp	14,2	15,0	0,8	0,03	-
Lys	273,0	322,4	49,4	2,05	2
Arg	294,6	295,0	-0,4	-0,02	-
nmoles per residue phosvitin S1**		24,8		Total 11,88	12

* Average of 3 analyses

** Calculated for a molecular weight of 20400, i.e. only amino acid residues, excluding phosphate groups of phosphoserine [197]

Table 5.2. Amino acid composition of bovine serum albumin and Niv-15-25-BSA conjugate.

Amino acid	nmol/ml*			Amino Acid Composition of Peptide	
	Conjugate	BSA	Peptide	Analysis	Theoretical
Asp	668,5	634,3	34,2	0,84	1
Thr	396,3	385,6	10,7	0,26	-
Ser	329,6	313,1	8,8	0,22	-
Glu	967,7	930,9	36,8	0,91	1
Pro	774,1	731,8	42,3	1,04	1
Gly	251,9	172,2	79,7	1,96	2
Ala	492,1	494,6	-2,5	-0,06	-
Cys	271,3	232,1	39,2	0,97	1
Val	366,9	375,3	-8,4	-0,21	-
Met	82,1	42,0	40,1	0,99	1
Ile	137,1	139,7	-2,6	-0,06	-
Leu	676,8	633,8	43,0	1,06	1
Tyr	250,2	208,9	41,3	1,02	1
Phe	333,5	282,2	51,3	1,26	1
His	178,0	174,3	3,7	0,09	-
Trp	9,4	9,3	0,1	0,00	-
Lys	652,2	573,2	79,0	1,95	2
Arg	261,4	263,6	-2.2	0,05	-
nmoles per residue BSA**		9,11	Total	12,29	12

* Average of 3 analyses

** Calculated for a molecular weight of 69000 [198]

With molecular weight of BSA and the peptide as 69000 [198] and 1368, respectively, the conjugation efficiency was also calculated as:

> 0,08 mg Niv -15-25 per 1 mg BSA. The conjugation efficiency to BSA of all the peptides ranged between 0,05-0,1 mg peptide per 1 mg BSA.

In 4.3 it was found that N.nivea $V^{II}1$ and PhA could be detected by their respective antisera diluted by as much as 2000 times. No positive interaction between antisera and any of the peptides, either in the free or conjugated form could be detected, even when only diluted 1:50. Less dilute sera could not be used, because background readings were then found to be too high.

Only when peptides were polymerized, was some interaction with antibodies detectable. The best repeatable results were obtained when the ELISA was carried out in duplicate with antisera diluted 1:50, 1:100 and 1:200. The result of such an ELISA, with antisera diluted 1:200, is given in Table 5.3. Inspection of these results showed that the anti-cardiotoxin serum interacts with Niv-1-10, and anti-PhA serum with Niv-30-40, and PhA-13-30 and 90-109. However, the control antisera, from guinea pigs immunized with poly-L-lysine, also respond to these, as well as to some of the other, polymerized peptides. One possible explanation is the presence of poly-L-lysine, which could have been formed when the polymerization was terminated with L-lysine hydrochloride. Antibodies raised to poly-L-lysine, the carrier molecule used for immunization with N.nivea $V^{II}1$, would interact with that lysine polymer. This, however, does not explain the reactivity of anti-phospholipase serum

Table 5.3. Reactivity of antisera and control sera to proteins and peptides as determined by ELISA.

Protein or Peptide	Antiserum		Control serum			
	Niv	PhA	1*	2*	3*	4**
N.nivea V 1	+++	-	-	-	-	-
Phospholipase A	-	+++	-	-	-	-
Niv 1-10	++	-	++	-	++	-
Niv 5-15	-	-	+	+	-	-
Niv 10-20	-	-	-	-	-	-
Niv 15-25	-	-	-	-	-	-
Niv 20-30	-	-	-	-	-	-
Niv 25-35	-	-	-	-	-	-
Niv 30-40	-	++	+	+	+	-
Niv 35-45	-	-	-	-	-	-
Niv 40-50	-	-	-	-	-	-
Niv 45-55	-	-	++	+	-	-
Niv 50-60	-	-	-	-	-	-
PhA 1-12	-	-	-	-	-	-
PhA 13-30	-	++	-	+	-	-
PhA 31-42	-	-	-	-	-	-
PhA 43-54	-	-	-	-	-	-
PhA 55-74	-	-	-	-	+	-
PhA 75-85	-	-	-	-	+	-
PhA 90-109	-	++	-	-	+	-
PhA 113-125	-	-	-	-	-	-

All the sera were diluted 1:200
Scale used for absorbance at 405nm:
+++, above 2,0 O.D.
++, 1,0-1,5 O.D.
+, 1,0-0,4 O.D.
-, < 0,4 O.D.

* Control serum from guinea pigs immunized with EDC-treated poly-L-lysine

** Control serum from an unimmunized guinea pig.

to these polymerized peptides, since poly-L-lysine was not present in the immunization mixture. The possibility of other non-specific interactions between antibodies and polymerized peptides could thus not be excluded. This showed that the use of various control experiments are very important to avoid artifactual results in the determination of antigenic sites by peptide mapping.

Since no definite positive interaction between antibodies and either of the peptides, whether conjugated to a carrier or not, could be detected, further experiments, e.g. inhibition [173] or competition [65] immunoassays were not done.

CHAPTER 6 CONCLUDING DISCUSSION

The aims and scope of this study were set out in 1.4. Briefly they were to do an initial investigation of the location of antigenic sites of a snake venom cardiotoxin, Naja nivea $V^{II}1$ with a future view of applying the knowledge obtained in the design of synthetic vaccines, for raising antiserum against snake toxins. The present methods of treating victims of snake bite have several drawbacks [11]. Parallel to the investigation of these properties of the cardiotoxin, a similar study was to be carried out on bovine phospholipase A_2, which was used as a control in the experimental work. Since a member of this enzyme family also occurs in snake venoms, the knowledge obtained here could be of value for repeating and comparing with the toxin enzyme. The results obtained will be briefly discussed. Some future prospects for studies on snake venom proteins would also be discussed in the light of the findings obtained.

The experimental work in this investigation consisted of:

1. Chemical synthesis of peptides with overlapping sequence derived from cardiotoxin $V^{II}1$ of Naja nivea , as well as peptides derived from bovine phospholipase A_2.
2. A structural characterization of these peptides by CD-spectroscopy for relating immunological properties to epitope structure.
3. Raising antiserum to the cardiotoxin and the phospholipase.
4. Searching for immunochemically reactive peptides by determining the reactivity of the antiserum to the above synthetic peptides by enzyme linked immunosorption assay (ELISA).

From the review presented in Chapter 1, it was concluded that only limited information about the immunological properties of snake venom cardiotoxins is available [81-87,128,129], methods of predicting antigenic sites in proteins [51,52,58] are unreliable, the use of synthetic peptides as vaccines to raise antibodies against native proteins give either positive or negative results, differing from protein to protein [9,10], and the information on antigenic properties obtained for one protein cannot readily be applied to another to predict the possible immunological properties. Before attempting the design of synthetic peptide vaccines for cardiotoxins, the immunological properties had to be investigated.

The results obtained in this work showed that the cardiotoxin antiserum did not interact with peptide sequences derived from the toxin, which suggested that continuous epitopes are absent in cardiotoxins.

The synthetic peptides were shown to adopt random structure in the aqueous solvents in which the immunological properties were investigated. An alternative explanation for the unreactivity of the antisera with these peptides could thus be provided. If these peptide conformations are all random in aqueous solutions and do not resemble the conformations adopted in the native cardiotoxin or PhA, lack of epitope rcognition by antiserum would be anticipated. It was mentioned in Chapter 1 that most of the peptides derived from N.nivea $V^{II}1$ are involved in β-pleated sheet structures in the native protein [62], and peptides 1-12, 43-54 and 90-109, derived from phospholipase A_2, in α-helical structures [100]. Previous researchers [199] have reported unreactivity of denatured cardiotoxin towards

cardiotoxin antiserum, presumably resulting from the generation of random structure within the intact denatured polypeptide. An equally valid explanation for this observation is that of epitope concealment through conformational change. The unreactivity of the antibodies with these peptides could also suggest that conformation is important for antigenic sites in cardiotoxins and phospholipase A2 , in contrast with results obtained for other proteins [4,16,53].

In solutions containing TFE peptide sequence 15-25 of N.nivea $V^{II}1$ displayed α-helical structure, despite being derived from a protein with β-sheet and no α-helix content [62]. It was shown that this peptide, in the α-helical form, is amphipathic, i.e. having distinct charged and hydrophobic faces [74]. Since TFE was shown to be a structure promoting solvent [108], and able to mimic the solvent properties of bilayer membranes [109], it is possible that this peptide fragment could be embedded in a cell membrane in the helical form, after processing of the cardiotoxin by a macrophage. This would suggest that peptide 15-25 of N.nivea $V^{II}1$ fulfills the criteria of De Lisi and Berzofsky for a potential T-cell epitope [74].

Two of the three peptide sequences derived from helical regions in the control protein PhA [100], sequences 43-54 and 90-109,should, by theoretical analysis, be able to form amphipathic helices. Only peptide 90-109 was able to adopt α-helical conformation in TFE. A third peptide, sequence 1-12, although not predicted to be an amphipathic helical structure, did adopt α-helical structure in TFE. Sequence 90-109 was thus shown to be a potential T-cell epitope for this enzyme.

Since members of the homologous family of phospholipases A_2, some of which are toxic, are found in snake venoms [97,98], the work done on the bovine enzyme in this study, could be of importance and worth repeating for comparison with enzymes found in venom.

The results on the immunological properties of N.nivea $V^{II}1$ and phospholipase A_2 , suggest that it is inadvisable to use linear peptides in vaccines to raise antibodies against these proteins. The peptides shown to be potential T-cell epitopes, Niv -15-25 and PhA-90-109, could be of possible use for such purposes, but not in the linear form. The helical conformations would have to be stabilized for use in aqueous solvents, e.g. by the substitution of some of the hydrophobic amino acid residues by 2-aminoisobutyric acid [176;179]. Since it was shown that T-cell and B-cell epitopes often coincide [5,66], these two peptides, when stabilized and used as synthetic vaccines, could also be able to act as B-cell stimulators to raise antibodies. A further suggestion is that peptides 21-38 and 42-53, derived from N.nivea $V^{II}1$, could be stabilized by cyclization with disulfide bonds to mimic loops II and III of the native toxin, respectively.

It was shown in a previous investigation by the present author that cardiotoxins differ in stability towards denaturation in acidic aqueous acetonitrile [109], and that these toxins could be classified into different structural groups [81,109]. In the abovementioned investigation it was found that N.nivea $V^{II}1$ belongs to the group of toxins with a less stable conformation. It is suggested that a cardiotoxin from the group with a more stable conformation be selected for future investigations of the present kind. The

possibility of obtaining peptide sequences with more stable conformations in the isolated form derived from such a toxin should be investigated.

Furthermore, the structural analysis of the peptides also showed that peptides in the isolated form do not always adopt the same structure they have in the parent protein. Niv-15-25 from a β-structured protein adopts α-helical structure, and PhA-43-54, derived from an α-helical segment, adopts no hydrogen bonded structure at all. Similarly the helical wheel prediction method [184], and therefore the potential T-cell epitope prediction method [74], were shown not to be very reliable.

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