An investigation into the inducible antibacterial peptides in *Calliphora vomitoria.*

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2.0 Abstract

Insects of many dipteran species have recently been shown to respond to bacterial challenge by a rapid synthesis of a number of antibacterial peptides that are secreted into the haemolymph. Presented here is a report on the inducible antibacterial peptides produced by the blow fly *Calliphora vomitoria*. The larvae of *Calliphora vomitoria* where inoculated with bacteria and incubated. The haemolymph of the larvae was collected and assessed for antibacterial activity. The antibacterial molecules were investigated and purified. Isolated by this study is a potent peptide that has antibacterial activity against gram positive bacteria. This peptide shows no antibacterial activity towards gram negative bacteria. This peptide is heat stable and relatively pH stable. The purified sample of this antibacterial peptide shows many similarities with other peptides of the insect defensin family.

3.0 Introduction

Insects comprise more than half of the total identified species of lifeforms living on the planet. Insects evolved around 450 million years ago, and have survived in a myriad of forms to the present day. Insects have diversified to fill large numbers of ecological niches, and have adapted to many climates. Insects therefore have become regarded by some as the most successful class of life known.

3.1 An overview of the insect immune system

Since the first single-celled organisms evolved the problem of infection and parasitism by other organisms has been ubiquitous. Infectious organisms are many, and varied, all with different methods of colonisation and modes of attack. It is therefore vital for any successful species that it has an immune system capable of both preventing pathogenic organisms from invading the host, and destroying, or neutralising any that do. All organisms have some sort of immune system to combat infection and research performed on immune systems of different species has shown superficial similarities between. The major thrust of immunological research has been towards the mammalian and, more specifically, human immune system.

Mammals have developed a complex immune system comprising three main elements: - physical and chemical barriers, cellular immunity and humoral immunity. Barriers, e.g. skin and mucus layers, play an important role in the prevention of infection, as a potential pathogen needs to gain access to the internal environment of an animal to cause harm. Both the cellular and humoral types of immunity are mainly involved in the neutralisation and / or destruction of any pathogens penetrating the barriers. Cellular components of mammalian immunity include phagocytic cells e.g. neutrophils and macrophages, which recognise foreign particles and cells, and engulf, then destroy them. The other main components of mammalian cellular immunity are cytotoxic T-cells. These cells again recognise foreign cells and cause them to lyse. These cytotoxic T-cells are able to identify virally infected cells and tumour cells, thus the lysis brought about by the T-cell destroys the living environment of the virus and helps destroy tumours.

Humoral immunity in mammals is conveyed by molecules produced by the host and released extracellularly into the blood, or lymph. The two major components of mammalian humoral immunity are antibodies and complement. Antibodies, or immunoglobins have the ability to recognise specific foreign antigens and bind to them. As huge numbers of antibody molecules circulate in the body at any one time a foreign cell will encounter enough antibody molecules specific to its antigens that it will become coated with them. This antibody - foreign cell complex is exploited in the following three ways: - an antibody-coated molecule is easily identified and engulfed by phagocytic cells, antibody molecules activate compliment, and, if other foreign cells are present, antibodies can crosslink to other cells forming large complexes which render the pathogen immobile.

Complement is a series of nine related proteins circulating in the blood and lymph at all times. When complement proteins are activated, either by antibodies as above, or on encountering foreign cells, a membrane attack complex is formed which inserts into the bacterial cell membrane forming a pore. This pore allows cellular contents to leak from the cell causing it to lyse. Other aspects of mammalian humoral immunity are non-specific antibacterial molecules e.g. lysozyme, which is present in large quantities in conjunction with the barriers mentioned above. Regulating all aspects of mammalian immunity are a complex system of helper T-cells, and humoral factors known as cytokines.

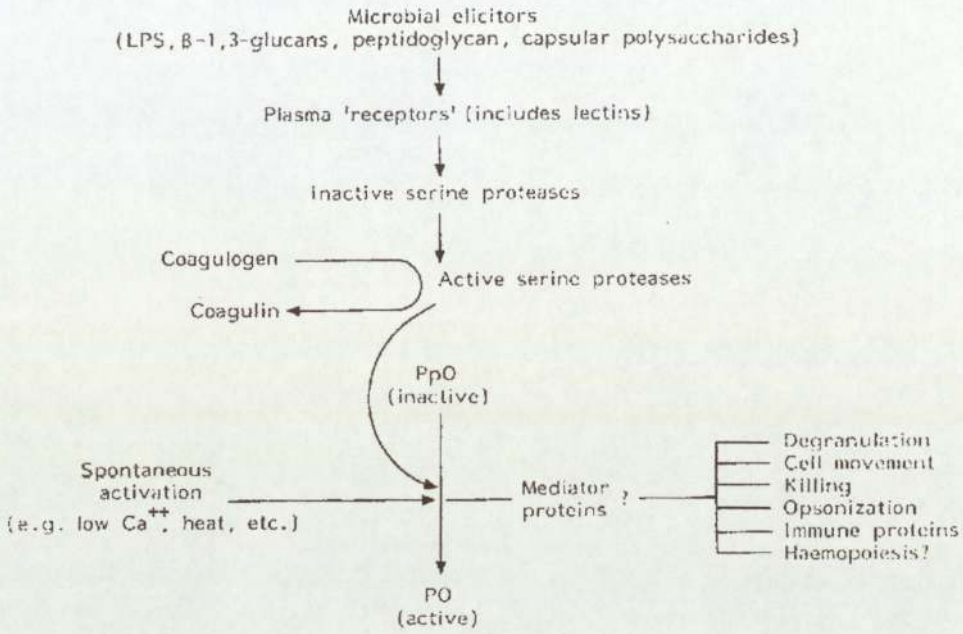
As a whole the mammalian immune system is a complex system of cells and molecules working in a complimentary fashion with many tight regulatory systems controlling the expression of the various parts[1].

The three main elements involved in mammalian immunity (physical and chemical barriers, cellular, and humoral immunity) are seen in many other class of animal. While there have been many studies into the immune systems of various invertebrate species most of them have looked at insects and molluscs. Insects, as invertebrates, lack the more complex aspects of the mammalian immune system, but have evolved an effective system that deals with their natural pathogens. The insect immune system is superficially similar to that of mammals as it posses the three elements discussed above.

External and internal barriers are non-specific in their nature, consisting of: - the hard, impermeable exoskeleton (made of cuticle) the cuticle of the fore and hindgut, and the mucus layer such as the peritrophic membrane of the midgut. These barriers are physical layers through any potential pathogen has to pass in order to infect the host. These mucus layers contain antibacterial agents such as protease inhibitors, lectins, lysins, and antifungal quinones, to kill microorganisms trapped by the mucus[2].

The responses involved in insect cellular immunity are: - phagocytosis, nodule formation and encapsulation, cytotoxicity, and wound repairing. Cellular components of insect immunity are conveyed by the haemocytes (found in the haemolymph) of which there are five types: - the prohaemoctyes, spherulocytes, plasmocytes, granulocytes, and coagulocytes. It is the last three of these that are involved in cellular immunity in insects. Phagocytosis, as in mammals, is the primary cellular response to invasion by a pathogenic organism. Phagocytosis is one of the major mechanisms of host defence performed throughout the animal kingdom and is used by protozoa as a feeding mechanism. Phagocytosis occurs is a similar way to that seen in mammals, whereby the phagocytic cells, or organisms, recognise the pathogenic organism as foreign, engulf, ingest, and kill it. In insects phagocytosis is performed by many types of haemocytes, principally the plasmatocytes, conferring non-specific immunity. Attraction or chemotaxis of the phagocytic cell is promoted by components of the prophenoloxidase system, and by insect agglutinins acting as opsonising agents. The killing agents inside the phagocytic cells are: - the ubiquitous lysozyme, the activated agents of the prophenoloxidase system, and low pH, see fig 1.

Fig 1:- The prophenoloxidase activating system[2]



Adapted from R.J.Turner

The processes of nodule formation and encapsulation evolved to deal with large pathogens and parasites, such as nematode worms, that could not be simply phagocytosed. The process is essentially the same regardless of whether a nodule (small) or a capsule (large) is formed. A granulocyte or coagulocyte contacting the large pathogenic organism causes the surrounding haemocytes to rupture, thus releasing factors into the haemolymph. These factors cause a very rapid localised coagulation of the haemolymph. Plasmatocytes, attracted to the area due to the release of chemotactic factors, attach to the outside of the coagulum forming either a nodule or a capsule surrounding the pathogenic organism(s). Any organisms trapped within a capsule are killed by melanization of the internal space, the nodule is then excreted. Organisms trapped within a capsule are killed by activated components of the prophenoloxidase system, and lack of oxygen. Capsules, because of their relative size, are retained within the body of the insect for the rest of its life [3].

Humoral immunity is present in many of the invertebrate phyla, reviews of the antibacterial peptides that have been isolated from non-insect invertebrate species is covered by the following references [4,5,6,7,8]. Unsurprisingly studies have shown that insects do not posses the typical humoral immune responses of mammals (antibodies

and complement), they instead have a more non-specific type of humoral immunity comprising : - agglutinins (also known as lectins) together with soluble antibacterial peptides and proteins. Agglutinins are proteins or glycoproteins with multiple binding sites for specific carbohydrate antigen. Agglutinins are in no way related to vertebrate immunoglobins. Agglutinins are ubiquitous throughout the invertebrate world but vary in both size and structure. In insects they are synthesised in the fat body, and are present as freely dissolved molecules in the haemolymph. The functions of agglutinins are to coagulate foreign particles, facilitate lysis of foreign cells, activate haemocyte coagulation[8], inhibit proteases, act as chemoattractants, and (to act) as oppsonins, serving as recognition and effector molecules in defence reactions[6,11,12].

The other component of insect humoral immunity, and the one upon which this study is based, are the antibacterial, soluble proteins and peptides. Proof of an insect humoral defence system was demonstrated by work published around 1920. Up until the end of the 1950's experiments had been carried out on a number of insect species and the conclusions drawn from these experiments were that: - there is no anti-bacterial activity present in insect larvae before the host is challenged. That anti-bacterial activity appears within a few hours of challenge, increasing until it reaches a plateau after about 24 hours. The duration of this activity is species-dependent, for most species this plateau of high anti-bacterial activity lasts for another 24 hours before slowly receding. This reaction is induced by many stimuli and has wide ranging anti-bacterial activity directed against both Gram-positive and Gram-negative bacteria. After the pioneering studies of Briggs[13] and Stephens[14] in the late 1950's little interest was shown in the field of insect humoral immunity. This fact, together with the lack of appropriate methodologies for structural characterisations at the micromolar level, hindered progress in the field until Steiner[15] et al published the full characterisation of two inducible anti-bacterial peptides in 1981. Since then, advances in technology have led to a host of new inducible antibacterial peptides being discovered, leading to the conclusion that all insects, when challenged by a bacterial infection respond, by producing a battery of antibacterial peptides[16].

These molecules have been loosely divided into five classes: -

The Lysozyme family.

Short, α-helical peptides.

Insect Defensins.

Proline-rich peptides.

Large Glycine-rich peptides.

A brief distillation of the work carried out on these molecules reveals that lysozymes and insect defensins act against gram-positive bacteria, the proline and glycine rich antibacterial peptides act against gram-negative bacteria, and the short α-helical peptides have activity against both gram-positive and gram-negative bacteria..

No insect order has examples of all five classes of inducible antibacterial peptide for example, there are no defensins in moths, no proline rich peptides in flies, and no short α-helical peptides in bees. It is known, however, that all insects produce multiple antibacterial peptides. These peptides are drawn from some of the different classes above (some having multiple members of the same class) and together they provide an effective means of defence against bacterial infection.

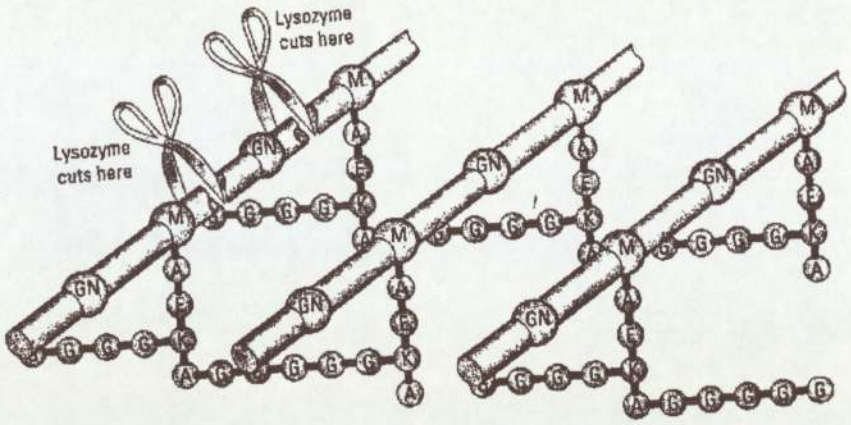
3.2 The Lysozyme Family

As early as 1931 it was reported that insects produce a non-specific bacteriolysin upon challenge with bacteria[20]. Further studies investigating this activity revealed that the molecule responsible for this activity was a heat stable peptide of low molecular mass. Conclusive studies using *Bombyx* and *Cecropia* showed this peptide to be lysozyme. It has subsequently accepted that lysozyme is widely distributed across invertebrate phyla. Lysozyme was already known to be present in human secretions e.g. tears, saliva, and nasal secretions, and was also found to be present in the plasma. The discovery of insect lysozyme, and the results of the early studies, led Mohrig and Messner[74] to wrongly conclude that lysozyme was responsible for all humoral immunity in insects. It was subsequently discovered that lysozyme works synergistically with other antibacterial peptides by attacking bacteria already damaged by other agents[17]. This research described how lysozyme is non lethal by itself and can only reach its target after damage by another antibacterial agent.

The Lysozyme family of related peptides are basic, around 120 amino acids in length, and have a 13.8 kDa relative molecular mass. Analysis of insect and human lysozyme shows some differences in amino acid sequence but show they have key disulphide bridges, and substrate binding sites conserved. This fact is not unusual as there is a marked variance between species and sometimes two isoforms of lysozyme can be present within the same animal.

Lysozymes exhibit N-acetyl glucosaminidase activity attacking the β-1,4 glycosidic bonds between the acetyl amino sugars N-acetyl glucosamine and N-acetyl muramic acid. These sugars form an integral part of the peptidoglycan layer of gram-positive bacteria. The degradation of this peptidoglycan by lysozyme causes the lysis of the infected bacteria (see fig 2).

Fig 2: Site of action of Lysozyme.



GN- N-acetyl glucosamine

M- N-acetyl muramic acid

Adapted from MD Schwatz[18]

The actions and activity of lysozyme have been well documented, and will not be covered in great depth here, for reviews see references [18,19].

One interesting recent study has shown that lysozyme is not only used by insects as an antibacterial peptide. Work headed by Ito in 1995[21] demonstrated a member of the lysozyme family present in the gut of the house fly *Musca domestica* acting as a digestive enzyme. This molecule is now being used to study the molecular evolution of lysozymes from lower invertebrates to higher mammals by the comparison of their peptide sequences.

3.3 Short, α- helical peptides

A major leap forward in the field of insect humoral immunity occurred in 1981 when Steiner et al[15] published the full characterisation of two inducible antibacterial peptides. These peptides were isolated from the Cecropia moth, Hylophora cecropia, and were therefore named Cecropin A and Cecropin B[22]. These new peptides were initially shown to have potent antibacterial activity against E. coli and other gram negative bacteria. Cecropin A was also shown to be specifically active against bacterial cells showing no activity against eukaryotic cells. Further studies showed cecropin molecules to be active against both gram positive and gram negative bacteria. Cecropins were later isolated from other lepidopteran insects, *Manduca sexta* and *Bombyx mori*, and also from the dipterans Sacrophaga and Drosophila.

Fig 3:- Amino Acid sequence of Cecropins[23]

Species (cecropin strain)	Peptide Sequence
Hyalophora cecropia (D)	W--NPFKEL EKVGQRVRDA VISAGPAVAT VAQATALAK
Athera pernnyi (D)	W--NPFKEL ERAGQRVRDA IISAGPAVAT VAQATALAK
Manduca sexta (B-2)	W--NPFKEL ERAGQRVRDA VISAAPAVAT VGQAAAIAR
Manduca sexta (B-3)	W--NPFKEL ERAGQRVRDA IISAGPAVAT VGQAAAIAR
Manduca sexta (B-4)	W--NPFKEL ERAGQRVRDA IISAAPAVAT VGQAAAIAR
Manduca sexta (B-5P)	W--NPFKEL ERAGQRVRDA VITSAAAVAT VGQAAAIAR
Hyalophora cecropia (A)	KW--KLFKKI EKVGQNIRDG IIKAGPAIAV VGQATQIAK
Hyalophora cecropia (B)	KW--KVFKKI EKMGRNIRNG IVKAGPAIAV LGEAKAL
Antherea pernyi (B)	KW--KIFKKI EKVGRNIRNG IVKAGPAVAV LGEAKAL
Bombyx mori (CMIV)	RW--KIFKKI EKVGRNIRDG IVKAGPAVAV VGQAATI
Bombyx mori (A)	RW--KLFKKI EKVGRNIRDG LIKAGPAIAV IGQAKSL
Bombyx mori (B)	RW--KIFKKI EKMGRNIRDG IVKAGPAIEV LGSAKAI
Drosophila melanogaster (A)	GWLKKIGKKI ERVGQHTRDA TI-QGLGIAQ QAANVAATAR
Drosophila melanogaster (B)	GWLRKLGKKI ERIGQHTRDA SI-QVLGIAQ QAANVAATAR
Sacrophaga peregrina (IA)	GWLKKIGKKI ERVGQHTRDA TI-QGLGIAQ QAANVAATAR
Sacrophaga peregrina (IB)	GWLKKIGKKI ERVGQHTRDA TI-QVIGVAQ QAANVAATAR
Sacrophaga peregrina (IC)	GWLRKIGKKI ERVGQHTRDA TI-QVLGIAQ QAANVAATAR
Sacrophaga peregrina (ID)	GWIRDFGKRI ERVGQHTRDA TI-QTIAVAQ QAANVAATLK
Porcine (PI)	SWLSKTAKKL ENSAK-KR-- -ISEGIAIAI QGG-----PR

It can be seen in fig 3 that cecropin molecules vary in chain length depending upon the species from which they are isolated. Dipteran cecropins are 39 amino acids in length, whilst lepidopteran cecropins vary between 35 and 37 amino acids in length. The structures of all cecropins have the following common features: - they have a strongly basic N-terminal region followed by a long, hydrophobic C-terminal section. As highlighted in Fig 3 several of the amino acids are common to all, these

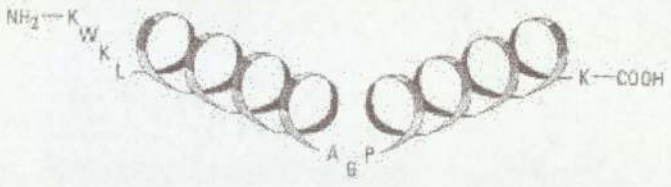
being a tryptophan at position 2, single or double lysines at positions 5, 8, and 9, and an arginine at position 18.

Analysis has been carried out using a cDNA library to examine the nucleotide sequence encoding for the cecropin gene. The discovery that although the two cecropins isolated from Dipteran insects were identical in amino acid sequence they were only 73 % identical in the nucleotide sequence coding for the cecropin which was something of a surprise. It was thus concluded that there are strong selection pressures that conserve certain cecropin sequences in different types of insect.

Since the discovery of the first cecropin in 1981 a mammalian cecropin-like peptide has been found to be present in pig intestine[24]. Porcine cecropin (PI) shows the characteristic cecropin amino acid sequence i.e. tryptophan at position 2, lysine at 5, 8, and 9, and arginine at position 18. The discovery of a mammalian cecropin has aided the understanding of the general properties of cecropins, their tertiary structure, and mechanism of action[25].

After the identification of the cecropin molecule and discovery of its activity against both gram positive and gram negative bacteria work was performed to identify the gene coding for the peptide. cDNA studies showed that the cecropin molecule was synthesised as a large precursor molecule 62-64 amino acids in length. The 24-26 amino acids at the N-terminus are not present in the active protein. Thus investigation into the tertiary structure was carried out using synthetically engineered peptide. In 1982 Steiner observed that there was a regular pattern of hydrophobic and polar side chains at the N-terminal region of mature cecropins[26]. He hypothesised that this distribution would form an almost perfect amphipathic α helix. Further studies showed a second slightly less amphipathic α helix at the C-terminal end of the peptide. Thus the mature cecropin molecule shows two amphipathic α helices interrupted by a 'hinge' region made up of a Glycine-Proline sequence, see fig 4. The discovery of the tertiary structure of cecropins was key to the subsequent investigation into the mechanism of action of the peptide.

Fig 4 :- The tertiary structure of cecropins

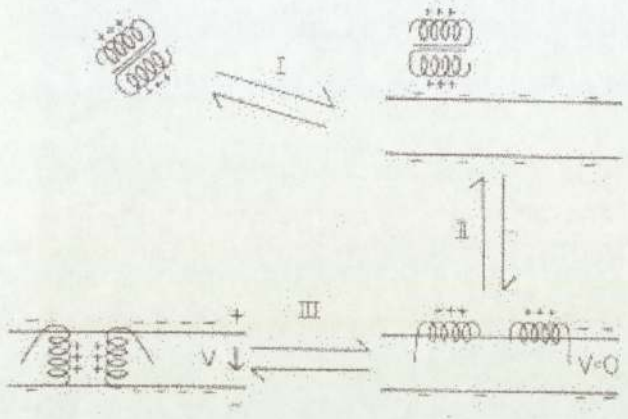


Andreau[75] et al in 1985 investigated a possible link between the tertiary structure, and the mechanism of action of cecropin A. This study formed the basis of subsequent investigations into the mechanism of action of cecropins. Work performed by Christensen et al in 1988[27], and Gazit et al in 1994[28] and 1995[29] used artificial lipid membranes to examine the ion channel forming properties of the cecropin molecule. Christensen found that the positively charged residues on the cecropin α helices would adsorb onto the surface of a negatively charged lipid membrane. Once adsorbed the largely hydrophobic C-terminal helix inserts into the hydrophobic membrane core, pivoting on the Glycine-Proline hinge. These first two steps were not observed to be voltage-dependent. The third, voltage-dependent, step involves the insertion of the polar (positively charged) N-terminal helix into the lipid. The cecropin monomers then move together in the lipid bilayer forming a central aqueous pore in the membrane. This model also shows a possible reason for the selective action of cecropins against bacterial cells, since when cholesterol (a component of eukaryotic cell membranes) was added to the lipid membranes, thus increasing the fluidity, the formation of pores was significantly reduced.

The studies by Gazit[28,29] and colleagues disagree with the above interpretation of results given by Christensen[27], and suggest a different method by which membrane permeation occurs. This study agrees on the mechanism by which the cecropin molecule adsorbs onto the lipid membrane, but it proposed that the peptide monomers do not form ion-channels in the membrane. Gazit suggests that the cecropins permeate the membrane and act on targets situated on the inner wall of the membrane. Cecropin action on these targets would disrupt the electron transport chain, and the enzymatic apparatus necessary for oxidative phosphorylation. These

actions cause the destruction of the energy metabolism of the target organism by increasing the permeability of energy-transducing membranes, see fig 5.

Fig 5:- Proposed mechanism of action of cecropins (adapted from Christensen)[27]



Tentative model for the interaction of cecropins with a lipid bilayer membrane. Aggregates to the bilayer-water interface by electrostatic forces (I). Only a diamer is sketched for the sake of simplicity, but larger aggregates are likely to occur. The next step (II) would be the insertion of the hydrophobic segment into the membrane core. Upon application of a voltage (positive on the side of the peptide addition), a major conformational rearrangement takes place (III), which results in channel formation. This rearrangement could be insertion of the positively charged amphipathic helix into the membrane or opening of preformed, closed channels.

When antibacterial peptides isolated from insects were first discovered they were quickly found to be produced by the fat body of the insect. It was shown by Trenizek and Faye[76] in 1988 that antibacterial peptides were produced by the insect fat body, in vitro, following contact with lipopolysaccharide. Merifield and colleagues in 1990 showed that cecropins were synthesised throughout the whole of the fat body of *Drosophila* with little activity elsewhere[30]. Lemaitre and colleagues working in 1997 on *Drosophila* found that as well as being synthesised in the fat body, cecropins were also synthesised in the midgut, malpighian tubules, salivary glands, muscle, pericardial cells, epidermal cells, and haemocytes[31]. In 1993 Brey et al published an investigation into the properties of the cuticle of the silkworm, *Bombyx mori*[32]. This work showed that cecropin molecules are synthesised by cells underlying the cuticle enabling the peptide to work as an antibacterial agent in the cuticle layers, thus

showing that cecropins play a major role in the antibacterial response of the insect both inside and outside the body.

It can thus be seen that cecropins are effective antibacterial peptides, with activity against both gram positive and gram negative bacteria. They form a double α-helical structure with a Glycine-Proline hinge that allows selective penetration into bacteria cell membranes causing a disruption of bacterial ion regulation by one of two proposed means. They are synthesised predominantly by the fat body when the insect is challenged by a bacterial pathogen and exist both as soluble factors in the haemolymph and as agents acting externally in the cuticle layer.

In 1995 a second inducible short α-helical, antibacterial peptide was characterised from the silkworm, Bombyx mori[33]. This peptide was named moricin and is 42 amino acids in length. Analysis of the amino acid sequence showed positively charged residues spaced 3-4 apart at the N-terminal end. This spacing suggested that the peptide is folded into an amphipathic α-helix in this region, with all the charged residues on one side of the helix, see fig 6.

Fig 6: - Amino acid sequence of moricin

AKIPIKAIKTVGKAVGKGLRAINIASTANDVFNFLKPKKRKH

From this the tertiary structure was estimated and it was proposed that, as with cecropins, the bacterial cell membrane is the target for the peptide. It has been predicted that the positively charged domain of the α-helix will adsorb onto the negatively charged bacterial cell membrane. Once adsorbed onto the membrane there is a conformational change in the molecule that brings about an increase in the permeability of the experimental liposome membrane. How this is achieved is not yet fully known, but the similarity of the molecule to cecropins suggests the two have similar mechanisms of action. The similarity of moricin and cecropins is also shown in their respective spectra of activity, as both are active against gram-positive and gram-negative bacteria. The difference between the two peptides is that cecropins have a greater activity against gram-negative bacteria, whereas moricin has greater activity against gram-positive bacteria. It is thought that the two molecules work in a

complimentary fashion in Lepidopteran insects since this is the only order of higher insects that do not synthesis insect defensins, another potent killer of gram-positive bacteria[34].

Finally much research has been performed in hybridising cecropin like molecules to enhance their antibacterial properties, summaries of this work are provided by the following references[35, 36,37,38].

3.4 Insect defensins

The third family of inducible antibacterial peptides is the insect defensins. They were first identified and fully characterised by Lambert and colleagues[39] in 1989, and named as a result of their similarity to mammalian defensins. This original study isolated two peptides with potent antibacterial activity against gram-positive bacteria. These peptides named Defensin A and Defensin B are 4 kDa in mass and differing by one amino acid, see fig 7.

Fig 7: - Peptide sequence of defensins A and B

Defensin A ATCDLLSGTGINHSACAAHCLLRGNRGGYCN**G**KGVCVCRN
Defensin B ATCDLLSGTGINHSACAAHCLLRGNRGGYCN**R**KGVCVCRN
Note: the only difference is at residue 32

Lambert went on to prove that there was no post-translational modification of the molecules either by glycosylation or phosphorylation, and that the 6 cysteine residues form 3 disulphide bridges. Lambert also concluded that the replacement of a glycine residue by an arginine residue at position 32, the only difference between the two peptides, resulted from a single base change in the DNA and thus this substitution is thought to have no functional significance. The name insect defensins was proposed after comparing the newly discovered peptides with anti-bacterial peptides found in rabbit lung macrophages. It can be seen (see fig 8) that half of the amino acids in the two molecules are identical, and that of the others, many are conservative substitutions.

Fig 8: - Comparison of mammalian and insect defensins

Peptide number	20	30
Defensin A	ACA-AHCLLRGNRGGYCNGKG	
MCP1 (rabbit)	ACRRALCLPRERRAGFCRIRG	

Mammalian defensins have been identified in many species[40]. To date, all have the characteristic 3 cysteine residues forming disulphide bridges. The major difference between mammalian and insect defensins is that insect defensins are inducible anti-bacterial peptides which appear free in the haemolymph, whereas mammalian defensins are synthesised in, and remain within the lysosomes of phagocytic cells e.g. macrophages and neutrophils.

By 1991 defensins had been characterised in all of the major families of the series Endopterygota except for the Lepidoptera. The latter, it was concluded, relying upon cecropins and lysozyme for defence against bacterial infection[41]. In 1992 an insect defensin was isolated from the dragonfly *Aeshna cyanea*, this showed that insect defensins were also present in the other major series of insects, the exopterygota[42]. This new defensin was shown to be synthesised in the fat body, and had a significantly higher antibacterial activity than defensin from Dipteran insects, despite the Dipterans being a more recent order of insect. It is thought that this comparatively high antibacterial activity is due to insect defensin being the only peptide produced by *Aeshna* capable of killing gram-positive bacteria. Thus *Aeshna* has evolved an insect defensin with a greater potency than insects e.g. the diptera, which rely upon many different anti-bacterial peptides. *Aeshna* defensin was shown to have anti-bacterial activity, as measured by a growth inhibition assay, at 1 pmol concentration, whereas defensin from *Phormia terranovae* (Dipteran) had no anti-bacterial activity at this concentration, see fig 9.

Fig 9: - Effective concentrations of the insect defensins isolated from *Aeshna* and *Phormia*

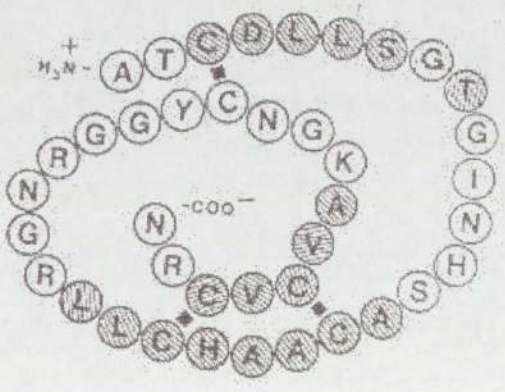
Activity spectrum of the inducible antibacterial peptide from *Aeshna* and comparison with insect defensin A from *Phormia*. Various amounts (1-10pmol) of the respective peptides were deposited on bacteria-seeded agar plates and the antibacterial activity was determined by plate growth-inhibition assay (expression in diameter of growth inhibition).

Bacterial Strains (All gram-positive)	Antibacterial activity (mm) from *Aeshna* (**A**) and *Phormia* (**P**)							
	10 pmol		5 pmol		2 pmol		1 pmol	
	A	**P**	**A**	**P**	**A**	**P**	**A**	**P**
M. luteus	10	8.5	8	7	5.5	4.5	3	2.5
A.viridans	15	11	13	9	11	6	9	0
P. acidilactici	8	3	5	0	3	0	0	0
B.megaterium	8	5	7	5	5.5	4	3.5	3
S. pyogenes	6	3	5	0	4	0	0	0
S. aureus	0	0	0	0	0	0	0	0

It has been suggested that the difference in antibacterial activity between Odontan and Dipteran insect defensin is due to a slight difference in the folding of the molecules. Odontan defensin is typically 38 amino acids in length and so folds tighter than Dipteran defensin which is typically 40 + amino acids in length.

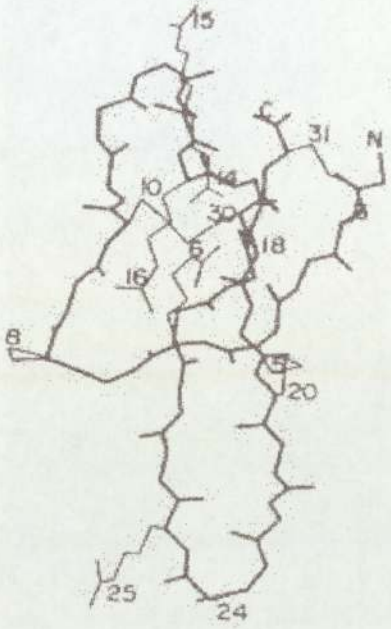
The nature of insect defensin was quickly investigated, and in 1991 two studies published full characterisation and tertiary structure of insect defensin[43,44]. It was shown by Lepage et al that, regardless of whether the insect defensin was natural or made from recombinant sources, the cysteine residues would pair up 1- 4, 2-5, 3-6 to form disulphide bridges. From these results a globular model for the folding of insect defensins was proposed. This model showed a structure in which an α-helix, and an anti-parallel β-pleated sheet are held together by the cysteine residues forming three disulphide bridges. Further studies identified only one disulphide bridge in Aeshna defensin. When the folding of this molecule was more closely examined there was enough evidence for the existence of a total of three disulphide bridges that the model of an insect defensin's tertiary structure below is accepted for all insect defensins discovered , see fig 10,11.

Fig 10:- Alignment of the disulphide bridges in insect defensins



Note: Hydrophobic regions are shadowed.

Fig 11:- Tertiary Structure of Insect Defensins



3D model of the polypeptide backbone of the defensin molecule

As early as 1989 cDNA clones were made for various anti-microbial peptides extracted from insects[45,46]. These were used to make synthetic peptides that allowed for more detailed investigations to be carried out on the molecules. Insect defensins are not modified at all after translation so the synthetic molecules behave in exactly the same way as the natural ones. It is for this reason that in only a short space of time the mechanism of action of insect defensins was discovered. Cocianich and colleagues in 1993 used recombinant insect defensin to show that the mechanism of action of the molecule is mediated by the formation of potassium channels in the cytoplasmic membrane of bacterium[47]. There is no firm evidence for the actual method by which these channels are formed but a number of models have been proposed. The first model suggests that the insect defensin molecules form oligomers in the surrounding fluid and then insert spontaneously into the cytoplasmic membrane of the bacterium. The second suggests that defensin monomers bind to the membrane independently of one another and diffuse laterally inside the lipid bi-layer to form

oligomers. Opinion is divided over which model is correct but however the oligomers are formed they give rise to potassium channels in the cytoplasmic membrane of Gram positive bacteria. These potassium channels cause a loss of about 500 mM cytoplasmic potassium that can not be replaced by active transport back into the cell. This loss of potassium causes the bacterial cell to become partially depolarised and inhibits respiration. Insect defensins also cause a depletion of intracellular ATP and it is a combination of these factors that destroys the bacterium. The bacterial cell membrane was identified as the target for insect defensins by use of inert, giant liposomes. It was demonstrated that potassium channels were spontaneously formed in liposomes treated with insect defensin, whilst no potassium conductance was observed in control liposomes. These liposomes were also used to discover that the potassium channels formed by insect defensins differ in their conductances, and in the duration of their opening and closing. It is thought that this difference is due to different numbers of insect defensin monomers combining to form the channel, thus a potassium channel formed of 5 insect defensin monomers will behave differently to one formed of 10 insect defensin monomers.

The inhibition of respiration, the secondary effect of insect defensins upon bacterial cells, has been shown not to occur as a consequence of the opening of potassium channels. It is therefore thought that insect defensins either affect the components of the respiratory chain, or cause the release of cofactors essential for respiration. The reduced ATP synthesis brought about by insect defensins is considered essential to the killing of the bacterial cells by the peptide. It is thought to be as a result of an efflux of inorganic phosphate though the channels formed, shifting the ATP hydrolysis equilibrium, and thus depleting intercellular ATP.

Once the insect defensin molecule had been characterised work was carried out to investigate the extent of defensin activity. It was shown that the disruption of the insect defensin molecule by the breaking of the disulphide bridges causes a loss of anti-bacterial activity due to the unravelling of the tertiary structure[47]. Insect defensin anti-bacterial activity is also reduced or removed by high concentrations of divalent cations, and by a decrease in temperature such that there is no anti-bacterial activity below 6°C. The activity of insect defensins has been shown to be concentration dependent[35], Cociancich's study showed that 105 molecules of insect defensin are

needed to kill one bacterial cell[40]. This does not compare favourably with another channel forming toxin, colicin A that requires a ratio of one molecule of toxin to one bacterial cell to provide sufficient potassium efflux to kill the cell[47].

The insect defensin molecules from many different species of insect have been investigated to gain an insight into the origins of the molecule. New insect defensins are being discovered all the time, but all have the characteristic 6 cysteine residues forming disulphide bridges, anti gram-positive activity and are about 4 kDa in molecular mass. Insect defensins vary in length between 37- 43 amino acids but are all very similar, see fig 12.

Fig 12: Peptide sequence of insect defensins [48,49,50,51,52]

Molecule	Peptide Sequence
A.d.defensin	VTCDLLSFEAKGFAANHSLCAAHCLAIGRRGGSCER-GVCICRR
Tenesin	VTCDILSVEAKGVKLNDAACAAHCLFRGRSGGYCNGKRVCVCR
Z.a.defensin	FTCDVLGFEIAGTKLNSAACGAHCLALGRRGGYCNSKSVCVCR
Z.a.defensin	FTCDVLGFEIAGTKLNSAACGAHCLALGRTGGYCNSKSVCVCR
P.a.defensin	ATCDILSFQSQWVTPNHAGCALHCVIKGYKGGQCKIT-VCHCRR
Phormicin	ATCDLLS----GTGINHSACAAHCLLRGNRGGYCNRKGVCVCRN
Phormicin A	ATCDLLS----GTGINHSACAAHCLLRGNRGGYCNGKGVCVCRN
Sapecin	ATCDLLS----GTGINHSACAAHCLLRGNRGGYCNGKAVCVCRN
D.n.defensin	ATCDLLS----KWNWNHTACAGHCIAKGFKGGYCNDKAVCVCRN

Note ; - spaces are introduced to obtain maximal sequence alignment

In 1994 further evidence was provided to confirm the wide distribution of defensins throughout insects, when a completely new insect defensin was isolated from the Hemipteran sap sucking bug *Pyrrhocoris apterus*[53]. This new insect defensin was seen to be similar to defensin A, but also had the characteristic long N-terminal loop of Coleoptera defensin. This similarity between the Hemipteran defensin and Coleopteran defensin is taken as a sign of the conservation of the insect defensin molecule as, Hemipteran insect pre-date all Endopterygotan insects by 100 million years[54]. As Coleoptera were the first of the Endopterygota to evolve so the similarity of the two molecules can be explained, see fig 13.

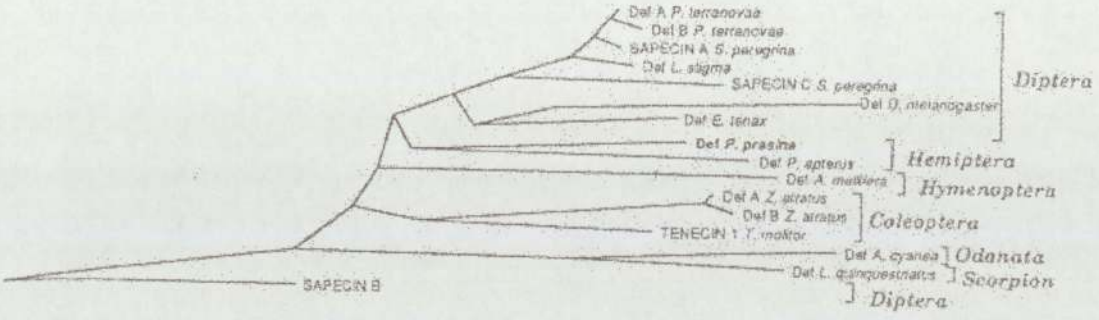
Fig 13:- Peptide sequences of Endopterygotan and Coleopteran defensin

```
Endopterygota     ATCDILSFQSQWVTPNHAGCALHCVIKGYKGGQCKIT-VCHCRR
Coleoptera        VTCDILSVEAKGVKLNDAACAAHCLFRGRSGGYCNGKRVCVCR
```

Note : Space - introduced to optimise alignment.

These discoveries have helped in the understanding of the evolution of the molecule so that a rough history of the evolution of the insect defensin can be presented, see fig 14 [4,55,56].

Fig 14:- Evolution of the insect defensin molecule



As work continues in this field new areas of research are opening all the time. In 1996 a peptide was isolated from the mussel *Mytilus galloprovincialis* with activity against gram positive and gram negative bacteria containing 8 cysteine residues (see fig 15) [57]. It was identified as part of the defensin family by computer searching. As a member of the defensin family it is unique both in having 8 cysteine residues, and for having activity against gram negative bacteria. This discovery will possibly lead to more defensins being identified from many invertebrate species with many possible benefits.

Fig 15:- Peptide sequence of *Mytilus galloprovincialis* defensin

```
Mytilus defensin      GFGCP-------NNYQCHRHCKSIPGRCGGYCGGXHRIRCTCYRC
Defensin A             ATCDLLSGTGINHSACAAHC-LLRGNRGGYCNG--KAVCVC-RN
```

Note : Spaces - introduced to maximise alignment

In 1999 a member of the insect defensin family was finally isolated from a Lepidopteran insect, *Heliothis virescens* [58]. This insect defensin, identified as such by the characteristic six cysteine residues forming three disulphide bridges, surprisingly showed exclusive antifungal activity, with no antibacterial activity at all. It was proposed that this antifungal activity is due to a peptide-receptor link, as experiments showed that the antifungal activity was not based on charge differences between the molecule and surface of the fungus. The lack of antibacterial activity of this lepidopteran defensin supports the widely held view that lepidopterans base their humoral immunity on cecropins, attacins, and lysozyme.

Potentially the most exciting discovery came in 1996 when it was shown that an insect defensin isolated from the beetle Allimyria dichotoma had anti-bacterial activity against methicillin resistant Staphylococcus aureus (MRSA)[59]. MRSA bacteria are resistant to almost all antibiotic drugs presently used and present a major threat to man's control of infectious disease. As insect defensins have been shown to be active against MRSA bacteria they could provide a novel solution to the problems of infection by MRSA bacteria. Chief amongst these could be the use of insect defensins as part of a skin antiseptic for the use of burns patients, probably the group most at risk.

3.5 Proline rich peptides

Most of the initial research into inducible antibacterial peptides in insects concentrated on moths and flies. This is because the large size of the larvae allows high yields of haemolymph to be obtained. Work published by Casteels et al in 1990[61] and 1993[60], specifically looked for inducible antibacterial peptides for insects outside these two classes. These studies focused on the honeybee, *Apis mellifera*, as these insects gather pollen from plants it was reasonably assumed that they could come into contact with plant micro-organisms. It was therefore thought that they would have a different range of pathogens, and peptides to combat these pathogens. Casteels characterised two novel inducible antibacterial peptides named apidaecin, and abaecin. These peptides are rich in proline residues, and show some sequence homology with each other, see fig 16. The proline residues are evenly spread along the whole 34 amino acid sequence of abaecin excluding an α helical conformation.

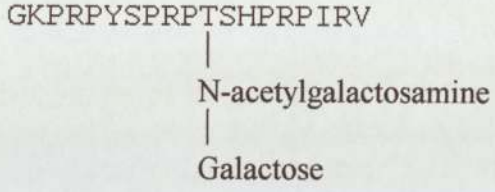
Fig 16:- Amino Acid Sequence of the Proline rich antibacterial peptides

Apidaecin Ia	GNNRPVYIPQ PRPPHPRI
Apidaecin Ib	GNNRPVYIPQ PRPPHPRL
Apidaecin II	GNNRPIYIPQ PRPPHPRL
Abaecin	YVPLPNVPQP GRRPFPTFPG QGPFNPKIKW PQGY
Drosocin	GKPRPYSPRP TSHPRPIRV

Apidaecin was shown to be rapidly bactericidal against gram negative bacteria, but did not have any activity against gram positive bacteria. Abaecin has only moderate antibacterial activity when compared to apidaecin, but shows antibacterial activity against both gram positive, and gram negative bacteria. The antibacterial activity of abaecin is significantly slower than that of apidaecin suggesting that the molecules could have different mechanisms of action. These mechanisms of action are, as yet, unknown, although the absence of an α helical structure suggests that the molecules do not interact with bacterial cell membranes, and may well involve a peptide-receptor recognition process.

In 1993 a novel proline rich peptide was identified from Drosophila, and proved to be the first identified member of the sub-family of O-glycosylated, proline rich, inducible antibacterial peptides[62]. This 19-residue peptide was named drosocin, and showed strong antibacterial activity against gram negative bacteria, with some slight activity against gram positive bacteria. Analysis of the peptide sequence showed a small relative molecular mass than the experimental data showed. Subsequent experiments showed that there was a post-translational modification of the molecule by way of a glycosylation of the threonine residue at position 11. This O-glycosylation involved the addition of an N-acetylgalactosamine-galactose disaccharide, see fig 17. It was noted that this glycosylation was important since antibacterial activity was impaired by the selective removal of this disaccharide. Thus the glycosylation must, in some way, be at least partly responsible for the mechanism of action.

Fig 17:- Glycosylation of Drosocin



A year later, 1994, a peptide was characterised from the sap-sucking bug *Pyrrhocoris apterus*. This peptide, pyrrhocoricin is 20 amino acids long, rich in proline residues, and shows sequence homology and comparable glycosylation with dorsocin, see fig 16. It is extremely hydrophilic and has slow, bactericidal activity against gram negative Bacteria. In 1995, and in 1998 two additional proline rich antibacterial compounds were extracted from the silkworm, *Bombyx mori*, and the bulldog ant, *Myrmecia gulosa*, respectively[63]. These peptides, lebocin and formaecin, again exhibited the same sub-family traits i.e. slow, bacteriostatic activity against gram negative bacteria, O-glycosylation of a central threonine residue, relatively short chain length, and a high percentage of proline residues, see fig 16.

The relatively slow antibacterial action and the inability to act on static bacteria suggest that the O-glycosylated peptide could act in one of the following

ways. It could act by way of membrane receptors. There may be an association with peptidoglycans. Or there could be competitive inhibition of the enzymes responsible for peptidoglycan synthesis. It is also thought that these O-glycosylated peptides do not work independently, rather they act synergistically with other antibacterial peptides produced by the insect in response to infection.

3.6 Glycine rich peptides

After the discovery of cecropins in the Cecropia moth other related studies were performed on a variety of insect species. The attacins were first isolated in 1983 during an investigation into cecropins. This group of related molecules was initially recognised because of their significantly larger molecular mass than that of cecropins. Attacins are 187-188 amino acids in length and exist in six isoforms, four basic, and two acidic. cDNA techniques were used to establish that only two genes were responsible for the six isoforms, one gene coded for the acidic, and one for the basic molecules, see fig 18[64]. Attacins were shown to act bacteriostatically on gram negative bacteria with a narrow spectrum of activity. Little is known about the mechanism of action of attacins, but it is thought that they act on the outer membrane of bacterial cells causing an increase in the permeability of the membrane of growing gram negative cells. The actions of attacin have been shown to facilitate the actions of both lysozymes and cecropins, thus enabling these three inducible antibacterial peptides to act in concert against all types of bacterial pathogens[65].

Fig 18:- Amino Acid sequence of the glycine rich peptides[66]

```
Acid attacin    DAHGALTLNSDGTSGAVVKVPFAGNDKNIVSAIGSVDLTDRQKLGAATAGVALDNINGHGLSLTDTHIPG
Basic attacin    QAGALTINSDGTSGAVVKVPITGNENHKFSALGSVDLTNQMKLGAATAGLAYDNVNGHGATLTKTHIPG

                FGDKMTAAGKVNVFHNDNHDITAKAFATRNMPDIWNVPNFNTVGGGIDYMFKDKIGASASAAHTDFINRN
                FGDKMTAAGKVNLFHNDNHDFSAKAFATKNMPNIPQVPNFNTVGAGVDYMFKDKIGASANAAHTDFINRN

                DYSLDGKLNLFKTPDTSIDFNAGFKKFDTPFMKSSWEPNFGFSLSKYF
                DYSLGGKLNLFKTPTTSLDFNAGWKKFDTPFFKSSWEPSTSFSFSKYF

Sarcotoxin IIA QKLPVPIPPPTNPPVAAFHNSVATNSKGGQDVSVKLAATNLGNKHVQPIAEVFAEGNTKGGNVLRGATVG
Sarcotoxin IIB QKLPVPIPPPSNPPVAVLQNSVATNSKGGQDVSVKLAATNLGNKHVQPIAEVFAEGNTKGGNVLRGATVG
Sarcotoxin IIC QKLPVPIPPPTNPPVAAFHNSVATNSKGGQDVSVKLAATNLGNKHVQPIAEVFAKGNTQGGNVLRGATVG
Sarcotoxin IID QKLPVPIPPPTNPPVAAFHNSVATNSKGGQDVSVKLAATNLGNKHVQPIAEVFAEGNTKGGNVLRGATVG

                VQGHGLGASVTKSQDGIAESFRKQAEANLRLGDSASLIGKVSQTDTKIKGIDFKPQLSSSSLALQGDRLG
                VQGHGLGASVTKTQ----------------------------TDTKIKGLDFQPQLSSSTLALQGDRLR
                VQGHGLGASVTKTQDGIAESFRKQAEANLRLGDSASLIGKVSQTDTKIKGIDFKPQLSSSSLALQGDRLG
                VQGHGLGASVTKSGNGIAESFRKQAEANLRLGDSASLIGKVSQTDTKIKGIDFKPQLSSSSLALQGDRLG

                -ASISRDVNRGVSDTLTKSVSANLFRNDNHNLDASVFRSDVRQNNGFNFQKTGGXLDYSHANGHGLNAGL
                GASISRDVN-GVSDTFTKSVSANVFRNDNHNLDATVFRSDVRQNNGFNFQKTGGXLDYSHANGHGLNAGL
                -ASISRDVNRGVSDTLTKSISANVFRNDNHNLDASVFRSDVRQNNGFNFQKTGGXLDYSHANGHGLNAGL
                -ASISRDVNRGVSDTLTKSISANVFRNDNHNLDASVFRSDVRQNNGFNFQKTGGXLDYSHANGHGLNAGL

                TRFSGIGNQATVGGYSTLFRSNDGLTSLKANAGGSQWLSGPFANQRDYSFGLGLDHNAWR
                THFSGIGNQANVGGSSTLFKSNDGSLSLKANAGGSQWLSGPFSNQRDYNVGLSLTHHGCG
                TRFSGIGNQANVGGYSTLFRSNDGLTSLKANAGGSQWLSGPFANQRDYSFGLGLSHNAWR
                TRFSGIGNQANVGGYSTLFRSNDGLTSLKANAGGSQWLSGPFANQRDYSFGLGLSHNAWR

Coleoptericin   SLQGGAPNFPQPSQQNGGWQVSPDLGRDDKGNTRGQIEIQNKGKDHDFNAGWGKVIRGPNKAKPTWHVGG
                TYRR

Diptericin A    DEKPKLILPTPAPPNLPQLVGGGGGNRKDGFGVEVDAHQKVWTSDNGGHSIGYSPGYSQHLPGPYGNSRP
                DYRIGAGYSYNF
```

In 1988 Dimarcq and colleagues isolated three related peptides that differed from cecropins, lysozyme, and attacins, the only known inducible antibacterial peptides at that time[67]. As these peptides had been isolated from the immune haemolymph of the dipteran flesh fly, *Sacrophaga peregrina*, they were given the name diptericins. Diptericins were shown to have antibacterial activity against E. coli and other gram-negative bacteria. This antibacterial activity was seen as an inhibition of the growth of the bacteria, with a minimum inhibitory concentration of 6-13 $\mu g\ ml^{-1}$. This relatively high concentration suggested either that the diptericin action held the bacterium in stasis, or that there is some change to the pure diptericin protein before it becomes active. The diptericin family of inducible antibacterial peptides form heat stable, cationic molecules of approximately 8 kDa relative atomic mass. It was shown, by Reichart and colleagues in 1989 using cDNA techniques, that upon inoculation of the animal with bacteria there is a rapid induction of the transcription diptericin mRNA in the fat body of the insect[45]. Thus confirming again that the fat body is the primary site of antibacterial peptide synthesis in insects. The full sequence of Diptericin A has been characterised and shows an 82 residue polypeptide with an N-terminal region rich in proline, and a glycine rich C-terminal region, see fig 18. Diptericin B and C have been partially characterised and have a very similar amino acid sequence to Diptericin A. A key feature of all diptericins being five successive glycine residues at positions 21-25[68].

Work published by Bulet et al in 1995 revealed clues to the mechanism of action of diptericin[69]. This liquid chromatography study showed that the diptericin molecule was indeed more complex than had previously been thought. It was observed that there is a glycosylation of the theronine residues in both the proline rich, and glycine rich domains, positions at 10 and 54 respectively. Both glycosylations consisted of identical trisaccharides:- glucose-galactose-N-acetyl-galactosamine - (threonine). It was shown that the removal of these trisaccharides completely removes the antibacterial activity of the diptericin molecule. As these trisaccharides were obviously essential to the antibacterial action of diptericin it was suggested that the glycosylation induces a conformational change in the peptide's tertiary structure. It is therefore thought that the molecule interacts with a specific receptor on the bacterial cell membrane that only accepts the active, glycosylated

form. As the glycosylation of diptericin confers protease resistance it is thought that the active molecule might act as a competitive inhibitor of the enzymes responsible for bacterial cell wall peptidoglycan synthesis. The mechanism of action proposed here is the same as that proposed earlier for the O-glycosylated proline rich antibacterial peptides. This is understandable as both glycosylated groups have the same spectrum of activity i.e. slow, bacteriostatic effects against gram negative bacteria. It has also been proposed by some researchers that some of the Proline rich and Glycine rich peptide families are versions of the same molecule as they have been characterised at different points in the life cycle of the insect, diptericin present in the larva and dorsocin found in the adult.

In recent years a number of inducible antibacterial peptides belonging to the Glycine rich peptide family have been identified. Work performed by Casteels et al published in 1993 reported the isolation of an inducible antibacterial peptide, from the honey bee, they named hymenoptaecin[60]. This 93 amino acid long peptide was seen to be hydrophilic with many glycine residues, having some sequence homology to diptericin, see fig 18. Experiments showed it to be active against gram negative bacteria at a minimum inhibitory concentration of 0.5 - 5.0 μg ml-1, with some activity against gram positive bacteria. The study also showed that the molecule permeated the bacterial cell membrane, to act on targets on the inner membrane, though a full mechanism has not discovered.

The Glycine rich peptides also includes coleoptericin, another large, glycine rich, anti gram negative peptide. Coleoptericin was characterised in 1991 by Bulet et al from the larvae of the large tenebricnid beetle, Zophobas atratus. It is 74 amino acids in length with no homology to the other inducible antibacterial peptides in this class[41].

All of the inducible antibacterial peptides discussed above, except the insect defensins, have been characterised from higher series of insects, the Endopterygota, and little characterised from the Exoptertgota. In 1994 Cociancich et al published a study investigating the inducible antibacterial peptides of a hemipteran insect, the sap sucking bug *Pyrrhocoris apterus*[53]. Cociancich isolated a new insect defensin, and also a 133 residue, glycine rich peptide active against gram negative bacteria. This molecule, named hemiptericin, is highly charged with a net overall positive charge; it

shows significant sequence similarity to diptericin in the central, glycine rich, and C-terminal domains; and shows some anti gram positive activity.

This overview of inducible antibacterial peptides is not complete, and will never be, as new molecules and mechanisms of action are being discovered all the time. It does however provide a background to the important molecules, and their functions.

3.7 Purpose of study

The purpose of this study is to investigate the inducible antibacterial response of the larvae of the blow fly, *Calliphora vomitoria*. *Calliphora vomitoria* is an insect of the Dipteran order, therefore this study will concentrate on looking for the presence of insect defensins, primarily, and also for cecropins and other inducible antibacterial peptides. Other studies into various aspects of insect physiology have been performed using *Calliphora vomitoria*. These studies have investigated the neuropeptides of insects principally the Callatostatins. Other work investigating *Calliphora vomitoria* has looked at the specificity of the agglutinin molecule. The larvae of *Calliphora vomitoria* are already produced on a commercial scale as they are the maggots used by fishermen, and therefore are a cheap, easily obtainable source of raw materials. Experiments were carried out on larvae as they can be induced to produce antibacterial peptides, and are more easily handled than the adult. It is therefore probable that with the inducible antibacterial response of *Calliphora vomitoria* characterised, any inducible insect antibacterial peptide discovered to have value in the pharmaceutical industry will have an existing mass produced source from which the peptide can be synthesised. This approach is particularly relevant as insect defensins have been shown to act against MRSA bacteria, therefore, as there are very few antibiotics left that have antibacterial activity against these bacteria, insect defensins may well prove useful in combating this problem.

The methodology used has been adapted from that first used by Lambert et al in 1989[32]. The objective of these experiments was to induce an antibacterial response in the larvae of the Blow Fly *Calliphora vomitoria*, following inoculation with bacteria. Once an antibacterial response had been observed it was the objective to

isolate the molecules causing the antibacterial activity and purify them. Once isolated the antibacterial molecules were then investigated to determine the following properties of the molecules: -

- Specificity of the antibacterial activity against gram-positive and gram-negative bacteria
- Stability of the molecule to heat and pH
- Duration of the antibacterial response from the insect in question.

4.0 Materials and Method

4.1 Standard conditions for all Experiments

4.1.1 Animals

Fourth instar larvae of the dipteran *Calliphora vomitoria* were purchased from the Birmingham Angling Centre, Erdington. The larvae were housed in a plastic containers with vented lids measuring 20cm x 20cm x 10cm and stored at 4° C in order to reduce metabolic rate and therefore delay pupation. As the larvae were used for experimental purposes a maximum of 14 days after purchase no food was provided, this also helped to reduce metabolic rate, and thus delay pupation. Preliminary studies performing antibacterial assays upon haemolymph from uninoculated animals prior to their being stored at 4°C showed that there is no detectable antibacterial activity present in the larvae before inoculation. Subsequent controls during the experimental procedures again showed no antibacterial activity post storage at 4°C and prior to inoculation.

4.1.2 Bacterial Strains

Bacteria were obtained from the collection held by the Microbiology Research group at Aston University: - Staphylococcus aureus NCTC 6571, also known as S. aureus ATCC 9144, and S. aureus NCIB 6571, was first deposited by NG Heatley in 1944 in Oxford[45]. Escherichia coli IC III, also known as E. coli W3110, is a K12 strain containing no plasmids or bacteriophages. First isolated by BJ Bachmann in 1972[44].

Bacterial strains were initially grown on agar plates containing nutrient agar (see appendix for list of ingredients). When samples were required for experiments a single colony was transferred into a sterile glass conical flask containing 50-100ml of sterile nutrient broth (see appendix from list of ingredients). The sample was then incubated for 16 hours at 37°C with constant shaking to ensure even growth.

4.1.3 Inoculation

To induce an anti-bacterial response within the larvae bacteria were introduced into the animals in the following way. A glass Pasteur pipette was heated in a Bunsen flame and the end drawn out to a fine, 'needle' point. These glass needles were loaded with bacteria by placing the needle into a culture of logarithmic phase *Staphylococcus aureus*, containing approximately 8 x 10^{11} bacteria per ml. Nutrient broth containing the bacteria was taken up by capillary action into the needle. Control needles were placed in sterile nutrient broth. The animals received injections of bacteria by inserting the pre-inoculated needles into the larvae between the 3rd and 4th segment. The volume of liquid administrated was between 3 - 5 µl per larva, and contained on average 2 x 10^5 bacteria. As more than 1000 individuals were inoculated per experiment this variance in dosage is considered unimportant. The inoculated larvae were placed in a glass conical flask sealed with aluminium foil, vented by means of small holes pierced through the aluminium foil and moistened with wet paper towel. The length of time spent incubating in this way was dependent upon the experiment carried out. For the majority of the following experiments the larvae were maintained for 24 hours at room temperature (20 °C +/- 4 °C).

In an initial test different batches of larvae were inoculated with logarithmic phase *Escherichia coli* (procedure carried out as above) or *S. aureus* to determine if there was any significant difference in the antibacterial activity induced by the different bacteria post inoculation. As no significant difference was observed, S. aureus was used for the resulting experiments.

4.1.4 Haemolymph Extraction

Haemolymph was extracted from immunised and control larvae at specific times post inoculation, usually 24 hours, in the following way. A Pasteur pipette was drawn out to a needlepoint as above and inserted into the animal between the third and fourth segment. The injection was performed at a shallow angle so as not to penetrate too far into the body cavity and pierce the gut thus releasing proteolytic

enzymes into the haemocoel. Haemolymph extraction was achieved by capillary action, typically 5-10 µl of haemolymph was obtained per animal. The haemolymph was pooled in a plastic centrifuge tube and containing 0.5 ml of Aprotinin (Sigma) and kept on ice. The aprotinin was used to prevent any proteolytic enzyme activity, which may occur due to a combination of proteolytic enzymes present in the haemolymph and those that could become present should the gut accidentally become punctured.

The haemolymph from 200 animals was pooled per experiment and centrifuged at 36000 x g for 20 min at 4° C to remove haemocytes and other cellular debris. The cell free supernatant (plasma) was frozen - 20 ° C until further use.

4.1.5 Anti-bacterial Assay

To determine whether the haemolymph extracted from the larvae had any anti-bacterial activity the following standard antibacterial assay was carried out. 10 ml of molten nutrient agar was added to sterile petri dishes 9-cm diameter and allowed to set. Logarithmic phase bacteria of the test strain were added to the surface of the agar so that a complete bacterial lawn would grow during a 24-hour incubation period. 2-mm diameter wells were cut into the agar using a gel cutter. 5 µl of cell free haemolymph taken from animals injected with bacteria as test samples and 5 µl of cell free haemolymph taken from animals injected with sterile medium as a control was added to the wells on the plates. Each sample and control was added to one well on three different agar plates to ensure that the results obtained were not due to a single occurrence of antibacterial activity on one agar plate. The plates were incubated for 24 hours at 37°C. At the end of this time zones of bacterial inhibition were recorded around each of the wells by means of measuring the diameter of the clearance zone seen across the centre of the well at the widest point. For the purposes of this study *S. aureus* was used as a typical gram-positive bacterium and *E. coli* as a typical gram negative species.

4.2 Nature of the Antibacterial Response

Anti-bacterial assays were used to determine the nature of the antibacterial activity seen in the animals post inoculation.

4.2.1 Investigation of antibacterial activity against gram positive and gram negative bacteria

300 larvae of the Blow Fly *Calliphora vomitoria* were injected with *S. aureus* following the method described in 4.1.3 above. After 24 hours at 20°C haemolymph was extracted as described in 4.1.4 above. Antibacterial activity against *S. aureus* and *E. coli* was then assessed according to the method described in 4.1.5 above. The above procedure was also performed using larvae injected with *E. coli* and control larvae were injected with sterile nutrient broth. As a further control the haemolymph from 300 non-injected larvae was extracted and subjected to the same antibacterial assays. The purpose of this experiment was to investigate whether there was any significant difference in the antibacterial activity of the haemolymph of larvae injected with gram positive or gram negative bacteria. Also it was the purpose of the experiment to investigate whether there is a significant increase in antibacterial activity of haemolymph taken from larvae that had been inoculated with bacteria and that in animals that had received a sham injection using a sterile needle. Thus this experiment examines whether the production of antibacterial activity is dependent on the presence of bacterial challenge, or is a non-specific reaction induced by wounding.

4.2.2 Investigation of the specificity of the antibacterial activity following challenge with *S. aureus*

300 larvae of the Blow Fly *Calliphora vomitoria* were injected with *S. aureus* following the method described in 4.1.3 above. After 24 hours at 20°C haemolymph

was extracted as described in 4.1.4 above. Antibacterial assays were performed (as described in 4.1.5) on the haemolymph extracted from these larvae. The bacterial strains used for these assays were: - *E. coli*; *Bacillus cereus* (both gram-negative); *S. aureus*; *Staphylococcus epidermdis*; and a methicillin resistant *S. aureus* strain (all gram-positive). The above procedures were performed on haemolymph from 300 control larvae that had not been inoculated. The purpose of these experiments was to investigate the specificity of the antibacterial activity produced by the larvae following challenged by gram-positive bacteria.

4.2.3 Investigation of the properties of the antibacterial molecules

4.2.3.1 Heat Stability

300 larvae of the Blow Fly *Calliphora vomitoria* were injected with *S. aureus* following the method described in 4.1.3 above. After 24 hours at 20°C haemolymph was extracted as described in 4.1.4 above. A 0.5ml sample of the haemolymph was placed in a plastic centrifuge tube. This centrifuge tube was placed in a boiling water bath for 4 minutes. Antibacterial activity was assessed on the heat treated haemolymph by performing the antibacterial assay described above in section 4.1.5 using *S. aureus* as the test organism. Control haemolymph taken from 300 larvae, that had not been inoculated with bacteria, was treated in the same way as the active sample haemolymph above. The purpose of this experiment was to investigate the heat stability of the antibacterial molecules and to show whether antibacterial activity is destroyed by heating to100°C

4.2.3.2 Protease Resistance

The remaining haemolymph collected in 4.2.3.1 above (both haemolymph with antibacterial activity and the control) was treated with 1ml of the proteolytic enzyme trypsin for one hour. A further sample of 300 larvae of the Blow Fly *Calliphora vomitoria* were injected with *S. aureus* in the method described in 4.1.3 above. After incubation for 24 hours at 20°C haemolymph was extracted as described

in 4.1.4 above with the exception that the haemolymph was not collected with aprotinin (a protease inhibitor). A control batch of haemolymph was extracted from another 300 C. vomitoria larvae that had not been inoculated with bacteria, this haemolymph was also collected with the protease inhibitor aprotinin being present. The haemolymph from all four samples (the two samples collected without aprotinin, and the two incubated with trypsin) were then assayed for antibacterial activity using *S. aureus* as a test organism. The antibacterial assay was also performed using trypsin and aprotinin as controls to establish that neither of these two compounds produced the results seen in the other antibacterial assays. The purpose of these experiments was to establish the fact that the antibacterial activity induced by the injection of bacteria into the larvae of *Calliphora vomitoria* was due to protein or peptide molecules. The experimental addition of trypsin to haemolymph with antibacterial activity was performed to discover whether the antibacterial activity was reduced due to the actions of a known protease. The collection of haemolymph without aprotinin was to show whether the antibacterial activity seen would be reduced due to the absence of a protease inhibitor, thereby allowing proteolytic enzymes from the animal to act on the molecules providing the antibacterial activity.

4.2.3.3 Investigating the pH stability of the antibacterial molecules

1000 larvae of the Blow Fly *Calliphora vomitoria* were injected with *S. aureus* following the method described in 4.1.3 above. After 24 hours at 20°C haemolymph was extracted as described in 4.1.4 above. 1ml aliquots of the cell free haemolymph were incubated at different pH levels ranging from pH 1-14 for 1hour. These experiments were performed in triplicate using 0.1M HCl and 0.1M NaOH were added to each sample as appropriate to create the difference in pH levels. As very small amounts of HCl and NaOH were need to effect this pH change the samples did not suffer any lose of activity due to dilution. After incubation at the pH level to be tested for one hour the pH was adjusted back to pH 7 for the antibacterial assay in to prevent any action on the bacteria used by the low pH.

Haemolymph was also extracted from another 1000 larvae that had not been injected with bacteria. This control haemolymph was treated in the same way as that described above. Antibacterial assays as outlined in 4.1.5 were then performed on the aliquots of active and control haemolymph using *S. aureus* as a test organism. The purpose of this experiment was to show whether pH had any effect on the antibacterial activity induced by the injection of bacteria into the larvae of *C. vomitoria* and to determine the optimum pH of the antibacterial molecules.

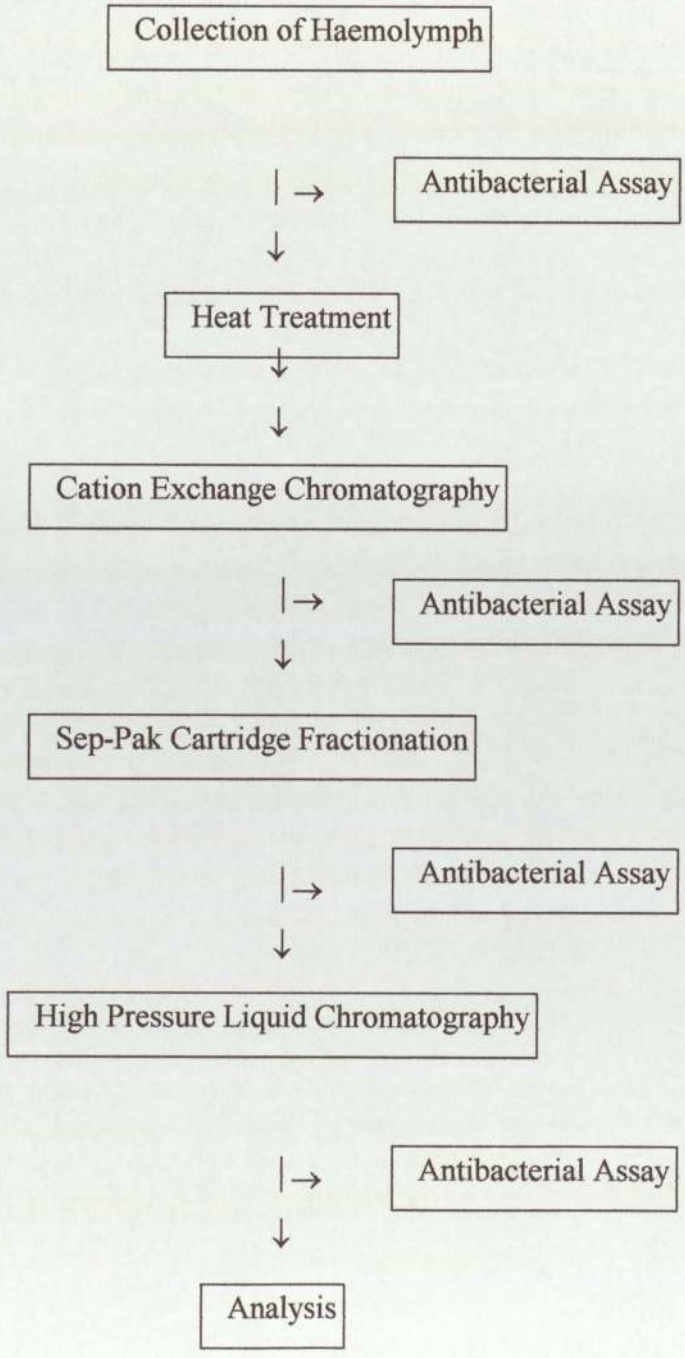
4.2.4 Investigating the kinetics of the antibacterial response

48 batches of 300 (a total of 14400 animals) larvae of the Blow Fly *Calliphora vomitoria* were injected with *S. aureus* following the method described in 4.1.3 above. For the first population of 24 batches haemolymph was extracted at specific time intervals (0 hours, 8 hours, 24 hours, 32 hours, 48 hours, 56 hours, 72 hours and 80 hours) in the manner described in 4.1.4 from three of the populations, resulting in triplicate samples of haemolymph collected at each of the time intervals noted above. The second population of 24 batches of animals had a second injection of *S. aureus* and haemolymph was extracted at the same time intervals and in the same manner as above. Animals used for controls were not injected with bacteria. Haemolymph from these control animals was collected in the above manner and at the above time intervals. All the haemolymph collected in this experiment was then assayed for antibacterial activity in the manner described in 4.1.5 above. The purpose of this experiment was to analyse the kinetics and duration of the induced antibacterial response produced by the larvae of *C. vomitoria* bacterial challenge.

4.3 Purification of Antibacterial Molecules

The following experiments were carried out in triplicate to identify the active components responsible for the antibacterial activity seen in the haemolymph of the blow fly *Calliphora vomitoria*..

Fig 19 Purification of the antibacterial molecules



4.3.1 Collection of the haemolymph

3000 larvae of the Blow Fly *Calliphora vomitoria* were injected with *S. aureus* following the method described in 4.1.3 above. After 24 hours at 20°C haemolymph was extracted as described in 4.1.4 above.

Haemolymph was collected from 3000 non-injected, control animals

4.3.2 Heat Treatment

Following the procedure described by the group headed by Lambert (1989)[32] the initial stage of separating the antibacterial proteins and peptides isolated from the larvae was to remove the non-heat stable proteins from the cell free haemolymph(see section 4.1.3). This was achieved by pooling the plasma extracted in 4.3.1 to give a final plasma volume of 30 ml. This plasma was diluted with 8 ml 1 M acetic acid (Sigma), and 16 ml 40 mm ammonium acetate (Sigma). This mixture was placed in a boiling water bath at 100°C for 4 minutes with constant stirring. It was then filtered and washed with 40-mM ammonium acetate to remove the precipitated material that consisted mainly of non-heat stable proteins. The pH of the filtrate was then adjusted to 6.8 with 40-mm ammonium acetate, with a resulting volume of 200 ml.

Haemolymph from the control animals in 4.3.1 was subjected to the same procedures as above.

4.3.3 Cation Exchange Chromatography

Previous studies referred to in the introduction have shown that many of the antibacterial molecules produced by insects are cationic peptides. Thus cation exchange chromatography was used as a first step for the attempted purification of the material in this study. This step was performed to separate the different, heat-stable proteins isolated in 4.3.2 above. Thus 200ml of heat step supernatant was loaded onto a column of CM Trisacryl C-25 2.5 cm x 18 cm (Pharmacia), and equilibrated with 40

mM ammonium acetate pH 6.8. 2000 ml of 40-mM ammonium acetate was then run through the column at 80 ml per hour to allow the sample to bind to the column and to remove the molecules not retained by the gel. Elution from the column was achieved by running 1600 ml of a linear gradient of ammonium acetate 40 - 500 mM through the column at a rate of 40 ml per hour. 15-ml fractions were collected throughout the loading, rinsing, and elution stages, therefore producing over 200 separate fractions. Each fraction was then assayed ultra-violet absorbance at 278 to determine the concentration of protein present. In addition the antibacterial assay described in 4.1.5 above was performed in triplicate for each fraction using both *S. aureus* and *E. coli* as test organisms. Control haemolymph was also treated in the same.

4.3.4 Sep-Pak Cartridge Fractionation

Once passed through the cation exchange column those fractions identified as containing protein and having antibacterial activity were concentrated using Sep-Pak cartridges. The antibacterial activity was shown to reside in three peaks which were labelled α, β, and δ. No significant antibacterial activity was observed in haemolymph from the control animals. Control samples were taken from the fractions in the control run in 4.3.3 that had the same retention time as the peaks α, β, and δ seen in the first runs using active samples. Sep-Pak cartridge fractionation was used to concentrate the samples as the volume of 15 ml obtained by ion exchange chromatography was too great for further analysis. The active fractions from the peaks labelled α, β and δ isolated by ion exchange chromatography were applied to Sep-Pak cartridges (C18 Waters) in the following way. 2 ml of acetonitrile (Sigma) was applied to the cartridge. Following the passage of the acetonitrile through the cartridge 1-ml of 0.4% trifluoroacetic acid (Sigma) was added to prime the cartridge. After this had run through the sample was loaded onto the cartridge. Elution was performed used 2 ml aliquots of step wise increases of acetonitrile in the proportions: - 25%, 50%, 75%, and 100%, all acidified with 0.1 ml of 0.4 % trifluoroacetic acid. The four fractions for each sample were then assayed for absorbance at 278 nm to determine in which of the four fractions the protein was eluted. The samples

containing the protein fractions were then vacuum dried using a rotary evaporator to remove the acetonitrile. An antibacterial assay using *S. aureus* and *E. coli* as test organisms was performed on each of the protein fractions to verify antibacterial activity before application of active samples to HPLC.

The above procedures were also performed on the control samples isolated as noted above. A second set of controls were also taken from fractions of the cation exchange chromatography that came from the active sample run but did not contain any significant antibacterial activity, i.e. not from peaks α, β, and δ.

4.3.5 High Pressure Liquid Chromatography (HPLC)

High Pressure Liquid Chromatography (HPLC) was used to purify the haemolymph taken from the Sep-Pak cartridges (4.3.4) so that the product could be identified as a single discrete peak on the HPLC trace thus indicating a pure sample. Work using HPLC techniques was performed using an automated HPLC 1000 system (Spectra Physics) with PC 1000 software. 100µl of the active samples taken after Sep Pak cartridge fractionation were loaded onto an HPLC column (C18 from HPLC Technology) and eluted with a linear gradient of acetonitrile. All of the HPLC work was performed with a flow rate of 1 ml per min. The initial loading of the sample onto the column was performed for 5 minutes with a mobile phase of water acidified with 0.1% trifluoroacetic acid. Elution was brought about by a mobile phase linear gradient of 0-50 % acetonitrile, acidified with 0.1 % trifluoroacetic. This elution was completed in one hour. Throughout the procedure UV absorbance at 254 nm was recorded by the software of the PC 1000 resulting in a trace recording UV absorbance against time for the entire procedure. 1ml fractions were collected continuously throughout the loading and elution phases at one-minute intervals. These samples were then assessed for antibacterial activity using both *S. aureus* and *E. coli* as test organisms as per 4.1.5 above.

Control samples were taken from those having under gone Sep-Pak cartridge fractionation as per 4.3.4 above. These controls came both from the animals that had not been injected with bacteria in 4.3.1 and also from the non-active fractions of

haemolymph from immunised animals isolated in 4.3.3 earlier. All control samples were subjected to the HPLC procedure as outlined above.

4.4 Analysis of Purification

4.4.1 Gel Electrophoresis

Pure samples taken from the HPLC run were analysed using gel electrophoresis in the following way. 100 µl of each sample was prepared for electrophoresis by warming in 200 µl denaturing sample buffer for 20 minutes. The denaturing sample buffer consisted of 2.5 ml 0.5M Tris HCl pH 6.8, 2.0ml Glycerol, 4.0 ml 10% SDS solution, 0.5 ml 0.1% (w/v) Bromophenol Blue, 0.5 ml 2-mercaptoethanol, and was made up to 10ml with deionised water. The Resolving Gel was prepared with the ProSieve Gel Solution (Flow Gen) according to the following formula;-

4.5ml Deionized Water
2.8ml ProSieve 50 gel solution
2.5ml 1.5M Tris-HCl, pH 8.8
0.1ml 10% SDS-solution
0.1ml 10% APS
4.0µl TEMED

The Resolving Gel was prepared by mixing the first five solutions in the above order. Two glass electrophoresis plates were used to run the gel measuring 8cm x 10cm and 6cm x 10cm. These plates were held 1mm apart in a vertical alignment. The 4 µl TEMED (setting agent) was added to the gel mixture and the gel was then poured into the 1mm gap between the two glass plates and allowed to set.

Once the Resolving Gel had been poured and set, the Stacking Gel was prepared according to the formula below. The Stacking Gel was mixed in the same way as the Resolving Gel. The majority of the solutions were mixed together first and the addition of the TEMED was left until everything was prepared so that the gel did not set during the pouring phase.

750µl Deionized Water

100µl ProSieve 50 gel solution

130µl 1M Tris-HCl, pH6.8

10µl 10 % SDS Solution

10µl 10 % APS

1µl TEMED

A plastic 'comb' was inserted on top of the Resolving Gel and the Stacking Gel was poured in around it. This plastic comb provided areas in the gel so that when it was removed there would be individual wells for loading the samples present at the top of the Stacking Gel. The Stacking Gel was then allowed to set. The samples, taken from the active peaks isolated by HPLC in 4.3.5 above, were then loaded into the wells on the Stacking Gel along with a molecular mass marker and 200 volts of electricity applied for up to 2 hours. Once the molecular mass marker had reached the bottom of the resolving gel the current was turned off and the gel stained with coomassie blue overnight. The stained gel was then washed with destain (70% Methanol, 20% Deionized water, 10% SDS) for 2 hours and then examined for signs of banding indicating the presence of a protein. Controls taken from non-active sections of the HPLC run in 4.3.5 above, from the original control (4.3.1) run on the HPLC, and controls taken from the peaks with no antibacterial activity after ion exchange chromatography (4.3.3). These were run on the HPLC and treated in the same way as the active samples for the purpose of Gel Electrophoresis.

4.4.2 Amino Acid Sequencing

The active fractions from the HPLC were sequenced with the help of Dr John Fox of Alta Bioscience at Birmingham University. The HPLC effluent was transferred onto PVDF (Polvinylidene Fluoride) membranes for microsequencing in the following way. The membrane was cut to size and pre wetted in 100% methanol for 3 seconds, distilled water for 3 sec's, equilibrated in CAPS buffer (10mM 3[cyclohexamino]-propane sulphonic acid, 10% methanol pH 11) for 15 minutes. A

5mm x 5mm square of PVDF membrane was then added to each 4ml sample inside a 15ml centrifuge tube. This centrifuge tube was then left on a shaker for 48 hours so that the PVDF membrane would become saturated with protein. Samples and controls were taken in the same manner as was described in 4.4.1 above.

The process of amino acid sequencing involved the use of a PE Biosystems 473A automatic protein sequencer. This instrument utilises the Edman[77] reaction to cleave the N- terminal amino acid from the protein chain. The resulting fraction then undergoes HPLC to identify the amino acid cleaved through this cycle. The machine then repeats the above procedure with the new N-terminal amino acid. In this way a whole peptide can be sequenced. The process is limited in its application as a maximum of 20 amino acids can be sequenced in this way as clarity of results is lost due to increase in background residues.

Fig 25: Cation exchange chromatography of control haemolymph containing no antibacterial activity

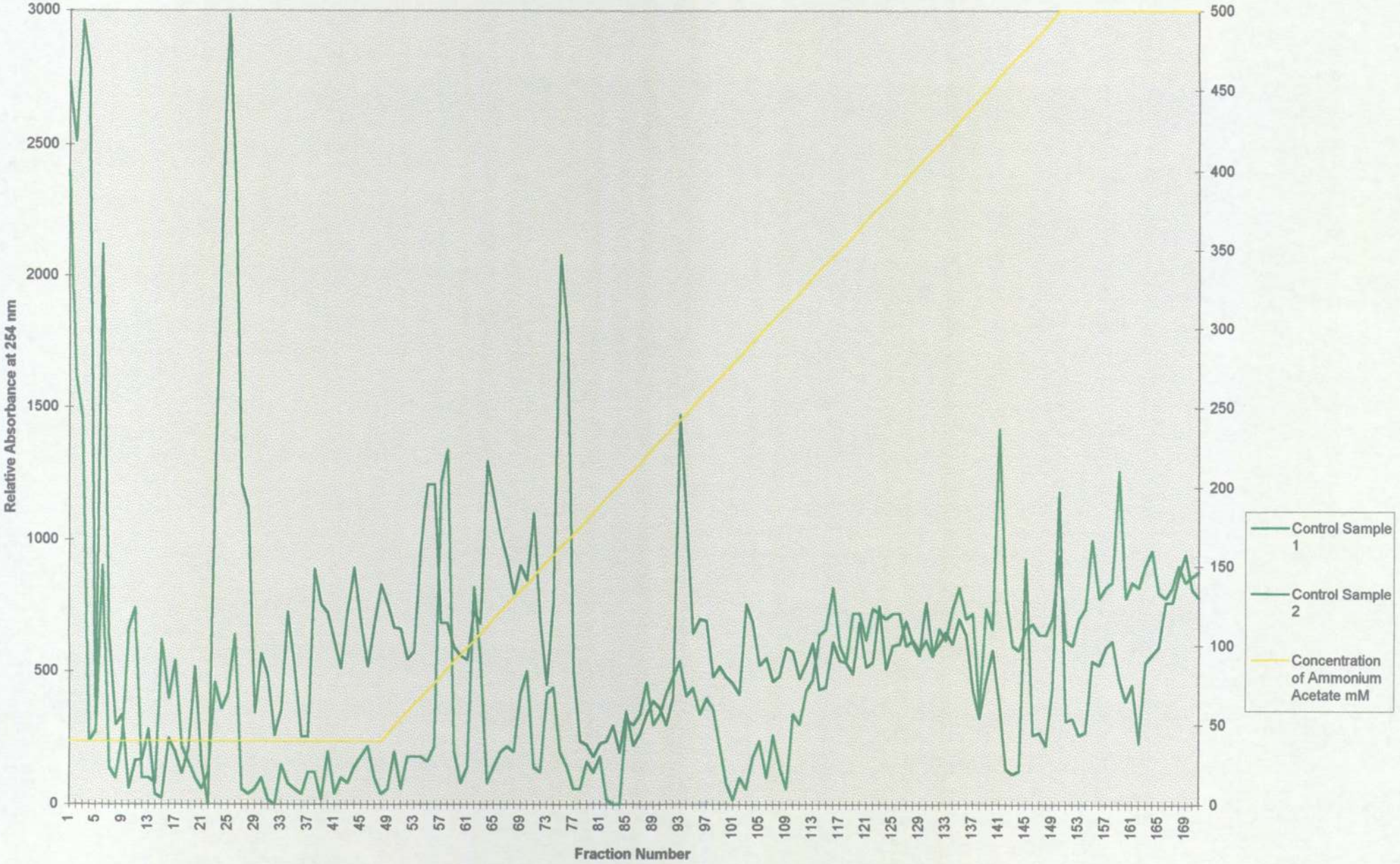


Fig 26: A Comparison of the cation exchange chromatography of sample haemolymph containing antibacterial activity and control haemolymph containing no antibacterial activity

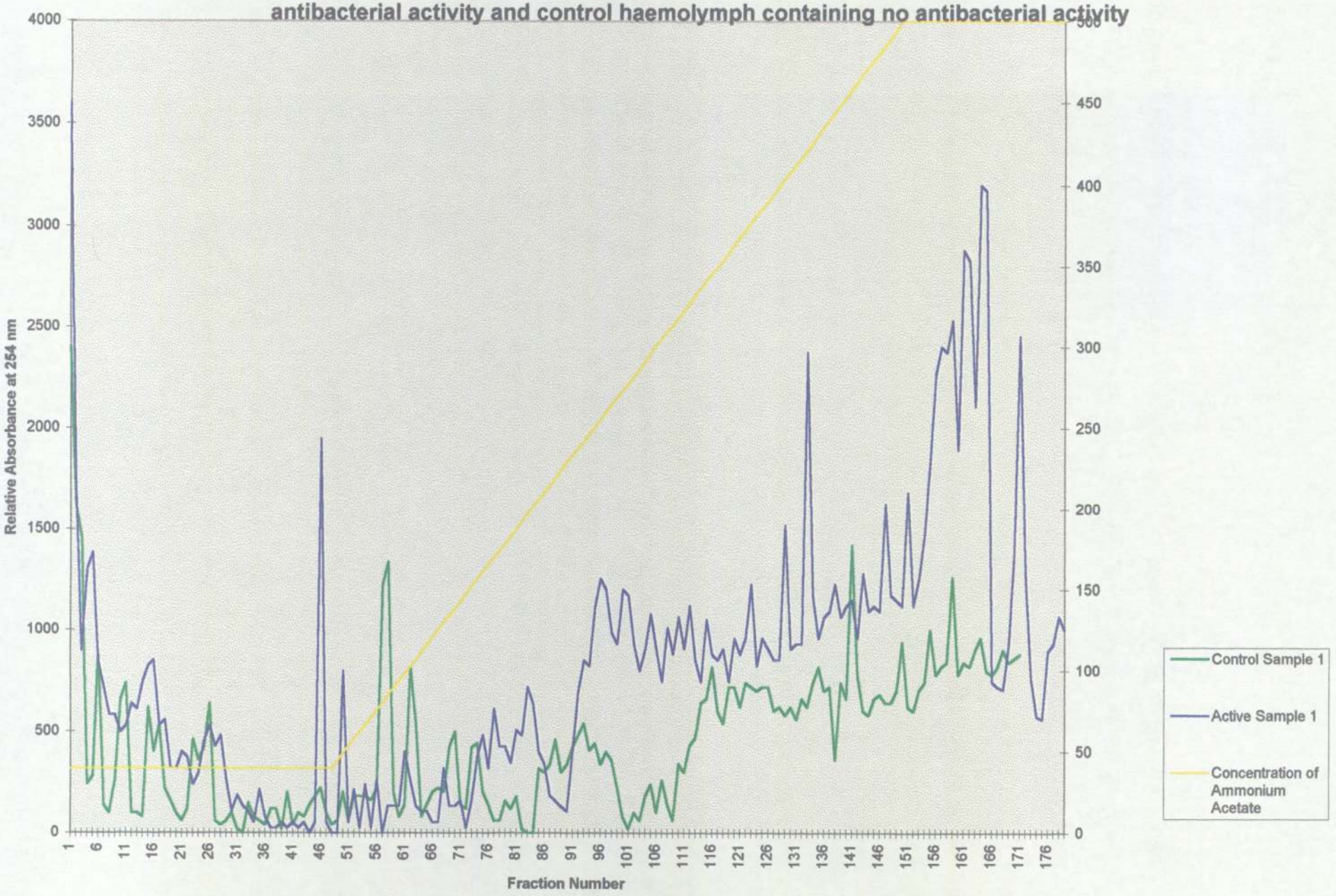


Fig 31: Analysis of protein content of the fractions in peak α after Sep-Pak cartridge fractionation.

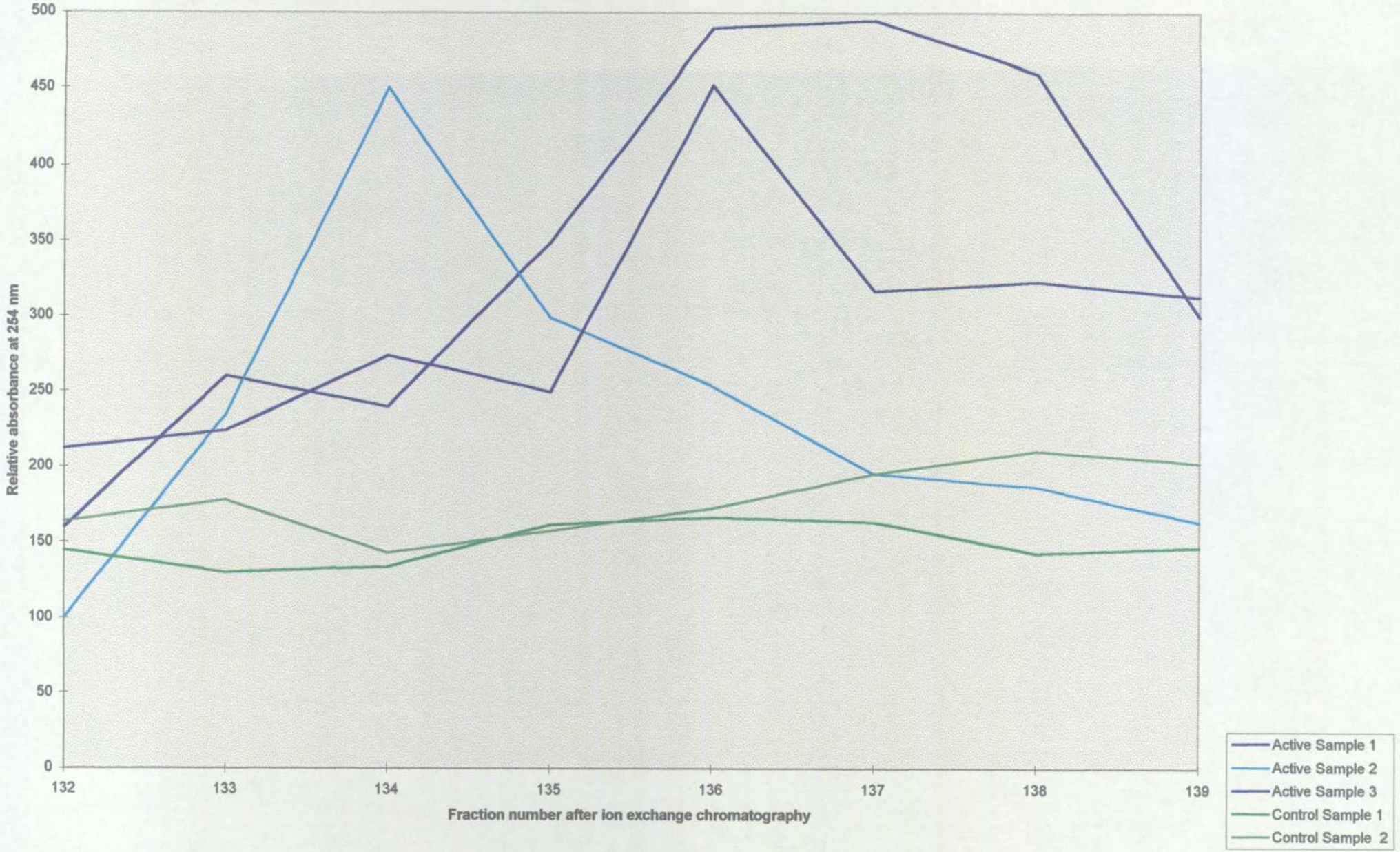


Fig 32: Analysis of the protein content of the fractions in peak β after Sep-Pak cartridge fractionation.

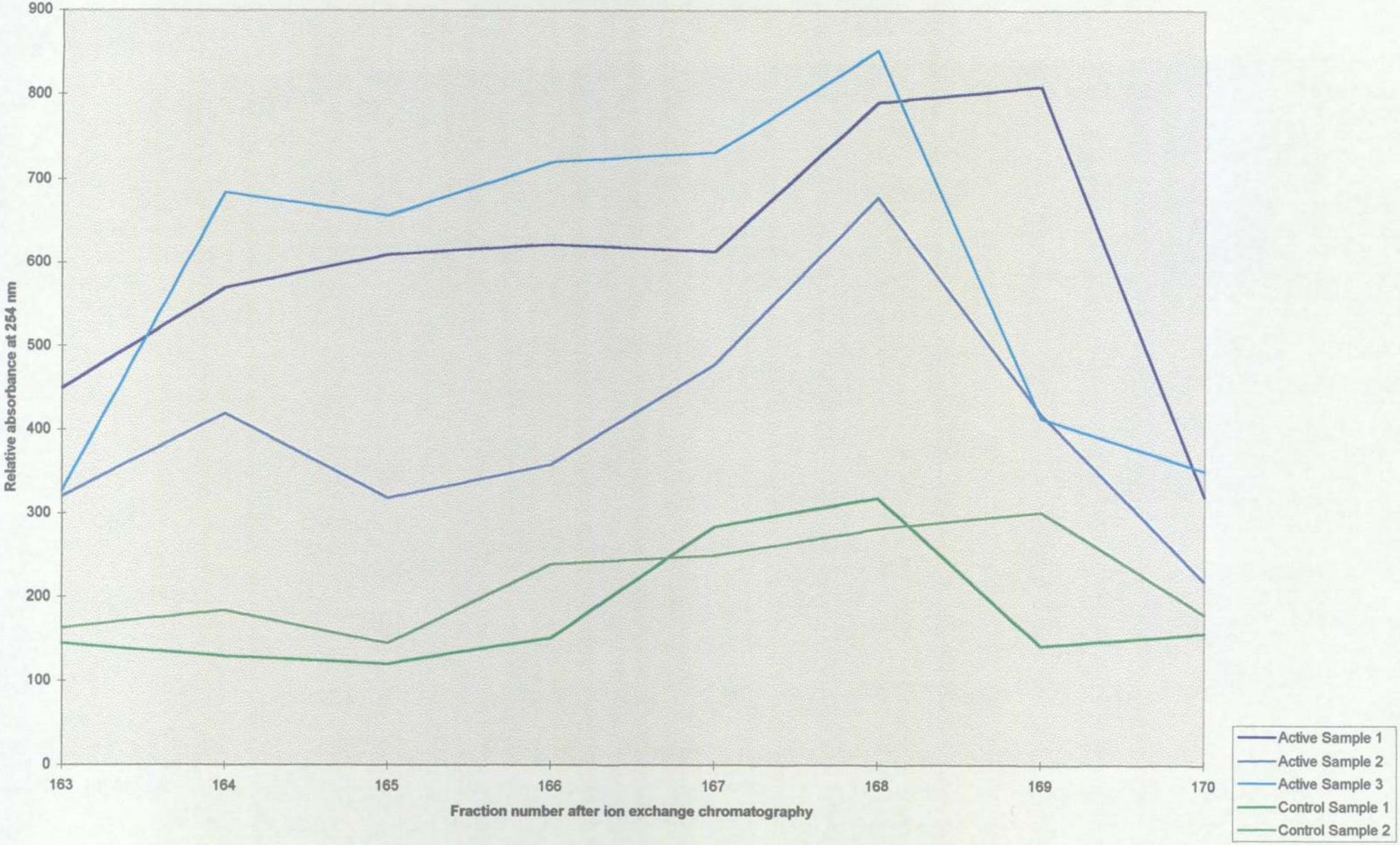
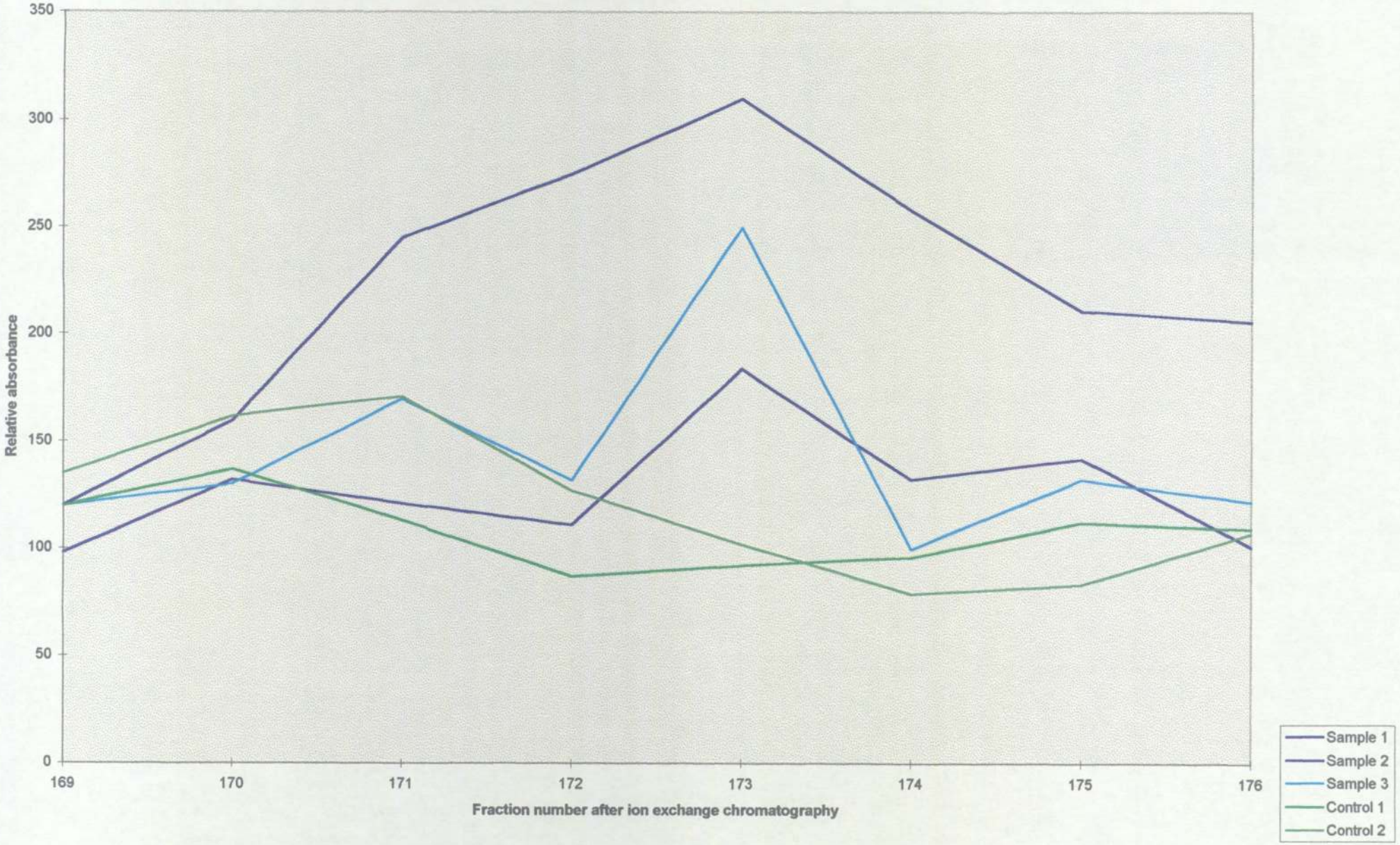


Fig 33: Analysis of the protein content of the fractions of peak δ after Sep-Pak cartridge fractionation.



5.0 Results

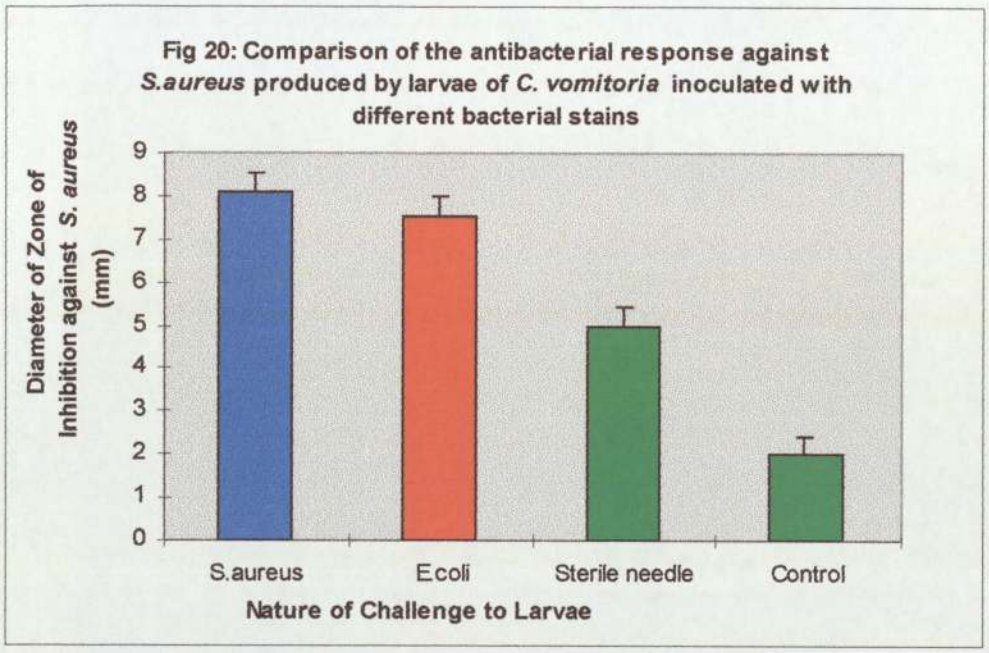
5.1 Kinetics of the Antibacterial activity

As stated in 4.2 above the antibacterial activity of the haemolymph extracted from the larvae of the blow fly *Calliphora vomitoria* was measured by means of growth inhibition assays. These growth inhibition assays (unless otherwise stated) were performed on samples of haemolymph collected 24 hours post inoculation. All assays were performed in triplicate, the results below are the arithmetic mean of the 3 assays.

All zones of inhibition (ZOI) were calculated by taking the mean of two diameters measured at right angles across the centre of the well. The diameter of the well (2mm) is included in all the measurements.

5.1.1 Antibacterial activity against gram-positive and gram-negative bacteria.

The methodology for the results described here can be found in section 4.2.1



+/- SEM, n=9

Fig 20 shows the antibacterial activity against *S. aureus* after challenge by various means.

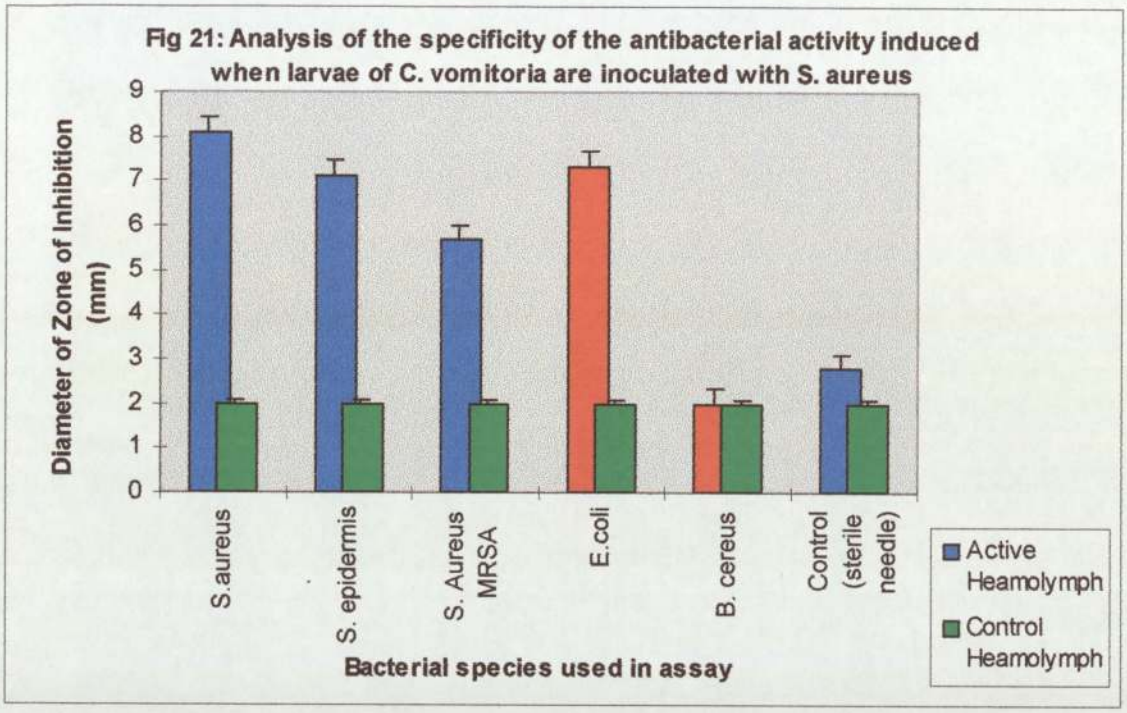
- There is a significant increase in antibacterial activity against *S. aureus* after challenge with either *E. coli* or *S. aureus*. This indicates a lack of specificity in the antibacterial response.

- There is some increase in the antibacterial activity seen against *S. aureus* after injury with a sterile needle, this increase is significantly smaller than that seen following a bacterial challenge.

- There is a small amount of background activity in the control.

5.1.2 Specificity of the antibacterial activity against gram-positive and gram-negative bacteria

The methodology for the results below can be found in section 4.2.2 above



+/- SEM, n=9

Fig 21 shows antibacterial activity against a range of bacteria following challenge with *S. aureus*.

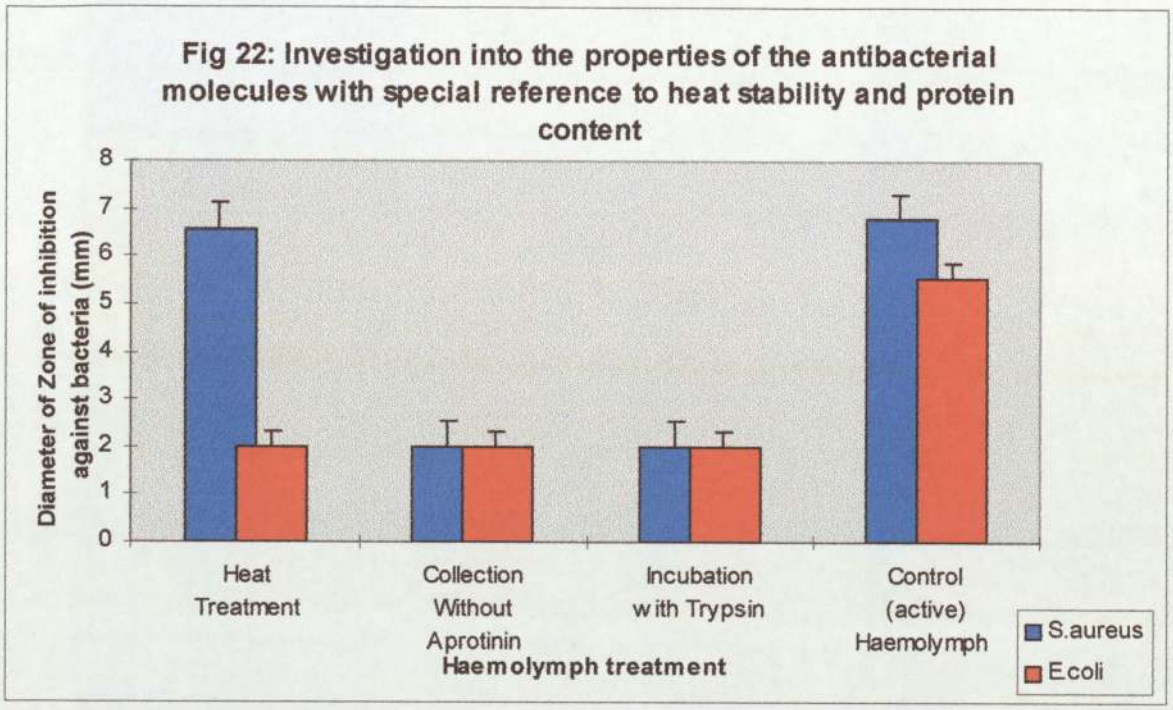
- There is a significant increase in the antibacterial activity against all of the bacterial strains except *B. cereus*.

- Although there is a lesser response seen against the MRSA strain of *S. aureus* the result is still significantly greater than that seen in the control.

- Whilst *S. aureus* is a gram positive strain the antibacterial response elicited does not appear to distinguish between gram positive (blue) and gram negative (red) bacteria.

5.1.3 Heat stability of and protease activity on the antibacterial molecules

The methodology for the results below can be found in section 4.2.3 above



+/-SEM, n=9

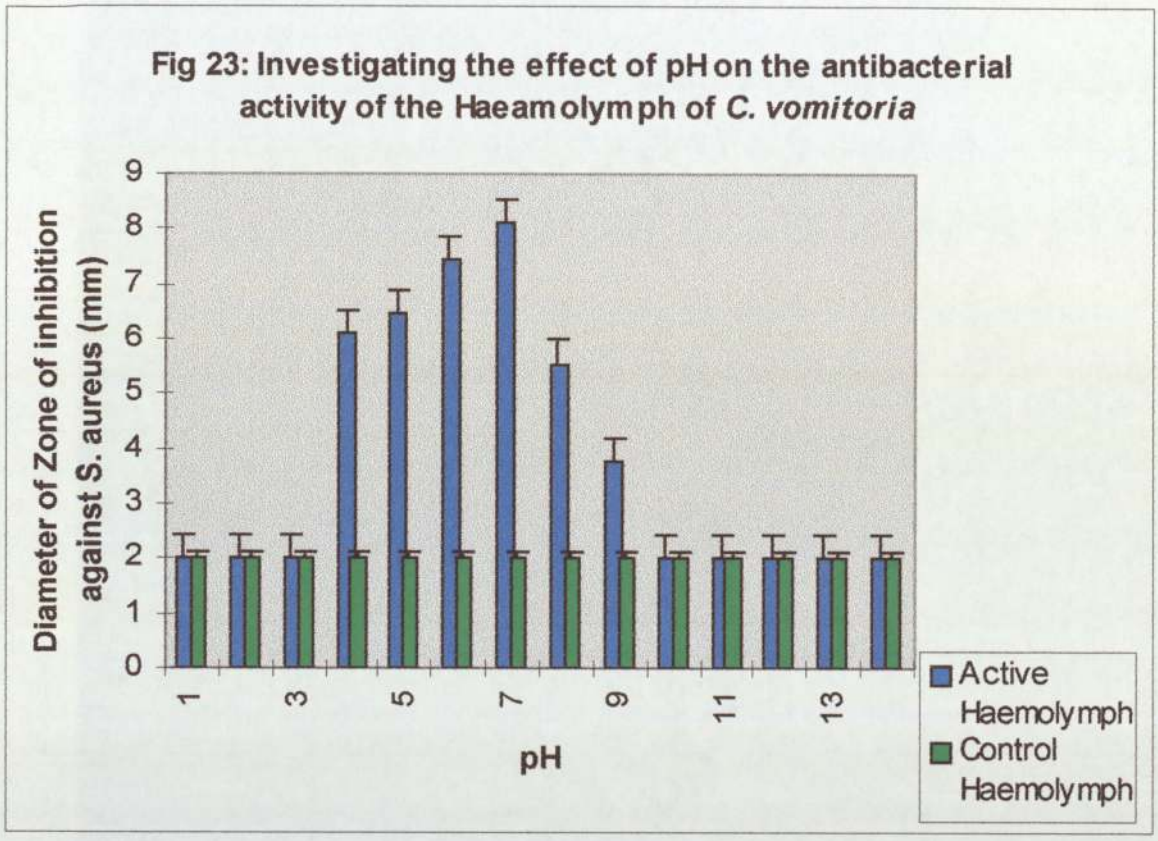
Fig 22 shows the effects of various treatments on the antibacterial activity of haemolymph collected from animals challenged with *S. aureus*.

- The antibacterial activity against *S. aureus* was not affected by heat treatment, however incubation with trypsin or collection without aprotinin significantly reduced antibacterial activity.

- Antibacterial activity against *E. coli* was significantly reduced following heat treatment, incubation with trypsin, or collection without aprotinin.

5.1.4 Effects of pH on the stability of the antibacterial molecules

The methodology for the results below can be found in section 4.2.3.3 above



+/- SEM, n=9

Fig 23 shows the effect of varying the pH on the antibacterial activity produced after inoculation with *S. aureus*.

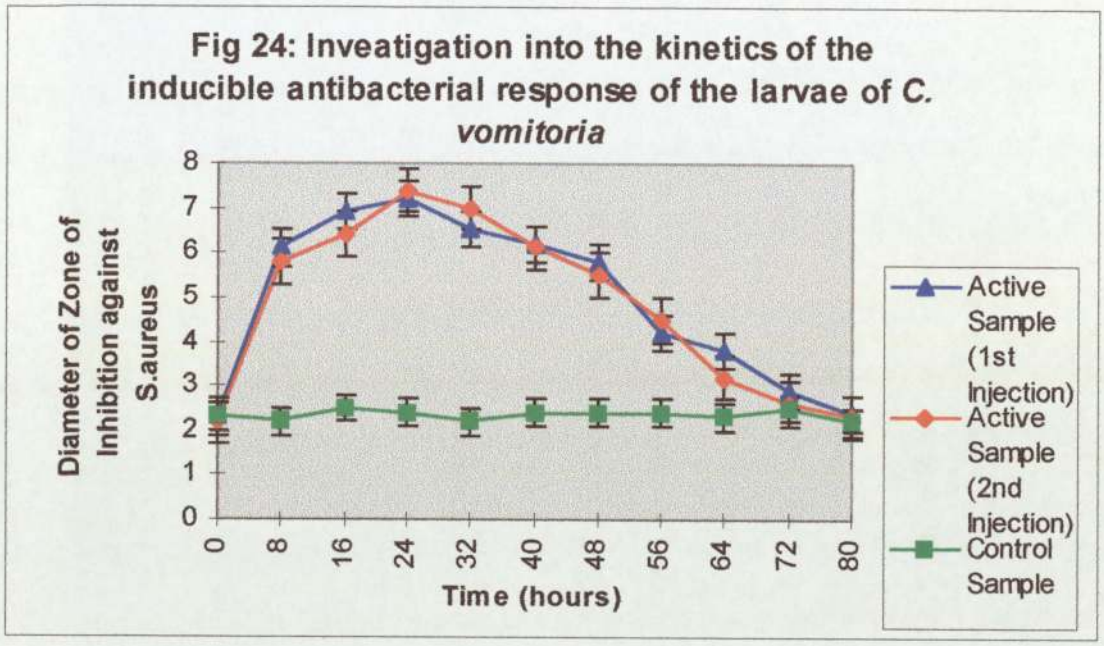
- There is no significant antibacterial activity at the extremes of both the acidic and basic pH scales.

- There is no sign of inhibition of antibacterial activity against *S. aureus* between pH 4 and 8.

- The optimum pH is pH 7

5.1.5 Investigating the kinetics of anti-bacterial response

The methodology for the results below can be found in section 4.2.4 above



+/- SEM, n=9

Fig 24 shows the level of antibacterial response over time for the larvae of *C. vomitoria* following inoculation with *S. aureus*. Fig 24 also compares the level of antibacterial response after injection of bacteria once or twice, thereby investigating if there is an immunological memory. Both samples are plotted on the same scale with time 0 being the time of the last injection.

- There is no significant antibacterial activity present before inoculation.

- Antibacterial activity rises to a peak, reaching an optimum 24 hours post inoculation.

- Antibacterial activity declines gradually over a period of 72 hours post inoculation.

- No significant difference between the antibacterial response of larvae injected with bacteria once or twice.

5.2 Purification of the antibacterial molecules

The haemolymph of the larvae containing inducible antibacterial activity was subjected to procedures to isolate the molecules producing the antibacterial activity. These procedures are described in detail in section 4.3 above.

5.2.1 Cation exchange chromatography

Haemoglobin was passed through the cation exchange column as a coloured marker to indicate flow rate, and the pump was calibrated so that the flow could be adjusted to the experimental level of 1 ml per min. Non specific binding sites were blocked with albumen. Due to the nature of the experiments, and to ensure the repetition of the results, haemolymph was collect from three different populations of larvae that had been inoculated with *S. aureus* using the methodology described in section 4.3.1 above. Samples of haemolymph having antibacterial activity were then labelled sample 1, 2 and 3. Two separate volumes of control haemolymph were collected by the same method (see 4.3.1) using animals that had not been inoculated. These volumes of haemolymph were subsequently labelled control 1 and 2. The samples and the controls were passed through the column and 15 ml fractions were collected, a detailed description of the methodology can be found in section 4.3.3 above. These fractions had their absorbance at 278 nm recorded, and were assayed for anti-bacterial activity against both E. coli and S.aureus.

Figs 25 - 30 show the absorbance trace and antibacterial activity for the two controls and the three samples.

Fig 25: Cation exchange chromatography of control haemolymph containing no antibacterial activity

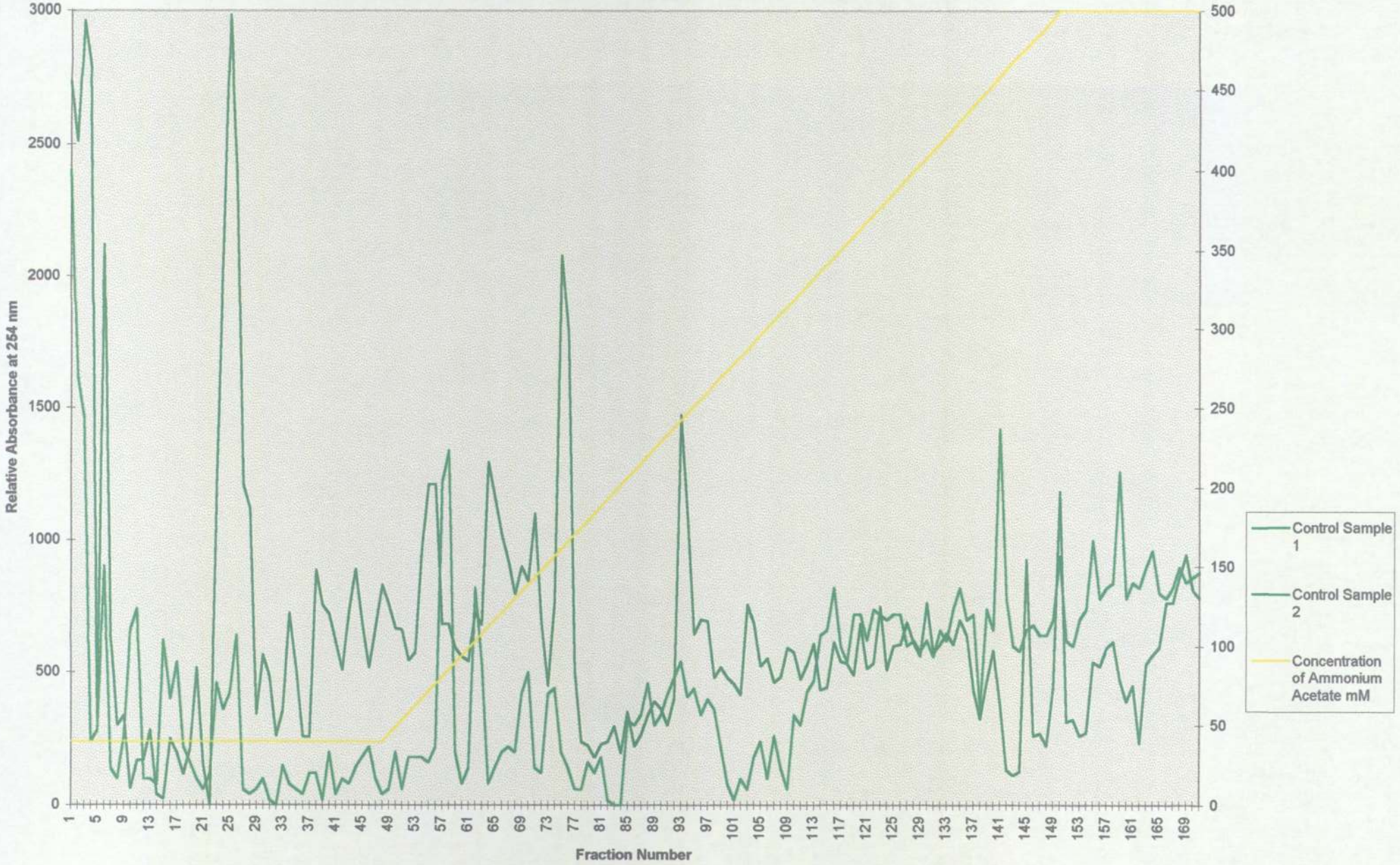


Fig 25

- In control haemolymph there is a large amount of protein (fractions 0-50) that has very little cationic charge therefore does not readily bind to the column.

- There are large peaks of protein concentration early in the ammonium acetate gradient (fractions 57-74) indicating that these proteins are weakly cationic.

- There are no large peaks of protein concentration at the higher concentrations of ammonium acetate indicating a lack of strongly cationic proteins.

- No antibacterial activity was observed in either of the control samples

Fig 26: A Comparison of the cation exchange chromatography of sample haemolymph containing antibacterial activity and control haemolymph containing no antibacterial activity

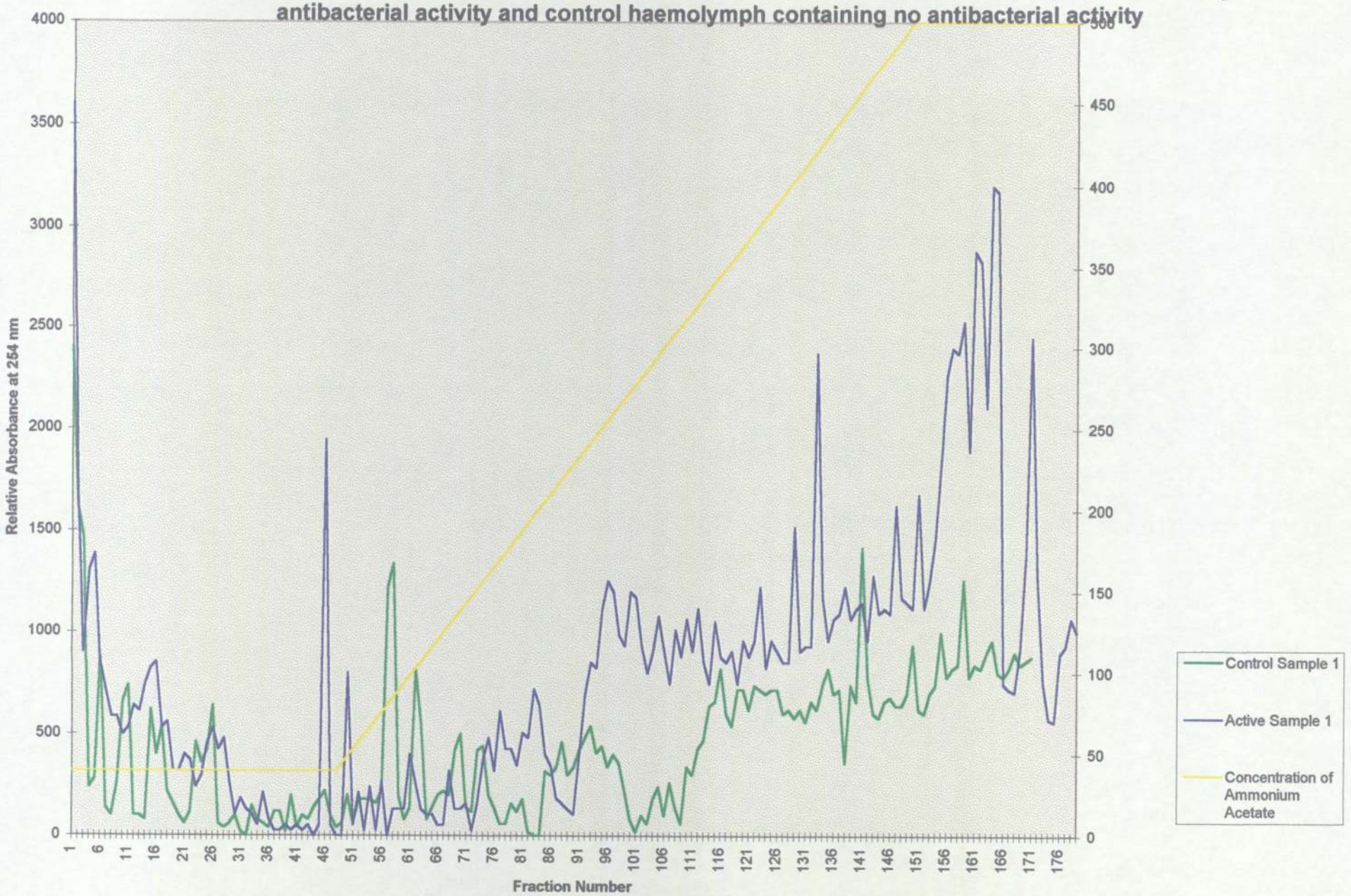


Fig 26

- Both active sample and control sample haemolymph eluted a large amount of non-cationic protein at the beginning of the chromatographic run (fractions 1-25).

- At the start of the ammonium acetate gradient both control and active samples have similar peaks that may well be identical the difference due to variations in the columns.

- There is a significantly higher amount of cationic protein eluted from the active sample than the control. It can be seen that there are three clear peaks of cation protein eluted from the active sample (factions 133, 156-64, and 171) that have no counterpart in the control sample. It will subsequently be shown that it is in these three peaks that the majority of the antibacterial activity was seen (figs 28-30).

Fig 27: Cation exchange chromatography comparing the three active samples of haemolymph inoculated with *S. aureus* and a control sample uninoculated.

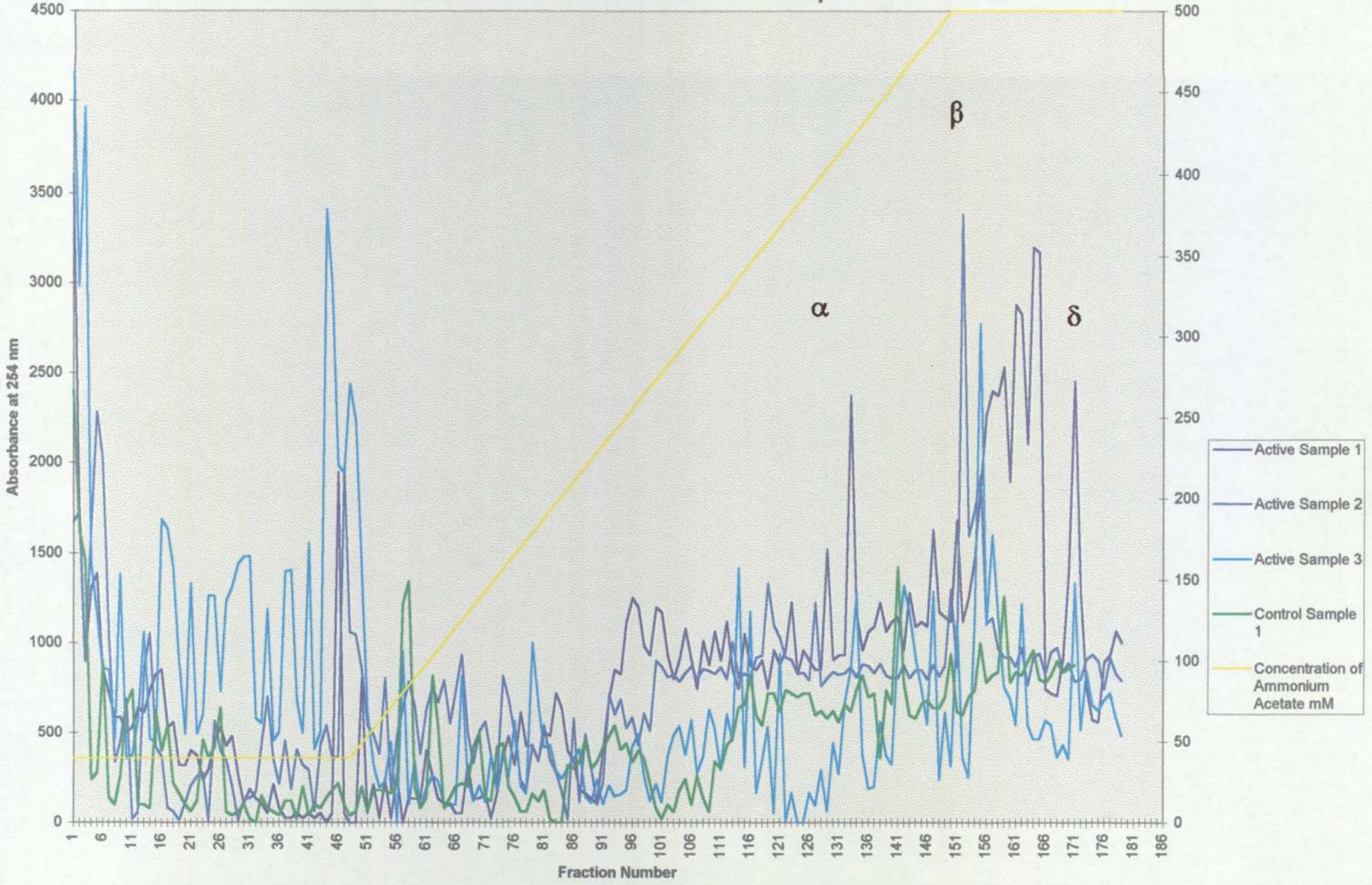


Fig 27

• There is a substantial amount of non-cationic protein eluted from all of the active samples and the control at the start of the chromatographic run (fractions 1-50)

• There is a large amount of cationic protein eluted from the column at fraction number 133 for both active samples 1 and 3, these fractions have been labelled peak α.

•All three active samples show elution of cationic protein between fractions 155-165, while no large concentration of cationic protein can be seen in the control. These fractions have been labelled peak β.

• There is a final peak of cationic protein eluted in fraction 171 in both active samples 1 and 3. There is no rise in cationic protein concentration eluted fro the column in this fraction in either the control or active sample 2. These fractions have been labelled peak δ.

Fig 28: Cation exchange chromatography for active sample 1 of haemolymph inoculated with *S. aureus.*

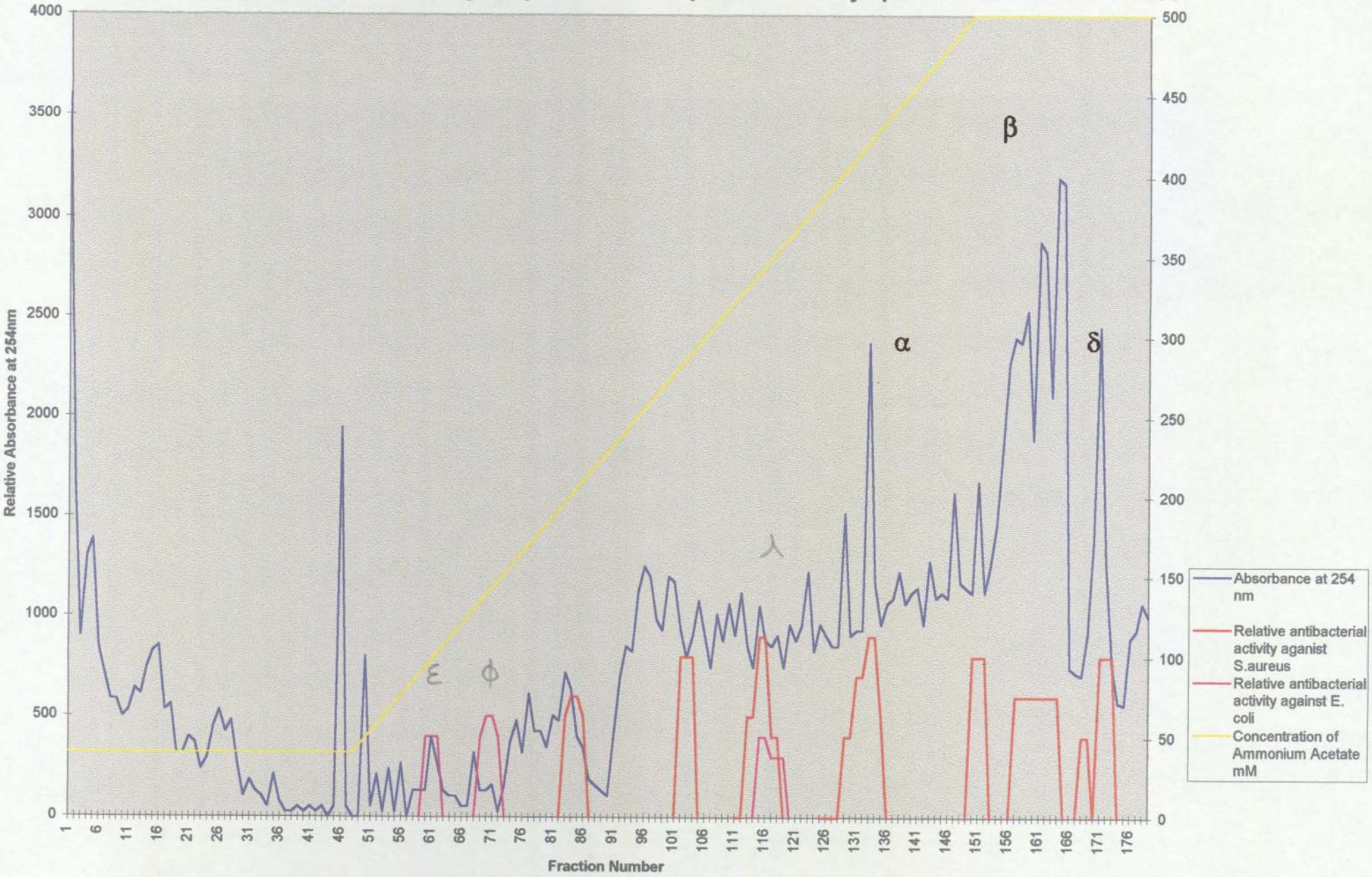


Fig 28

- Antibacterial activity against *E. coli* alone is apparent in fractions 62-3 and 71-2 (labelled ε and ϕ). As there is a low concentration of protein present in these fractions in can be concluded that the antibacterial activity seen here is quite potent.

- Antibacterial activity against both E. coli and S. aureus is present in fractions 113-119 (labelled λ). There is also a substantial concentration of protein eluted in these fractions.

- Strong antibacterial activity against S. aureus can only be observed in the high protein concentration peaks α, β, and δ.

Fig 29: Cation Exchange Chromatography for active sample 2 of haemolymph inoculated with *S. aureus*.

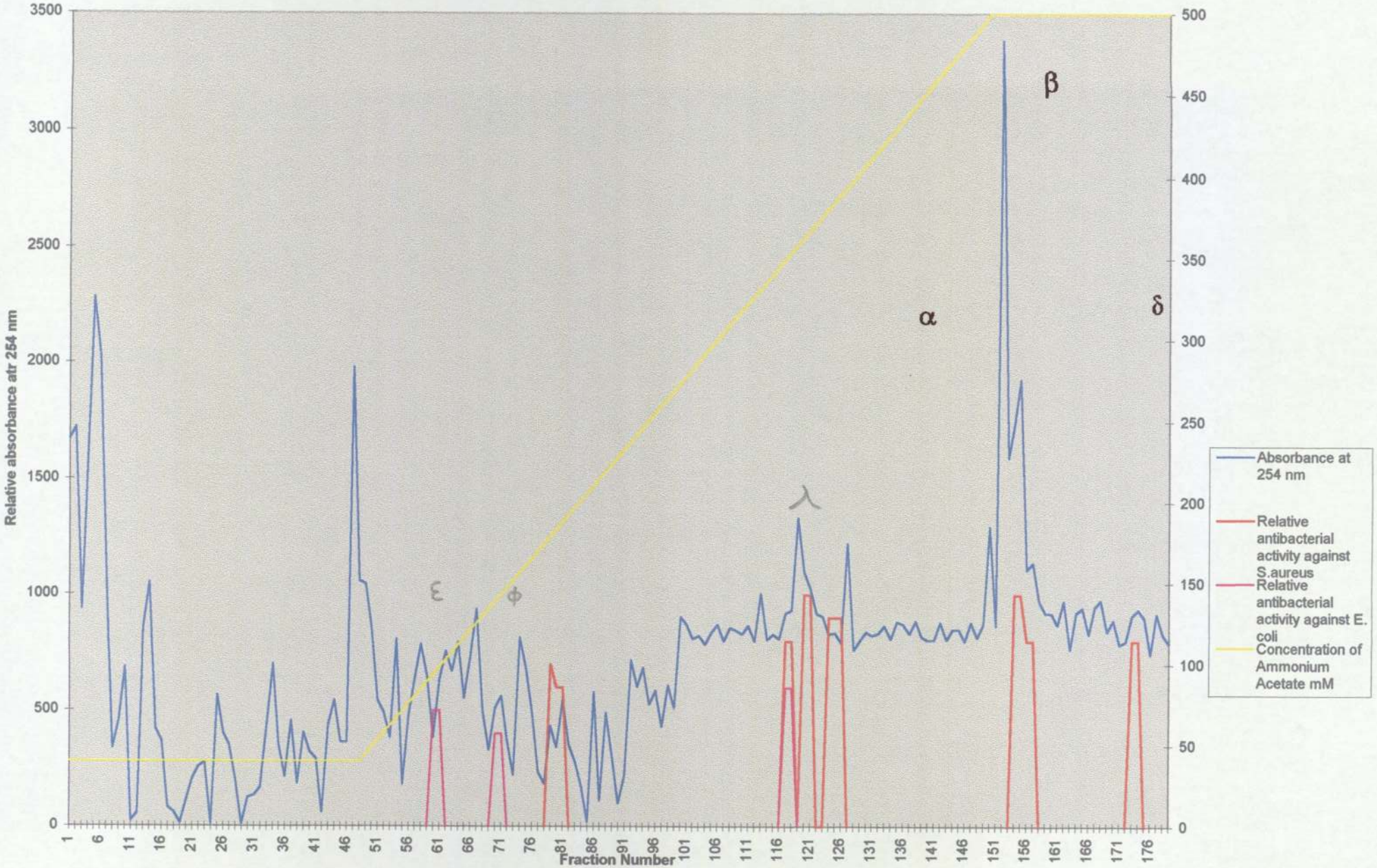


Fig 29

• There is antibacterial activity against E. coli only in fractions 60-1 and 70-1 (labelled ε and ϕ). This antibacterial activity is of a smaller magnitude to that against S. aureus and occurs in areas of low protein concentration.

• Antibacterial activity against both E. coli and S. aureus can be seen in fractions 116-27 (labelled λ) which also have high protein concentration.

• No antibacterial activity can be seen at fraction 133 where peak α is present in active samples 1 and 3 (figs 28 and 30).

• Antibacterial activity against S.aureus only can be seen in fractions 154-8, occuring in the peaks of protein concentration labelled β.

• Antibacterial activity against S. aureus only can be seen in fractions 172-5 (peak δ), there is however little increase in protein concentration in this active sample.

Fig 30: Cation Exchange Chromatography for active sample 3 of haemolymph inoculated with *S. aureus.*

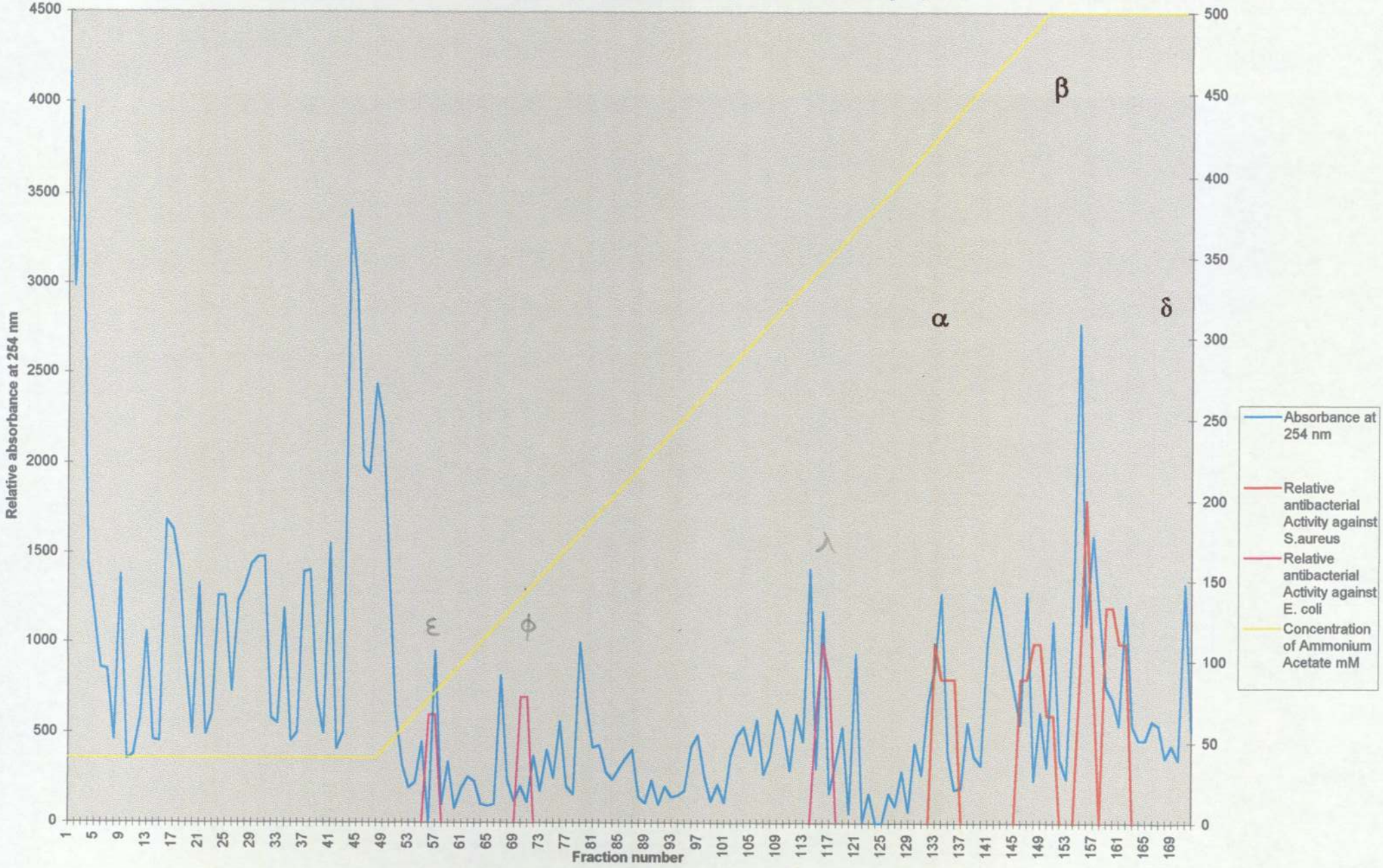


Fig 30

• There is antibacterial activity against E. coli only in fractions 55-61 and 71-4(labelled ε and ϕ). This antibacterial activity is of small magnitude compared to that against S. aureus and occurs in areas of low protein concentration.

• There is antibacterial activity against E. coli only in fractions 114-125 (labelled λ). This differs from the other two active samples where antibacterial activity was seen against S. aureus as well as E. coli in these fractions (see figs 28 and 29).

• Strong antibacterial activity against S. aureus only can be observed in the high protein concentration peaks α, β, and δ.

5.2.2 Sep-Pak Cartridge application

The 15ml fractions making up the three major peaks of anti-bacterial activity (the peaks labelled α, β, and δ) observed after cation exchange chromatography were individually applied to Sep-Pak cartridges, a detailed description of the methodology can be found in section 4.3.4 above. Four effluents were collected for each fraction and assessed for UV absorbance at 278 nm. The 25 % acetonitrile effluent was seen to contain the protein eluted from the cartridges and so the following profiles of the anti-bacterial peaks were compiled using this data.

See figs 31-33

Fig 31: Analysis of protein content of the fractions in peak α after Sep-Pak cartridge fractionation.

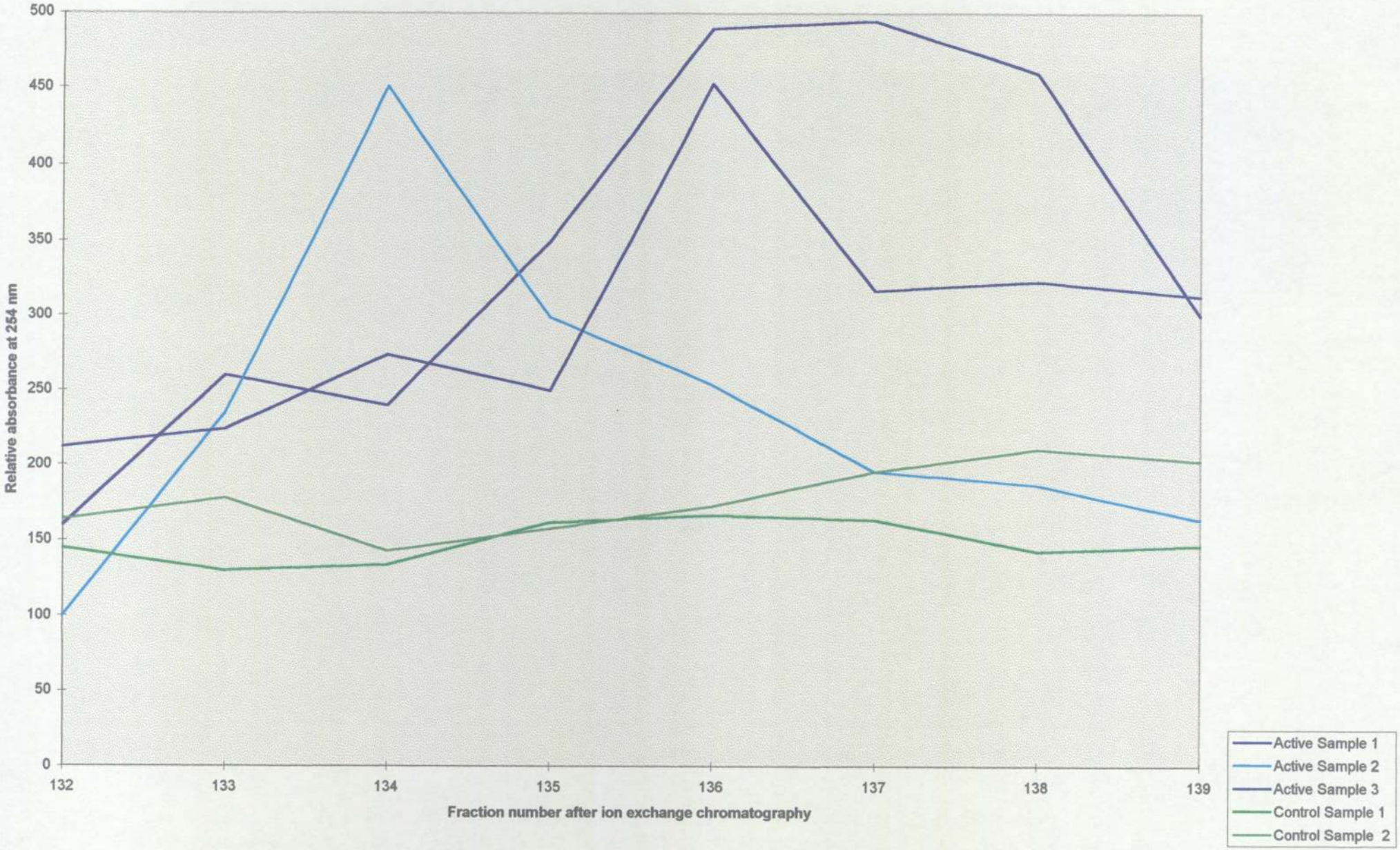


Fig 31

- All of the active samples reach a maximum relative absorbance of between 400 and 450

- Both the control samples stay constantly around 150 relative absorbance.

Fig 32: Analysis of the protein content of the fractions in peak β after Sep-Pak cartridge fractionation.

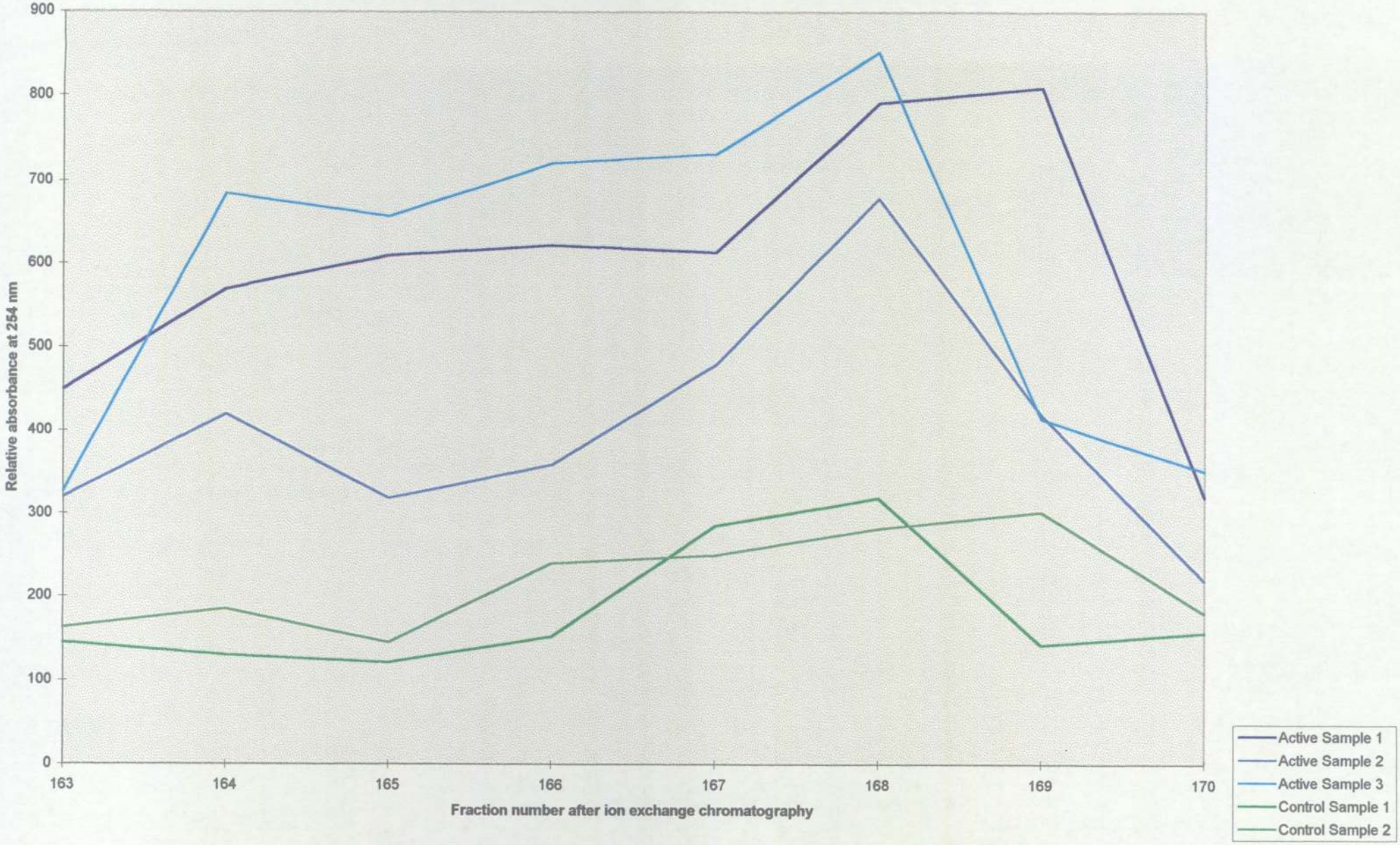


Fig 32

- All of the active samples reach a maximum relative absorbance of greater than 700. This indicates a relatively high concentration of protein present in these samples.

- Both the control sample traces remain constantly around 200 relative absorbance. This indicates that there is a relatively low concentration of protein present in these samples.

Fig 33: Analysis of the protein content of the fractions of peak δ after Sep-Pak cartridge fractionation.

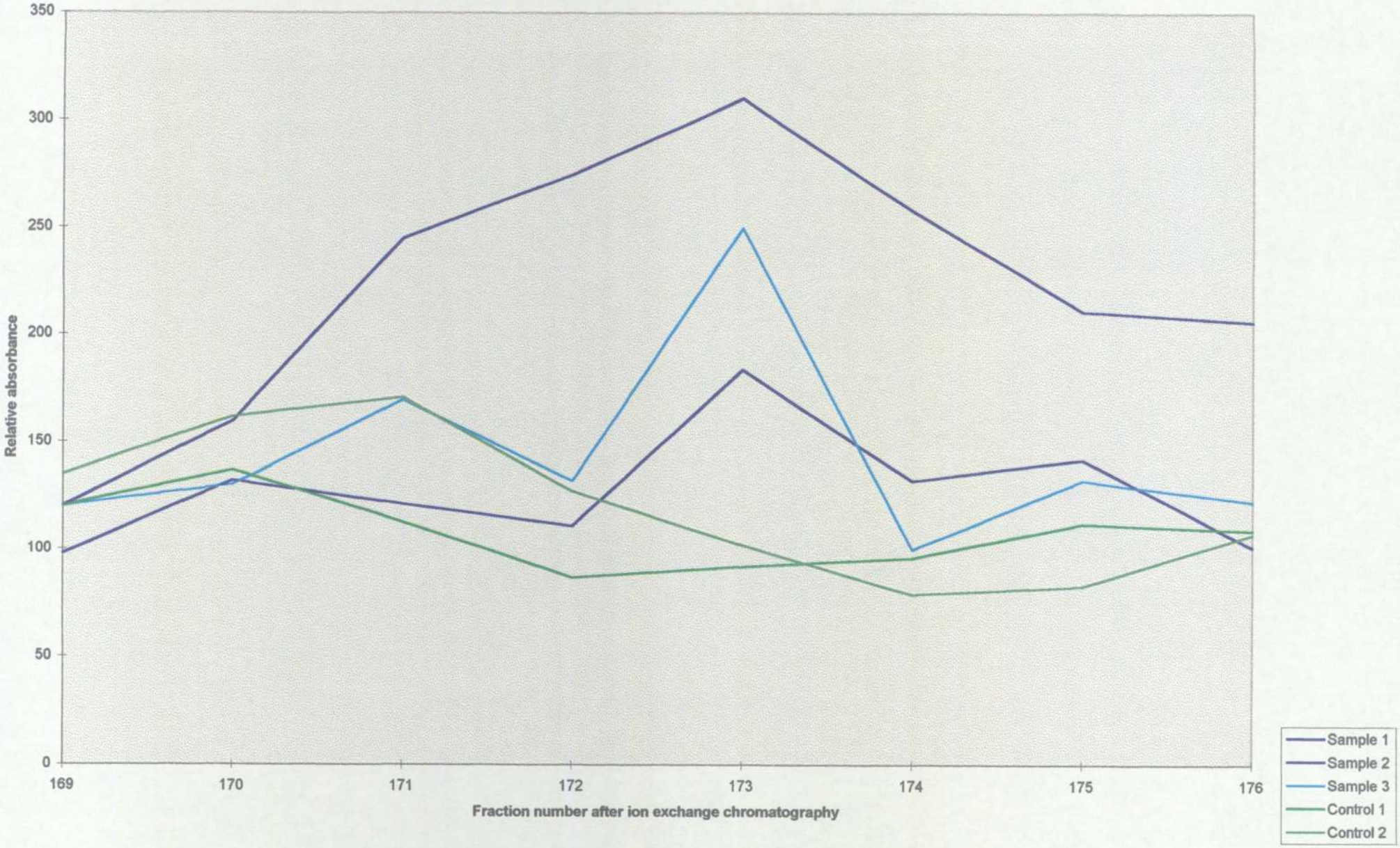


Fig 33

- Only active sample 1 shows a high relative absorbance compared to the other active samples and control samples.

- Active sample 2 has a relative absorbance that reaches 175 indicating a low relative concentration of protein.

- Active sample 3 has a relative absorbance that reaches 250 indicating a relatively small amount of protein present in this sample.

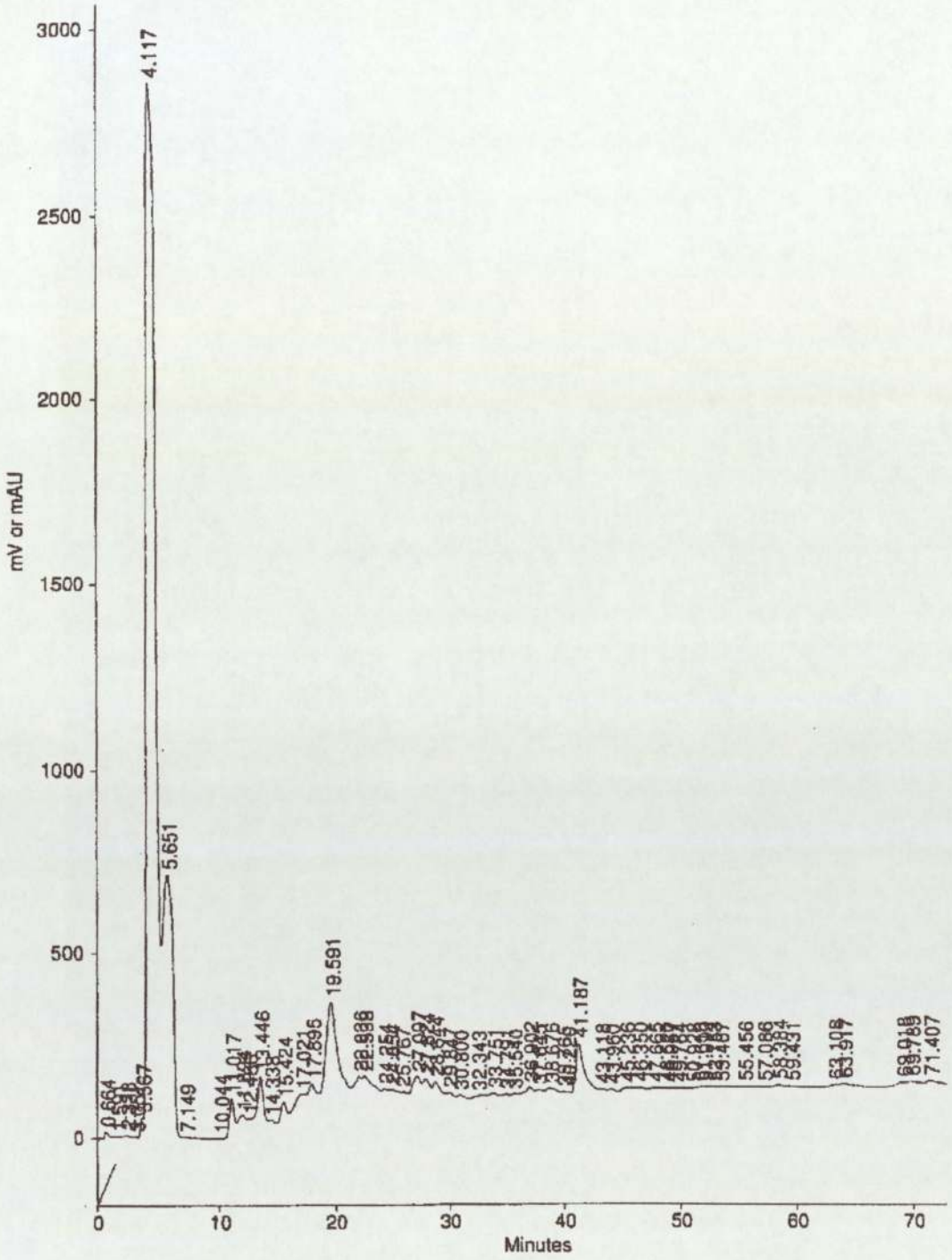
- Both the control samples remain constantly around 150 relative absorbance.

5.2.3 High Pressure Liquid Chromatography Results

Again the following narrative and diagrams will use the nomenclature outlined in 5.2.1 above in that the three volumes of haemolymph showing antibacterial activity will be labelled sample 1, 2, and 3, and the two volumes of haemolymph possessing no antibacterial activity will be labelled control 1 and 2. Also the major peaks of antibacterial activity observed after cation exchange chromatography will continue to be labelled α, β, and δ as noted in 5.2.2 above.

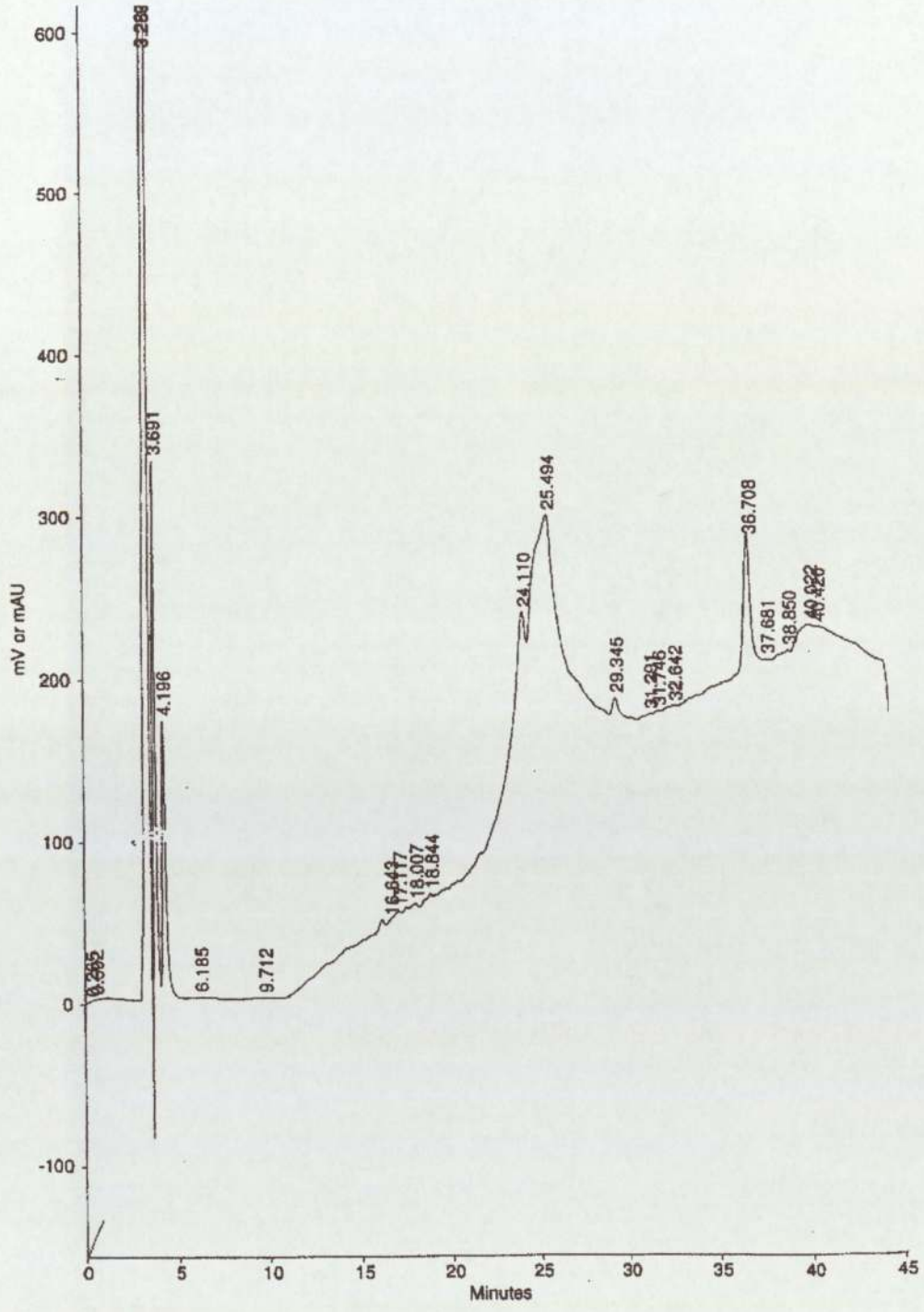
The following diagrams are traces from the PC 2000 software used to run the HPLC equipment used for these experiments. The methodology leading to these results can be found in section 4.3.5 above. All of the following diagrams show absorbance at 254nm against time for the following set of conditions.

Fig 34: HPLC Trace showing absorbance at 210nm against time for control sample 1, fraction 133 (from ion exchange chromatography). Note artificially high TFA level. This fraction is a control for the protein content seen in Peak α.



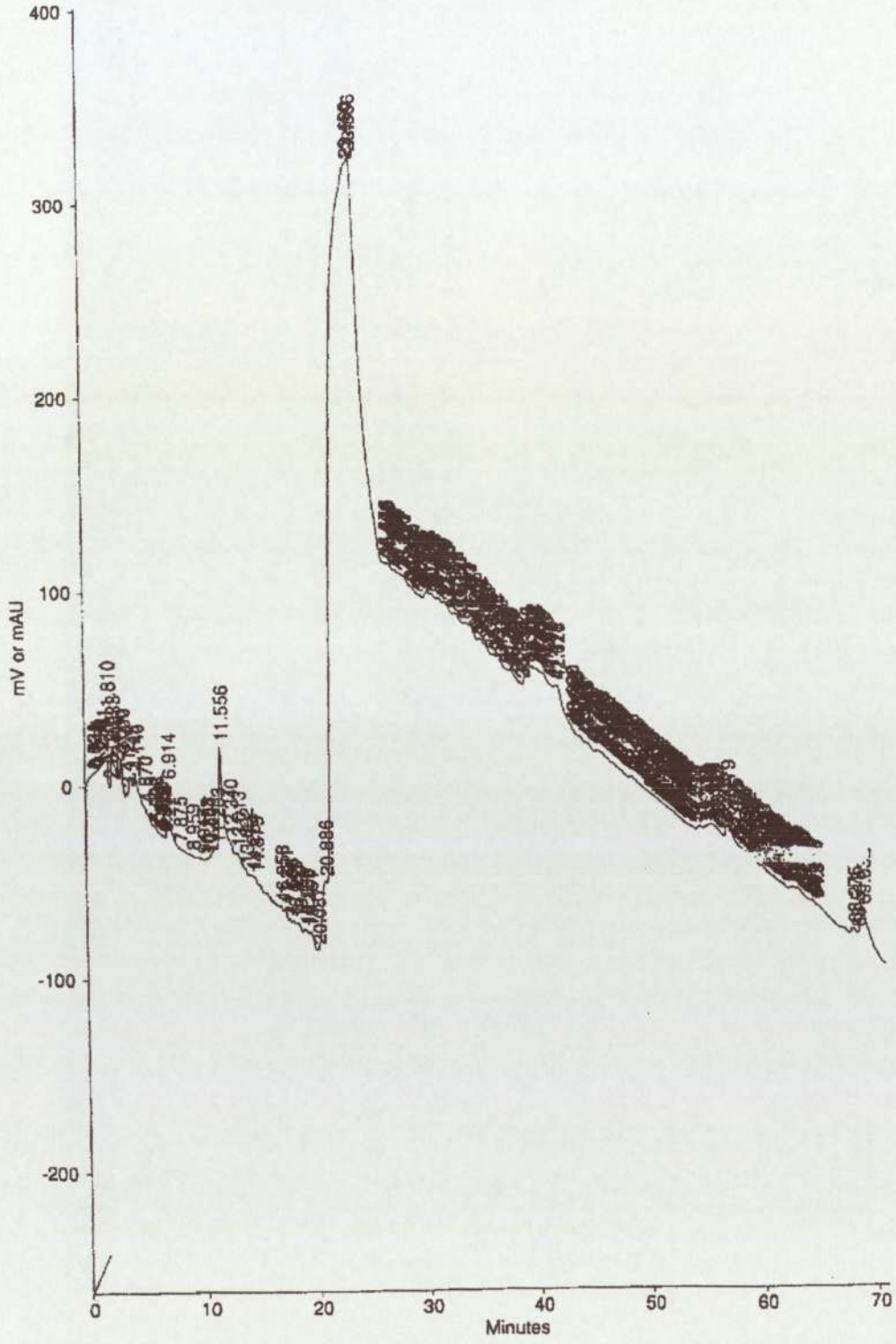
- Large TFA peak
- Small protein peak after 19 mins (about 400 mV)
- Small protein peak after 41 mins (about 300 mV)

Fig 35: HPLC Trace showing absorbance at 210nm against time for control sample 2, fraction 157 (from ion exchange chromatography).
This fraction is a control for Peak β



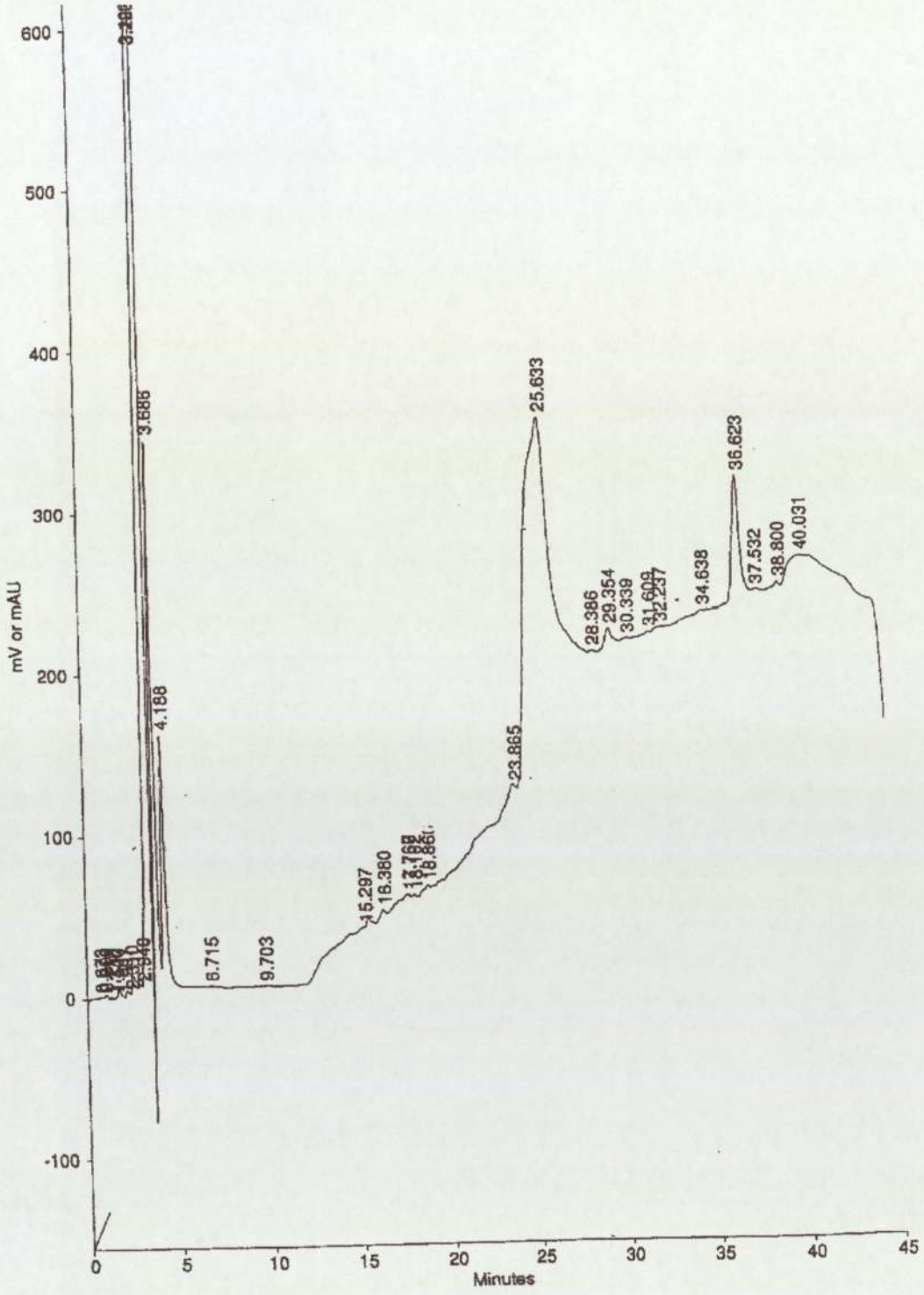
- Large TFA peak
- Small protein peak after 25 mins (about 300 mV)
- Small protein peak after 36 mins (about 300 mV)

Fig 36: HPLC Trace showing absorbance at 210nm against time for active sample 1, fraction 133 (from ion exchange chromatography).
This fraction is taken from peak α.



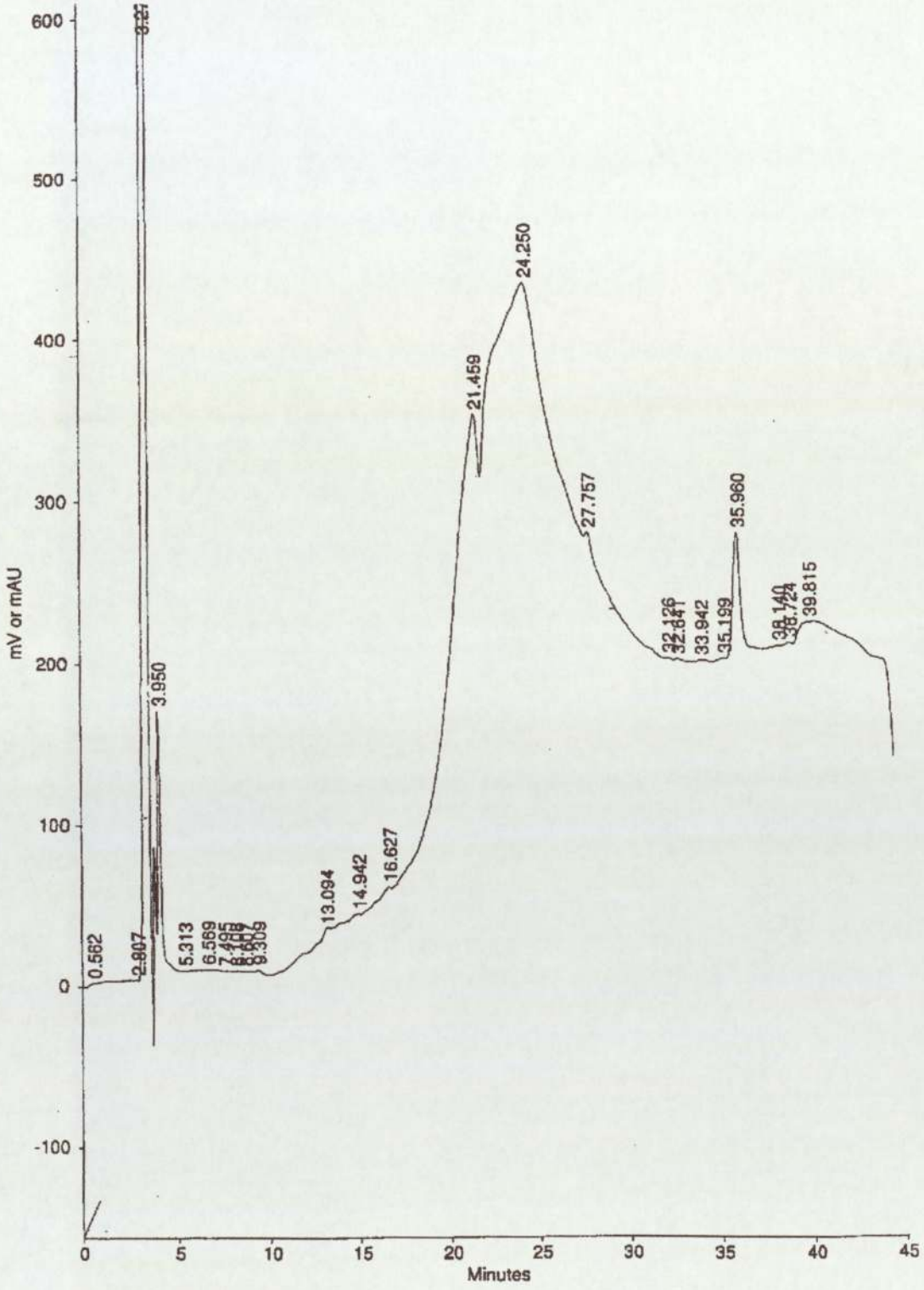
- Weak protein peak after 23 mins (about 350 mV)
- Very weak protein peak after 42 mins (about 120 mV)

Fig 37: HPLC Trace showing absorbance at 210nm against time for active sample 1, fraction 133 (from ion exchange chromatography)
This fraction is taken from peak α.



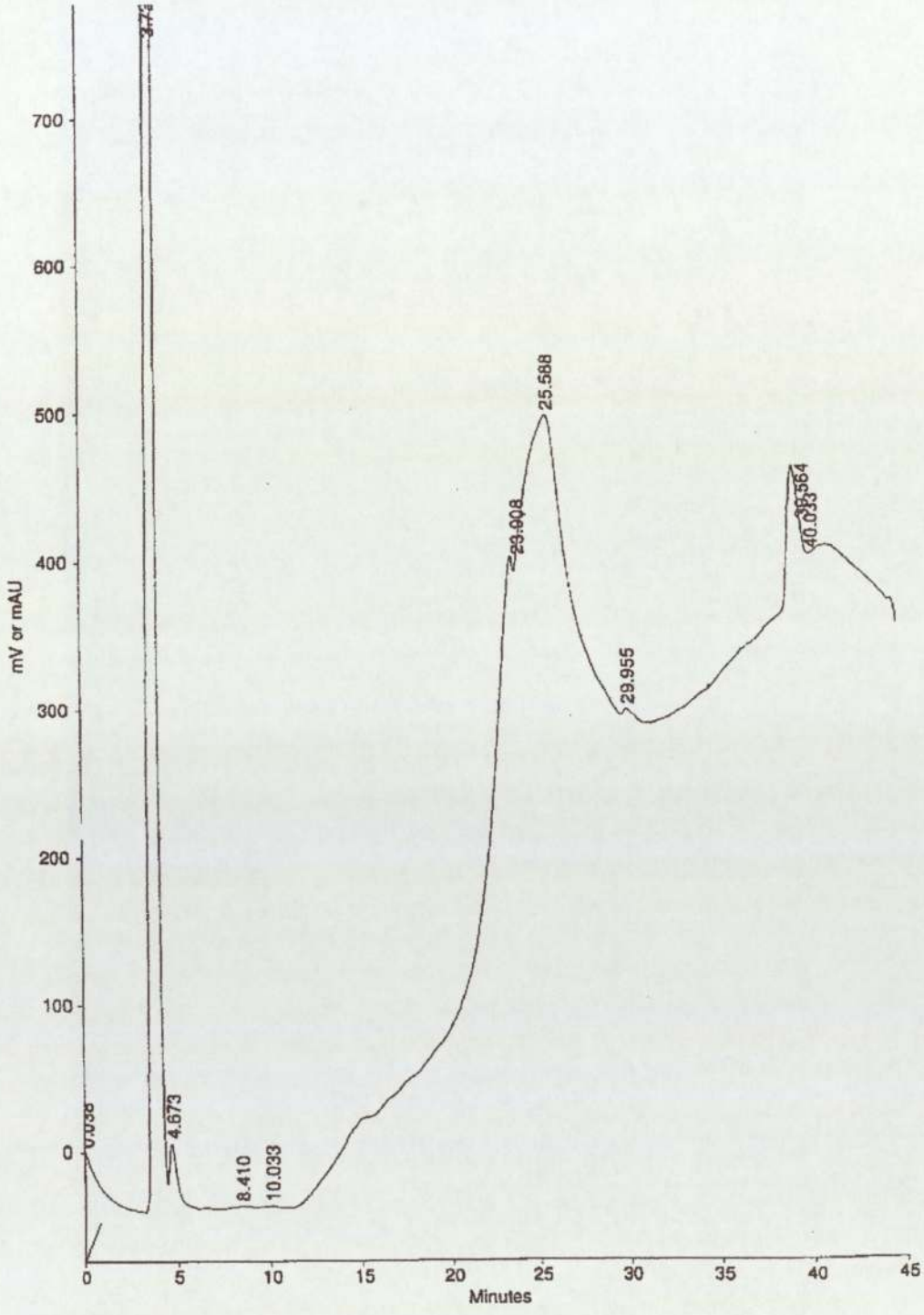
- Strong TFA peak after 3 mins
- Small protein peak after 25 mins (about 400 mV)
- Small protein peak after 36 mins (about 320 mV)

Fig 38: HPLC Trace showing absorbance at 210nm against time for active sample 2, fraction 133 (from ion exchange chromatography).
This fraction was taken from Peak α



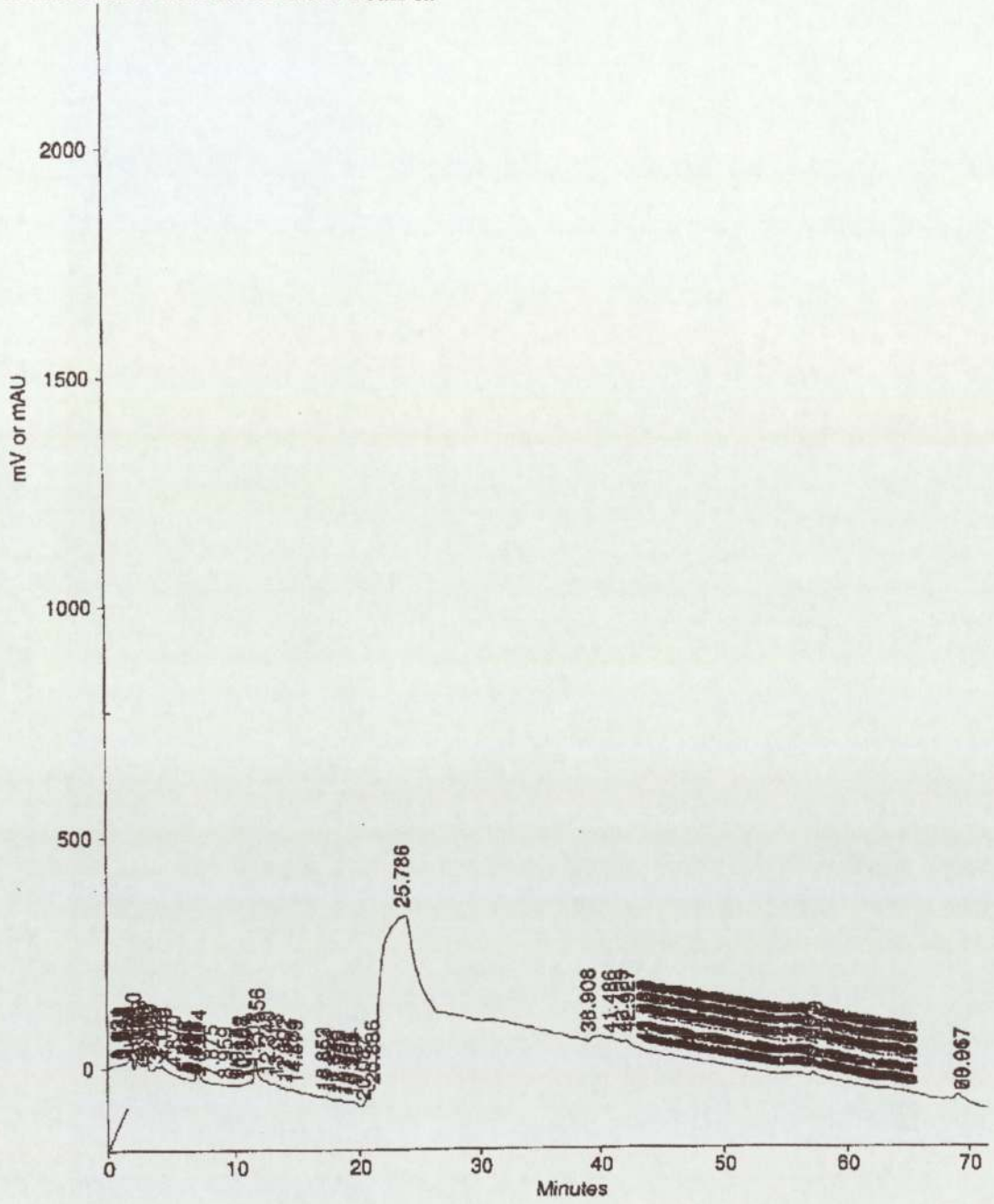
- Large TFA peak after 3 mins
- Broad protein peak after 24 mins (about 450 mV)
- Small protein peak after 35 mins (about 300 mV)

Fig 39: HPLC Trace showing absorbance at 210nm against time for active sample 2, fraction 133 (from ion exchange chromatography).
This fraction was taken from Peak α.



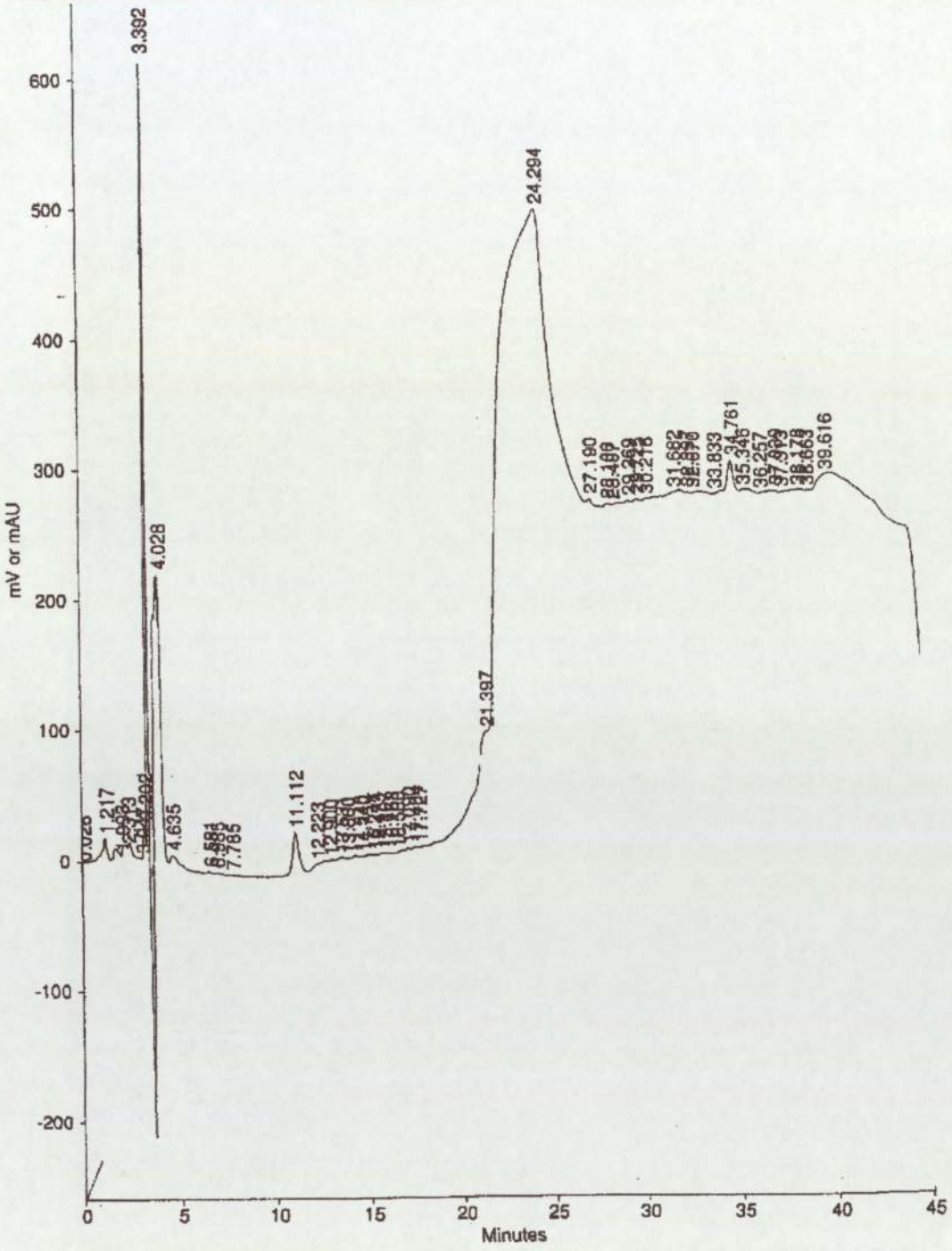
- Large TFA peak after 3 mins
- Broad protein peak after 25 mins (about 500 mV)
- Small protein peak after 39 mins (about 400 mV)

Fig 40: HPLC Trace showing absorbance at 210nm against time for active sample 3, fraction 133 (from ion exchange chromatography).
This fraction was taken from Peak α.



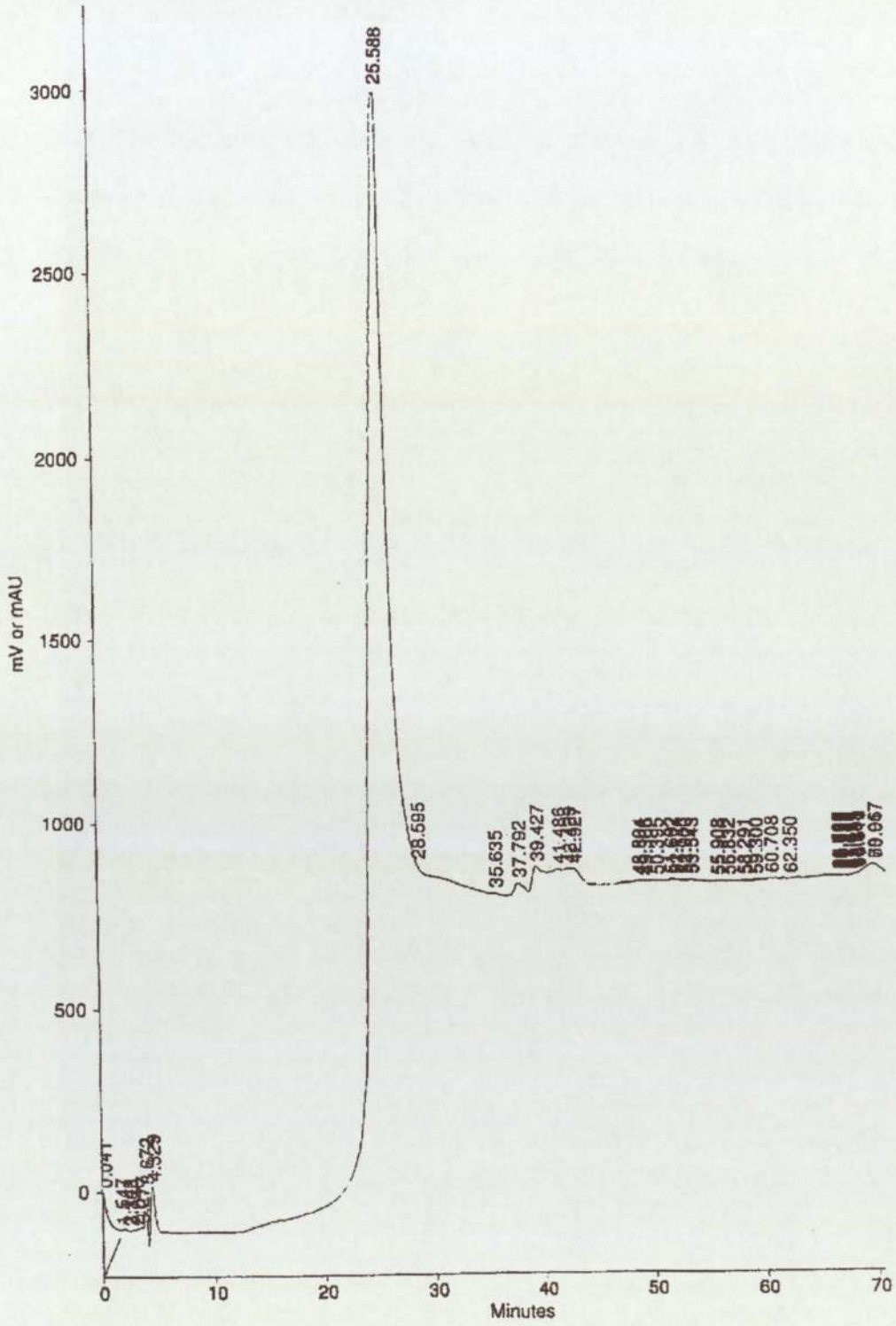
- Small protein peak after 25 mins (about 400 mV)
- Faint protein peak after 39 mins (about 200 mV)

Fig 41: HPLC Trace showing absorbance at 210nm against time for active sample 3, fraction 133 (from ion exchange chromatography).
This fraction was taken from Peak α.



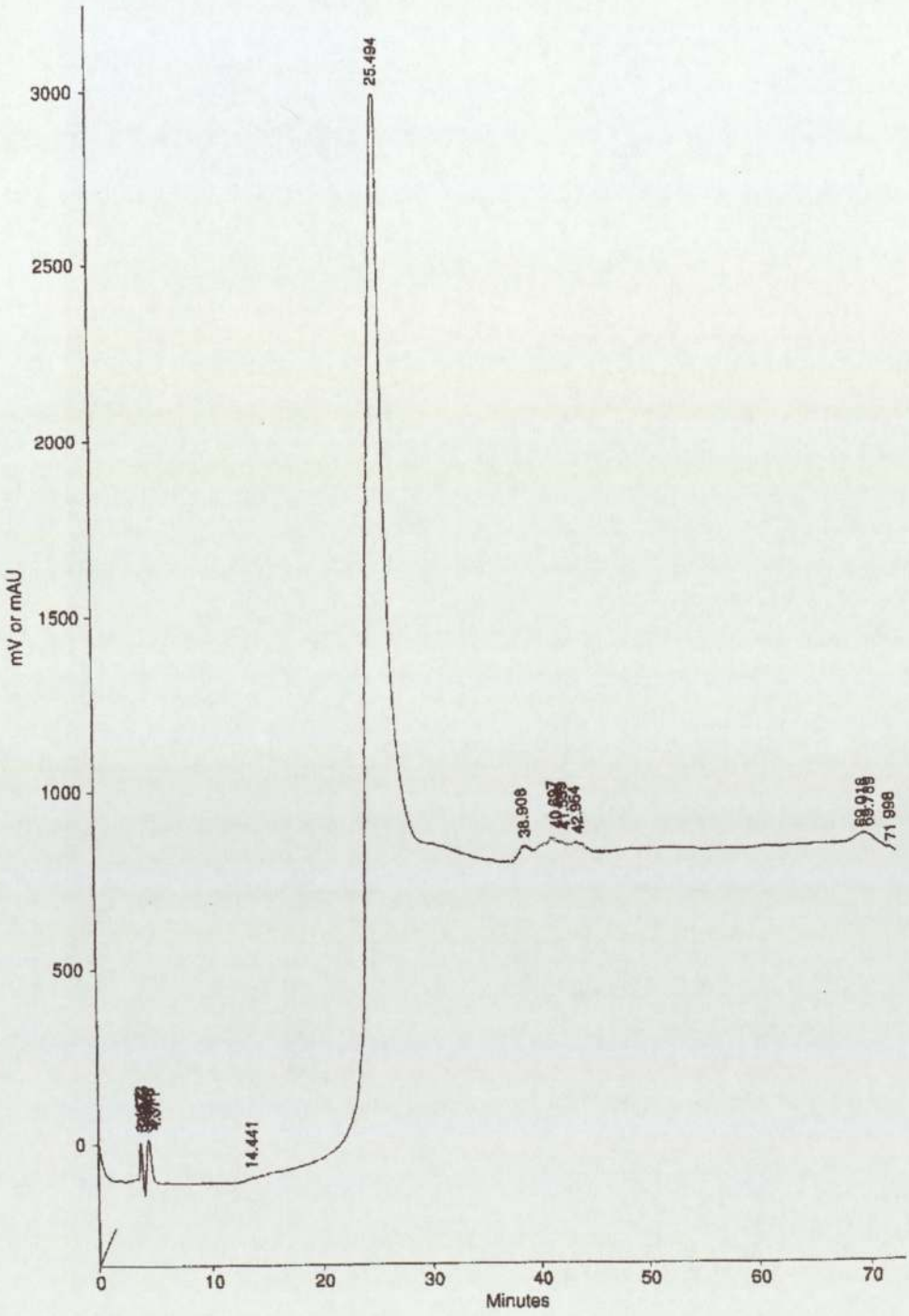
- TFA peak after 3 mins
- Tiny protein peak after 11 mins (about 50 mV)
- Broad protein peak after 24 mins (about 500 mV)
- Small protein peak after 34 mins (about 300 mV)

Fig 42: HPLC Trace showing absorbance at 210nm against time for active sample 1, fraction 157 (from ion exchange chromatography).
This fraction was taken from Peak β.



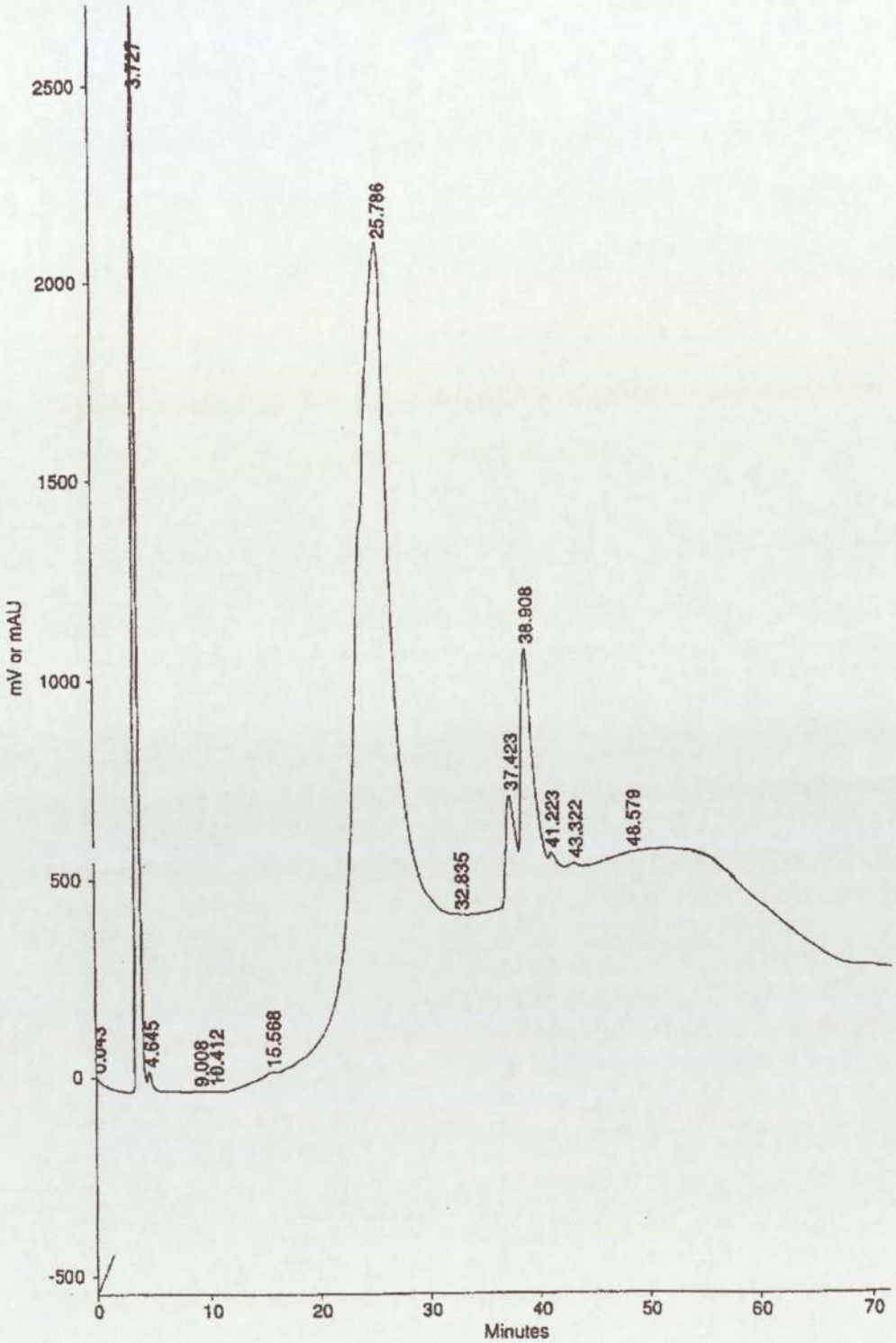
- Small amount of TFA after 3 mins
- Large discrete protein peak after 25 mins (about 3000 mV)
- Small protein peaks after 37, 39, and 42 mins (about 800 mV)

Fig 43: HPLC Trace showing absorbance at 210nm against time for active sample 1, fraction 157 (from ion exchange chromatography).
This fraction was taken from Peak β.



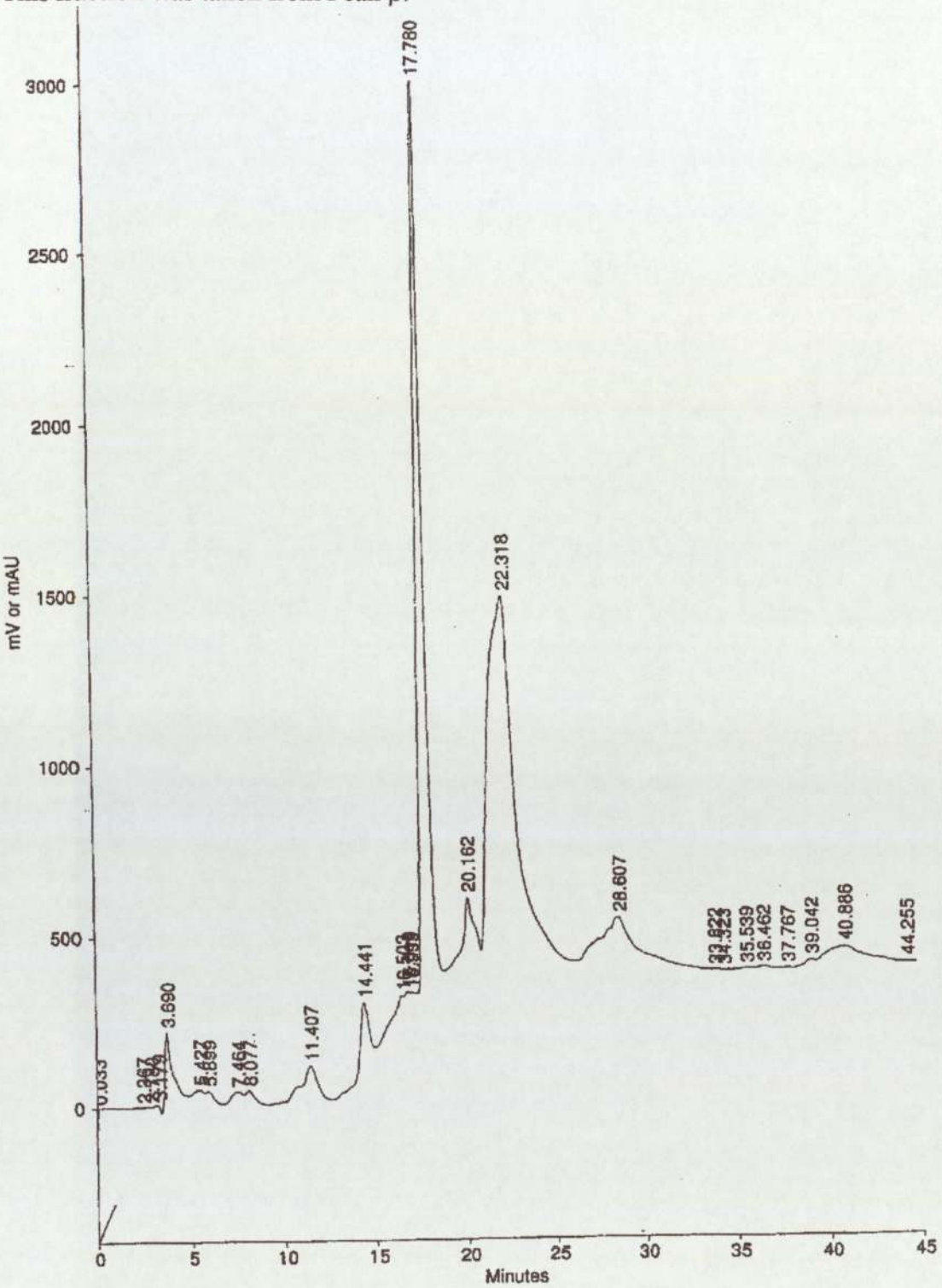
- TFA after 3 mins
- Large protein peak after 26 mins (about 2900 mV)
- Small protein peaks after 37, 39-41 mins

Fig 44: HPLC Trace showing absorbance at 210nm against time for active sample 2, fraction 157 (from ion exchange chromatography).
This fraction was taken from Peak β.



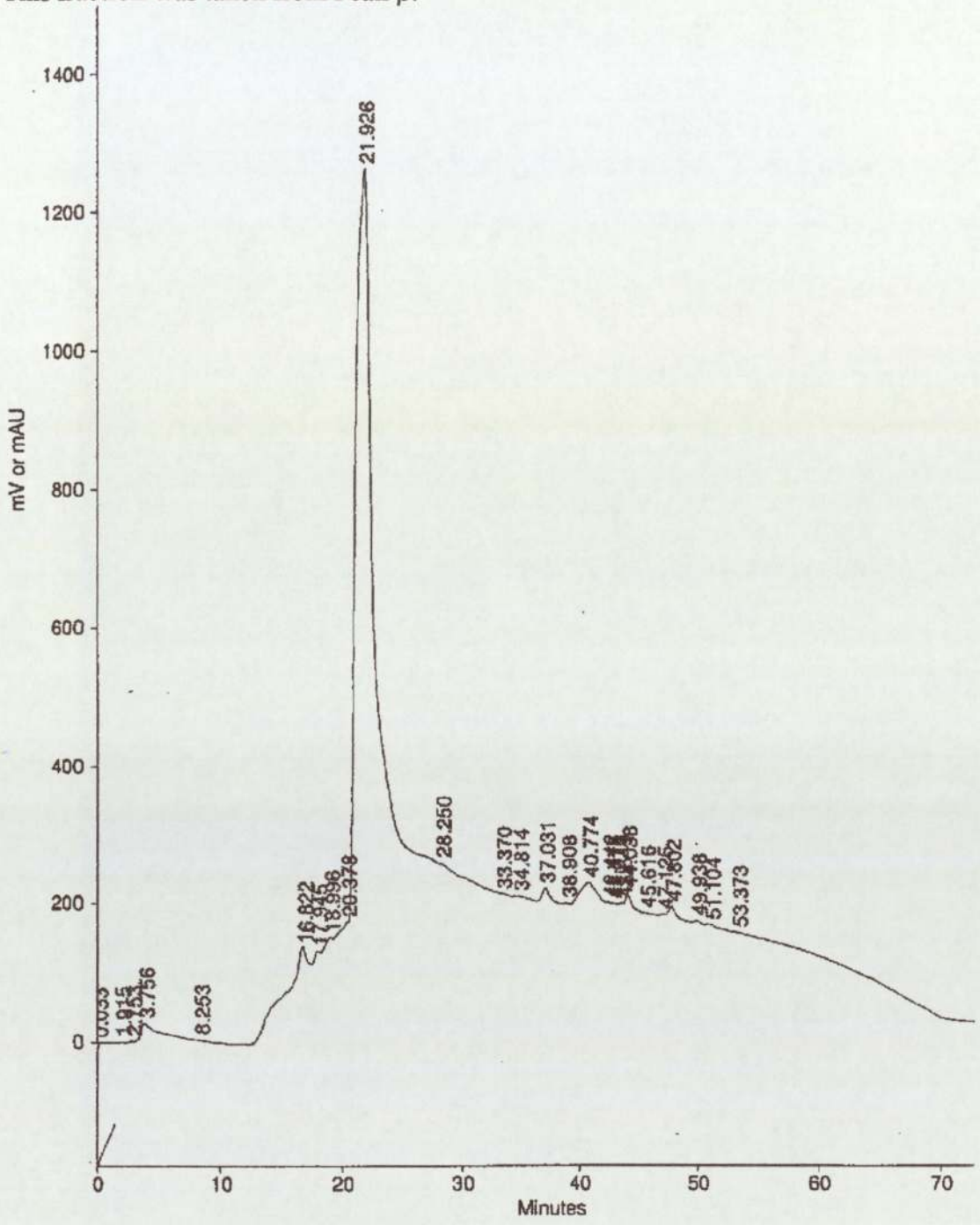
- Large TFA peak after 3 mins
- Large protein peak after 25 mins (about 2100 mV)
- Significant protein peak(s) after 37-38 mins (peaking at 1000 mV)

Fig 45: HPLC Trace showing absorbance at 210nm against time for active sample 2, fraction 157 (from ion exchange chromatography).
This fraction was taken from Peak β.



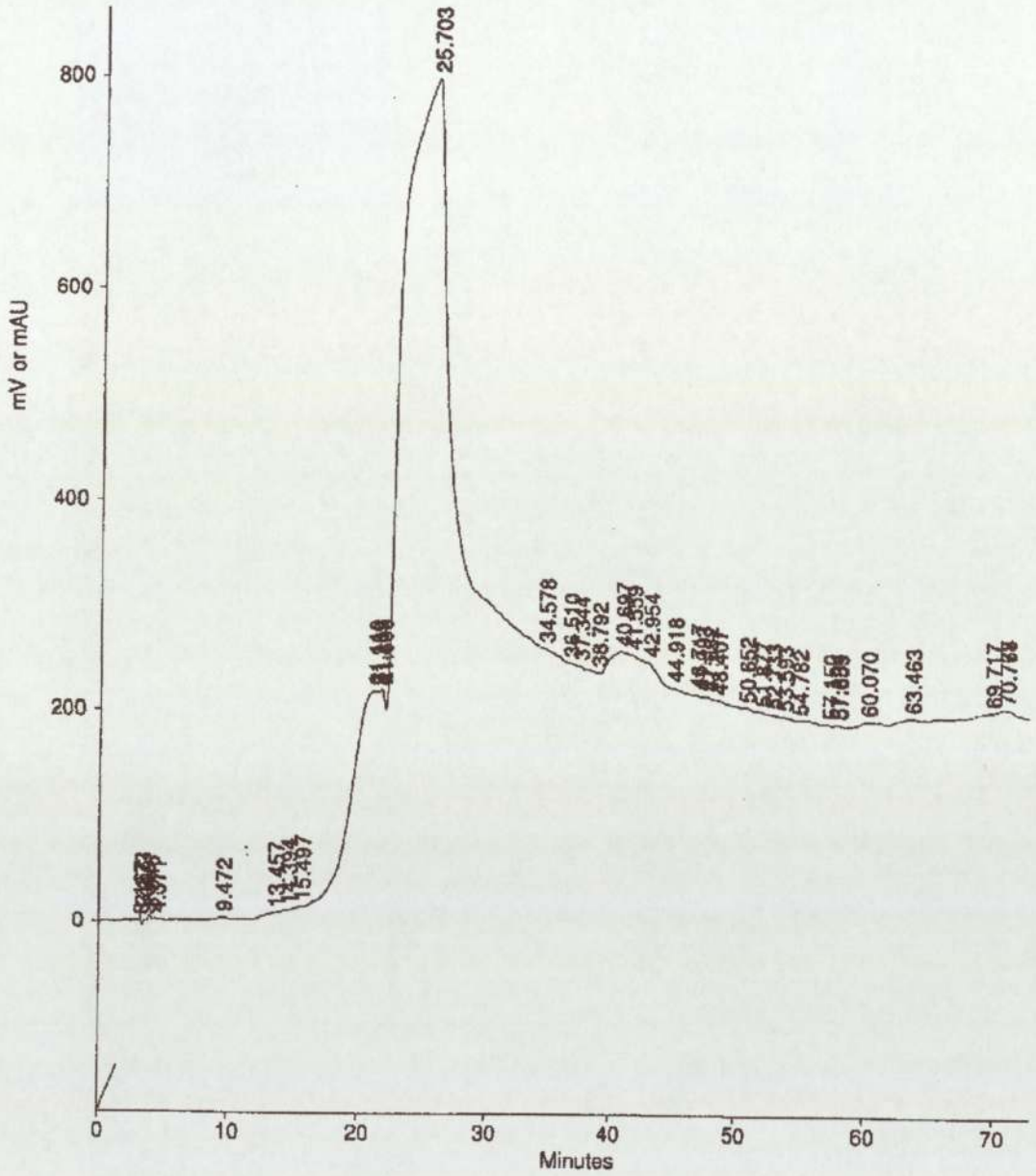
- Huge protein peak after 17 mins (about 3000 mV)
- Large protein peak after 22 mins (about 1500 mV)
- Small protein peak after 28 mins (about 600 mV)

Fig 46: HPLC Trace showing absorbance at 210nm against time for active sample 3, fraction 157 (from ion exchange chromatography).
This fraction was taken from Peak β.



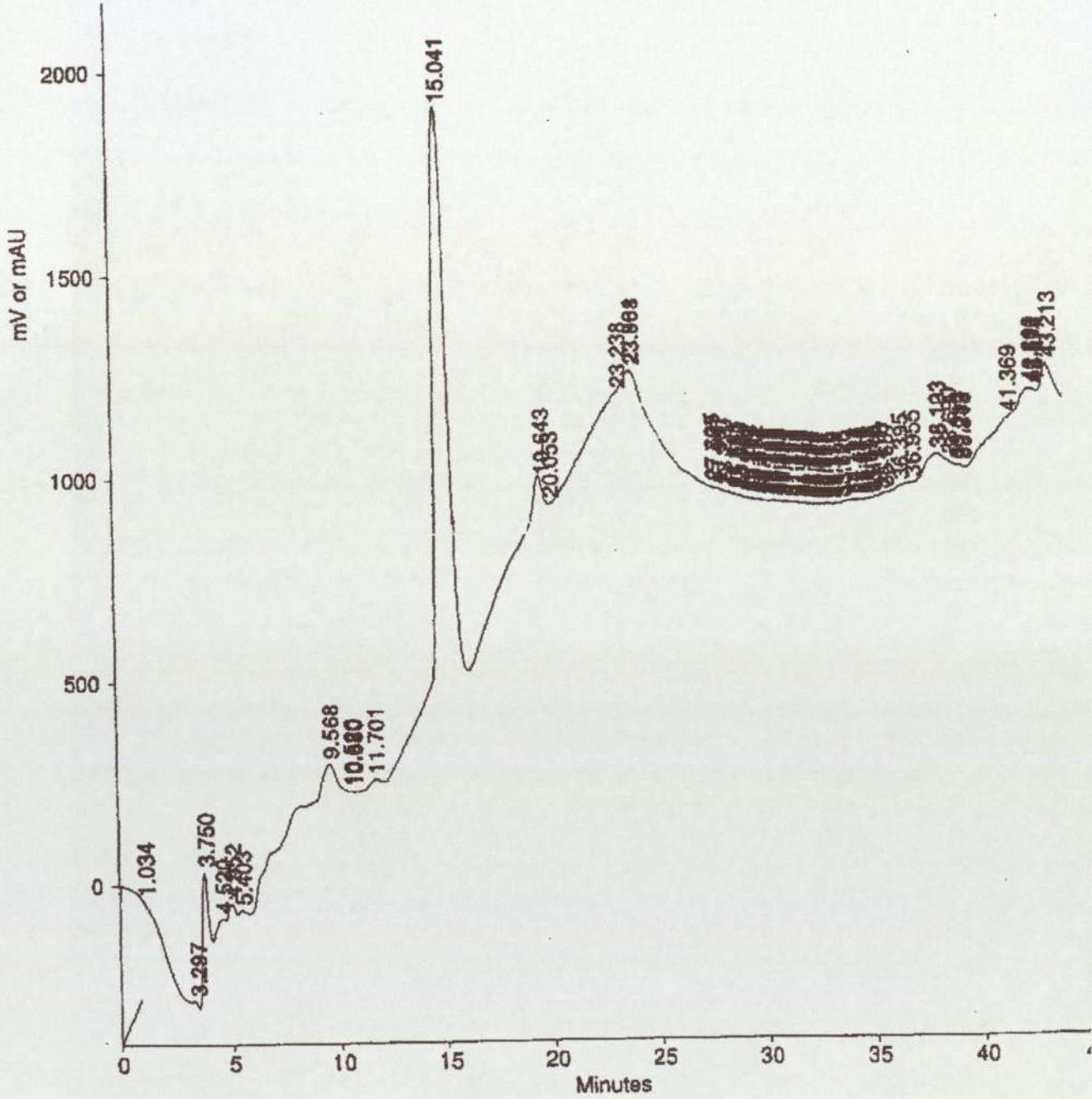
- Large protein peak after 21 mins (about 1300 mV)
- Small protein peaks after 37, 40, 44, and 47 mins (about 300 mV)

Fig 47: HPLC Trace showing absorbance at 210nm against time for active sample 3, fraction 157 (from ion exchange chromatography).
This fraction was taken from Peak β.



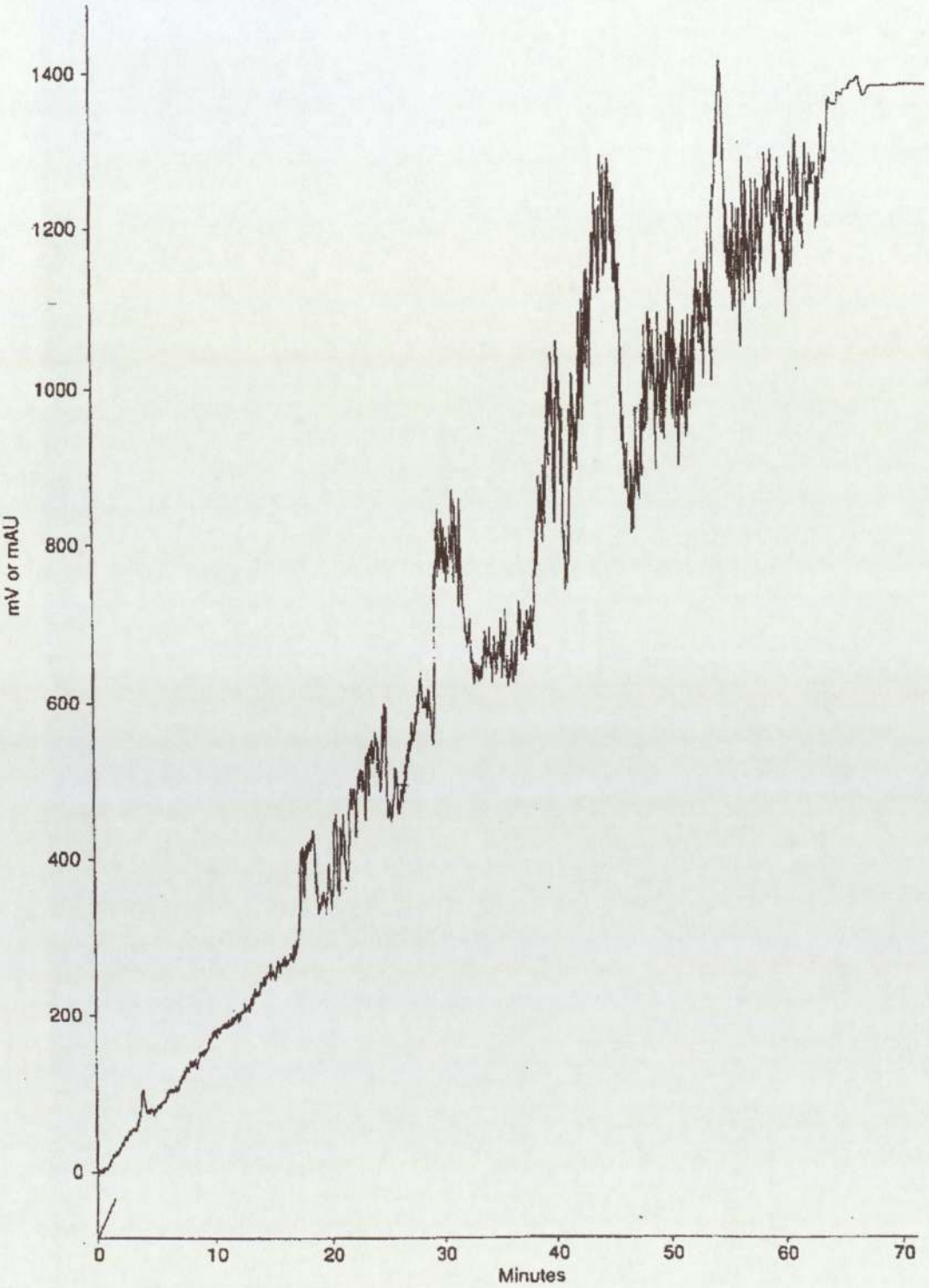
- Broad protein peak after 25 mins (about 800 mV)
- Small protein peak after 38-43 mins (about 300 mV)

Fig 48: HPLC Trace showing absorbance at 210nm against time for active sample 1, fraction 173 (from ion exchange chromatography).
This fraction was taken from Peak δ.



- Large protein peak after 15 mins (about 2000 mV)
- Large protein peak after 23 mins (about 1300 mV)
- Small protein peaks after 38, 41, and 43 mins (about 1000 mV)

Fig 49: HPLC Trace showing absorbance at 210nm against time for active sample 3, fraction 173 (from ion exchange chromatography).
This fraction was taken from Peak δ.



- No clearly defined protein peaks.

5.3 Analysis of the Purification

5.3.1 Gel Electrophoresis

After staining the resolving gels only one positive result was obtained showing a single faint band at a low molecular mass. Subsequent runs of the gel showed no bands of protein at all therefore the original result has no corroborating evidence to support it. It is not therefore possible to rely upon this one result as proof of the purity and relative molecular mass of the peptide identified in the HPLC step.

5.3.2 Protein Microsequencing

The methodology for the protein microsequencing can be found in section 4.4.2 above. The samples collected from the HPLC run and subjected to protein microsequencing gave the following positive results. Please refer to nomenclature for the haemolymph samples (5.2.1) and antibacterially active peaks (5.2.3).

Fig 50: Amino Acid Sequencing

Amino Acid Position Number	Sample 1 Peak β	Sample 2 Peak β	Sample 3 Peak β
1	-	-	-
2	Lysine or Glutamine	Lysine or Glutamine	Lysine, Glutamine, or Glutamic Acid
3	-	Proline	-
4	Proline or Phenalanine	Proline	Isoleucuine
5	-	-	-
6	Leucine	-	Glutamic Acid
7	Glutamic Acid	-	Valine
8	Proline	-	Alanine
9	Proline	-	Theronine
10	Tyrosine	Tyrosine	Isoleucine

6.0 Discussion

6.1 Characterisation and kinetics of Antibacterial Activity

The results in 5.1.1 show that antibacterial activity can be detected in the haemolymph of the larvae of *Calliphora vomitoria* after inoculation with bacteria. Inoculation with different bacterial stains showed that there was no significant difference in the resulting antibacterial activity observed in larvae inoculated with *Staphylococcus aureus*, a gram-positive bacterium, and those inoculated with *Escherichia coli*, a gram-negative bacterium. Larvae that had been sham-inoculated with a sterile needle showed some antibacterial activity that was greater than the control but less than that seen in inoculated larvae. There was no antibacterial activity observed in the haemolymph from non-immunised animals. The antibacterial activity observed in the sham-inoculated controls could be due to either or both of the following reasons:- Firstly there may be a mechanism in the animals by which antibacterial peptides are synthesised in response to an injury of any kind. Secondly a small proportion of the sham-inoculated animals could have had their wound infected during the 24 hour period post inoculation and prior to extraction of the haemolymph. As the haemolymph extracted from individual larvae was pooled there is a chance that antibacterial peptides synthesised in a minority of infected animals were present in the final sample volume. These results correlate strongly with those published previously using other dipteran species[22,39,52]. The results indicate that there is an up regulation of antibacterial peptides following contact with a bacterial species. This strong antibacterial activity is not caused by the simple wounding of the animal and it can be concluded that the up regulation of antibacterial peptides is due to the presence of bacteria in the larvae's haemocoel.

The results shown in 5.1.2 investigating the specificity of the antibacterial activity show that there was a wide range of antibacterial activity produced. It can be seen that there is antibacterial activity in the untreated haemolymph directed against both gram-positive and gram-negative bacteria. Antibacterial activity was observed against *S. aureus, S. aureus MRSA*, and *S. epidermidis* all gram positive bacteria, and also against *E. coli* a gram negative bacterial strain. There was no antifungal activity

observed against *Saccharomyces cerevisiae*. It can be concluded from these investigations into the antibacterial activity of untreated, immunised haemolymph that one or more antibacterial agents are produced that have activity against both gram positive and gram negative bacteria. These agents are likely to represent molecules from the groupings described in the introduction. As these results do not differ greatly from those of other studies into the inducible antibacterial peptides of dipteran insects[22,39,52] it is reasonable to conclude that there are insect defensins, cecropins, lysozymes and/or proline and glycine rich peptides represented in the active haemolymph extracted.

Studies presented here on the nature of the antibacterial molecules (5.1.3 and 5.1.4) indicate that the molecules are protein in nature as both the addition of trypsin (a protease) and collecting the haemolymph without aprotinin (a protease inhibitor) produced a loss of antibacterial activity. Haemolymph collected without aprotinin would presumably have been subjected to degradation by indigenous protease molecules of the larvae during the collection and storage phases and therefore would suffer the loss of its antibacterial activity. Since aprotinin alone showed no antibacterial activity, it can be concluded that aprotinin is not responsible for the antibacterial activity seen in the active samples. The reduction of antibacterial activity seen when the active sample is incubated with trypsin gives strong evidence that the molecules responsible for the antibacterial activity are protein in their nature. As trypsin is a potent protease the reason for the reduction of antibacterial activity will be due to the action of trypsin.

Heat treatment of the active haemolymph showed that the pattern of antibacterial activity observed changed after exposure to a temperature of 100°C for five minutes. Activity directed against gram positive bacteria suffered no significant change whereas the antibacterial activity directed against gram negative bacteria was seen to be significantly reduced to a level when no significant anti gram negative activity can be seen. The results indicate that there are different molecules responsible for the activity against the two classes of bacteria. The molecules responsible for anti gram positive activity are heat stable, whereas those responsible for anti gram negative activity are not.

The investigation into the pH stability of the peptides responsible for the antibacterial activity showed that antibacterial activity is not affected between pH 4 and pH 8. Once the pH is lowered below pH 4 all antibacterial activity is seen to become reduced to control levels. Once the pH is raised above pH 8 there is a diminution of activity and antibacterial activity disappears once the pH is raised above 10. These results are unsurprising as antibacterial activity is only seen across a pH range one would expect to find in vivo[3].

Proteins are complex molecules that are perform their roles primarily due to the way in which the molecule is folded. This three dimensional structure is subject to change as levels of temperature and pH vary. This is due to the molecule becoming unravelled and adopting a different three dimensional shape. As most proteins fold spontaneously into shape (due to hydrophobic areas 'moving' to the core of the molecule) once the effect of the heat is removed the molecules should revert to their original conformation and therefore become active again[75]. The results show that the molecules responsible for the antibacterial activity against gram positive bacteria did retain their antibacterial activity and therefore folded back into their original alignment after heat treatment. The molecules responsible for anti gram negative activity had the nature of their three dimensional folding changed in such a way by heat treatment that they were unable to form their original conformation and therefore no antibacterial activity could be seen.

The interactions of the individual amino acids in a peptide to the acidity of the solution in which it is placed also change the folding of a protein molecule. As a solution becomes more acidic there are more free H^+ ions in the solution and therefore able to interact with the protein molecule. It is the disruption of the internal hydrogen bonds in the protein caused by these ions that change the shape of the protein and thereby reduced antibacterial activity when the pH is changed.

The results of antibacterial assay investigating the kinetics of the antibacterial response 5.1.5 show that there is no significant antibacterial activity present in larvae before infection/inoculation. Antibacterial activity appears a few hours post inoculation and peaks after 24 hours. It is present at a high level for a further 24 hours before declining. The antibacterial activity was seen to be present but in decline 55 hours after inoculation but had reduced to background levels after 72 hours. The

conclusion reached to describe this pattern of synthesis of the antibacterial molecules is that an immune response is triggered by the presence of bacteria inside the larvae's haemocoel. This immune response takes the form of the synthesis of a battery of antibacterial peptides at an exponential rate. This log phase of peptide synthesis lasts for 24 hours and then as the bacteria are killed by the peptides produced the rate of synthesis decreases due to the removal of the original stimulus (the invading bacteria).

The results from the final antibacterial assay (investigating a secondary response, 5.1.5) show that there is no significant difference between the antibacterial activity produced by animals exposed to the same bacterial species twice and the response produced by animals inoculated only once. These results give evidence supporting the view that there is no immunological memory in insects such as mammals possess, i.e. there is no mechanism by which an organism recognised as having previously infected the animal will induce a response of greater magnitude and duration than the response from first contact with a pathogen[1].

When all of these results are viewed together they show strong correlation with results obtained in other studies of the inducible antibacterial peptides of dipteran insects[22,39,52]. In all cases larvae from the dipteran insect have produced antibacterial activity following inoculation with a bacterial species. Little or no activity was present prior to inoculation. This antibacterial activity was seen to be due to proteins or peptides that are active principally against gram positive bacteria, are heat stable and relatively pH stable. This activity was seen to reach its peak after 24 hours and remain for some time after before returning to the original levels. There was no evidence of an immunological memory such as mammals have, and there was no difference between the first response and the second in animals that had been inoculated twice.

There appears to be no specificity to the antibacterial peptide response as larvae inoculated with gram positive bacteria produced peptides that had antibacterial activity against gram negative bacteria and vice-versa. This could be due to the fact that there is no specific trigger mechanism for the production of these antibacterial peptides. The evidence of this study points towards there being a production of all

antibacterial peptides when the larva detects bacteria challenge, and also the production of small amounts of antibacterial peptide after wounding.

6.2 Purification of the Antibacterial Molecules

6.2.1 Cation Exchange Chromatography

The ultra centrifugation of the haemolymph was performed to whole cells and cellular debris from the haemolymph prior to storage. The heating and filtering of the pooled haemolymph served to remove any other large particles i.e. lysosomes and to further purify the sample before placing it onto the ion exchange column. Earlier results had indicated that the molecules of interest (inducible anti gram-positive peptides) were heat stable at a higher temperature than that used here there was no concern that the antibacterial activity would be impaired.

As explained earlier the methodology used here followed a revised version of that performed by Lambert and colleagues[39] in 1989. For ion exchange chromatography it was assumed that the antibacterial peptides shown to be present in the active haemolymph of Calliphora vomitoria would exhibit similar properties to the antibacterial peptides already discovered in other dipteran species. A cation exchange resin was therefore used for the separation. Once on the column the samples and controls were allowed the time to stick to the resin by way of a large volume of 40 mM ammonium acetate. The ammonium acetate moved the molecules throughout the column allowing all of the cation peptides to become bound to the ion exchange resin. This loading step also allowed any proteins that would not bind to the column, or which bind only weakly, to be eluted prior to the elution of the molecules of interest. After the loading step the gradient of increasing ammonium acetate was used to elute the peptides of interest off the column.

The results for the controls (fig 25) show that there is a large elution of protein molecules during the loading stage and during the first stages of the elution gradient. These proteins are those that would not have stuck to the ion exchange resin and therefore passed straight through. Afterwards there is a reduction in the amount of protein eluted from the column with a few peaks coming after the gradient has finished. No significant antibacterial activity was observed in any of the fractions collected from either of the controls. These results are to be expected as the earlier studies (5.1.1) have indicated the levels of antibacterial peptide present in an

unchallenged animal are relatively low and only become up regulated upon antigenic challenge.

When the controls are compared with the samples there is a difference in the traces (fig 26). Again at the start of the chromatography there is the elution of protein molecules during the loading step and at the start of the ammonium acetate gradient. As the gradient increases towards 500 mM it can be seen that more protein is eluted from the sample (active) haemolymph than from the control. There are defined peaks of protein concentration seen in the active sample and not the control sample. This pattern continues for the rest of the chromatography culminating in large peaks of protein seen in the sample after the ammonium acetate gradient had reached its maximum. These results indicate that there is an up regulation of antibacterial peptides due to antigenic challenge as there are protein peaks appearing in the places of the active sample that are not there in the control.

When the absorbance traces for the three samples are compared (fig 27) it can be seen that there is significant elution of protein during the initial loading stage as proteins that were unable to bind to, or had only a weak affinity with the ion exchange resin were washed through. Once the gradient of ammonium acetate started, only small volumes of protein were eluted from all three samples. As the concentration of ammonium acetate increased there were peaks of protein eluted from all three samples between fraction numbers 110 and 120. Also for samples 1 and 3 there were peaks of protein at fractions 133 - 134. After the gradient of ammonium acetate had reached its peak all three samples had large amounts of protein eluted between fractions 152 and 162, also samples 1 and 3 had peaks of protein absorbance in fraction 171.

When the samples are investigated for their antibacterial activity (figs 28-30) it can be seen that there is a small amount of antibacterial activity directed against *E. coli* concentrated around fractions 61 and 71 in all three samples. This antibacterial activity is surprising for two reasons. Firstly the antibacterial activity occurs in fractions that do not correspond to any great peak of protein concentration for any of the samples. Secondly when the initial antibacterial assays were performed the antibacterial activity against *E. coli* was shown to be non heat stable. This is relevant as during the preparation for ion exchange chromatography the samples were all

heated in a boiling water bath for 4 minutes, therefore according to the earlier results there should be no antibacterial activity observed against *E. coli*.

As the gradient of ammonium acetate increased the results show that there is some antibacterial activity against *S. aureus* seen in both sample 1 and sample 2 around fraction 81. Again this antibacterial activity does not coincide with a large peak of protein concentration for either of the two samples indicating that there is a small amount of antibacterial activity present in these early, small peaks of protein concentration. Attempts were made to isolate the molecules responsible for the antibacterial activity seen in these peaks but no positive results were obtained from the Sep-Pak cartridge fractionation.

There is a second peak of antibacterial activity directed against *E. coli* on all three active sample traces around fraction 116. This again is a small but this time it coincided with a peak of protein concentration. Along with this antibacterial activity against *E. coli* the results show that there is also antibacterial activity against *S. aureus* in both active samples one and two. The cause of this dual antibacterial activity could be because there is one antibacterial peptide present that has activity against gram positive and gram negative bacterial species; or that there are two antibacterial peptides present that were eluted from the ion exchange column at the same time one with activity against gram positive bacteria and one active against gram negative species. Due to the nature of the antibacterial molecules under investigation it is probable that the antibacterial activity seen here is due to one molecule having activity against both gram positive and gram negative bacteria. The results from 5.1.3 show that the strong antibacterial activity against gram negative bacteria was reduced due to heat treatment whereas that against gram positive bacteria remained. It is therefore reasonable to assume that any molecule retaining anti gram negative activity would be unaffected by the heat. As the molecules with primarily anti gram positive activity have been shown to be heat stable it is most likely that it is one of these molecules that has dual activity that would be present in this protein fraction[55,62,64].

The results show that a peak of anti *S. aureus* activity occur in all three active samples in fractions 126-131. This antibacterial corresponds to a peak in protein concentration. When this peak (labelled α) is compared with other results obtained on

inducible antibacterial peptides in dipteran insects[39] it can be seen that the peak is present at the same concentration of ammonium acetate as the peak labelled Peak A by Lambert and his group. In this study (which was the first to discover insect defensins) the protein fraction giving the antibacterial activity in peak A was purified and identified as an insect defensin. As the two peaks (A and α) occur at similar points in the ammonium acetate gradient and both have activity against gram positive bacteria it is reasonable to make a correlation between the two and hypothesise that the antibacterial peptides present in peak α are of the insect defensin family[39].

Finally, on all of the traces for all of the samples there are large peaks of anti gram positive activity and large peaks of protein concentration present after the ammonium acetate gradient had reached its maximum and had run for some time at 500 mM. These peaks occur between fractions 155 and 170 on all of the samples and may well be due to the same peptides or two closely related peptides being eluted at the same time. As all of the active samples contained haemolymph in which the production of antibacterial peptides had been upregulated it is reasonable to assume that this large amount of protein (compared to the control) with anti gram positive activity would contain the molecules of interest, therefore this group of peaks was named β. Comparing this data with that produced in the study headed by Lambert it can be seen that there is no analogous peak of anti gram positive activity after the ammonium acetate gradient had reached its maximum of 500 mM, it is therefore reasonable to draw the conclusion from this data that the antibacterial peptide(s) present in peak β are not similar to the insect defensins seen in the study by Lambert in terms of their performances in cation exchange chromatography.

It can be concluded from the data from ion exchange chromatography that there is an up regulation of antibacterial peptides in larvae inoculated with bacteria. These antibacterial peptides are not seen in the control fractions. This data from ion exchange chromatography show that the antibacterial peptides are cationic in nature with a varying degree of charge density. The largest amounts of antibacterial peptide isolated from the column are very cationic in their nature requiring a high concentration of ammonium acetate to remove them for the column. There are three significant peaks of protein concentration eluted from the column that also show significant antibacterial activity. It can be seen that one of these peaks of protein

concentration, when compared to other available reports into insect antibacterial peptides[39,42,55], shows similarity to the peaks identified as insect defensins in these other studies. This peak of protein concentration (peak α) also shows antibacterial activity against gram positive bacteria which again in an indicator of the presence of insect defensins.

6.2.2 Sep Pak Cartridge Fractionation

Application of the ion exchange chromatography fractions to Sep Pak cartridges involved binding the protein fractions onto the cartridge material. Using increasing proportions of acetonitrile (25%, 50%, 75%, 100%) the protein was then eluted from the cartridge. All fractions collected were read for ultra-violet absorbance at 278 nm. It was observed that the protein was eluted in the 25% fraction of each sample, thus the molecules of interest, the antibacterial peptides, were present in this fraction. The results also show that there is no significant reduction of the antibacterial activity of the peptides due to Sep Pak cartridge fractionation. Figs 31-33 show the relative absorbance at 278 nm for the 25% acetonitrile fraction for all the major peaks of interest from the ion exchange chromatography. It can be seen from all of the graphs covering the peaks of antibacterial activity and protein concentration that there is little difference between the results from Sep Pak cartridge fractionation and ion exchange chromatography. The relative absorbances of all the peaks after application to a Sep Pak cartridge are proportional to those seen after ion exchange chromatography. These results were as expected as Sep Pak cartridge fractionation was used as an intermediate step to concentrate the samples and to prepare them for HPLC.

6.2.3 High Pressure Liquid Chromatography (HPLC)

HPLC was used as a final step in the purification of the antibacterial peptides identified in the haemolymph of larvae of *Calliphora vomitoria* after inoculation with bacteria. The process of HPLC involved the use of an increasing gradient of acetonitrile to remove protein molecules that had adsorbed onto the column. Fig 34 shows the results for control haemolymph from a fraction from the control run on the ion exchange column analogous to one from peak α in the sample run. This control had added to it an artificially high concentration of trifluroacetic acid (TFA). The reason for the addition of the large amount of TFA was to show where the TFA would appear in an HPLC trace and therefore would not be interpreted as a peptide with antibacterial activity. The graph shows that there is a large peak of absorbance after three minutes and two subsequent small peaks of absorbance after 19 and 41 minutes. The large peak at three minutes represents the large amount of TFA added as TFA does not interact with the column in any way and thus travels straight through. There are two subsequent peaks corresponding to relatively small amounts of protein concentration. The peaks occur with retention times that are similar to those seen in the active samples and represent the level of the antibacterial peptides in the control animals prior to immune challenge.

Fig 35 shows the HPLC trace for control haemolymph fractions corresponding to peak β. Again in this trace there is a large TFA peak after three minutes and then two subsequent smaller peaks. As with Fig 34 the retention times for the smaller peaks are located in the same fractions as those that will be seen for the sample peaks of active haemolymph. When the relative absorbance is looked at it can be seen that the control values for both graphs, i.e. controls for peaks α and β, show an absorbance of between 400 and 500 mV for both of the HPLC peaks. These values were therefore taken to represent the level of antibacterial peptide present in non immunised larvae.

Figs 36-41 show the HPLC traces for the peaks labelled α identified in all three runs of ion exchange chromatography that purified haemolymph with induced antibacterial activity. The peptides in peak α were seen to have antibacterial activity against S. aureus. This peak corresponds with that of insect defensins isolated in other

studies[39,42,48,49]. The common feature throughout all of the graphs is a large peak of absorbance with a retention time of between 23 and 25 minutes. Figs 36 and 37 (from active sample 1) show this peak to be discrete and relatively self contained. The HPLC results taken from active sample 2 (Figs 38 and 39) however show a broader peak that tails off. This pattern leads to speculation as to whether there is a single peptide present or two or more related compounds. The HPLC results from active sample 3 (Figs 40 and 41) contrast with those seen for active samples 1 and 2 as the peaks seen are neither discrete nor broad. The whole of these results taken together lead to the conclusion that the antibacterial peptide(s) present may be from the insect defensin family as the insect defensins are a large family of related peptides leading to two or more defensin isomers having similar retention times. Studies that have identified insect defensins in other insect species have used HPLC to purify their samples. In the study by Lambert[39] that first identified insect defensins the acetonitrile concentration at which the defensin eluted was 29%. The concentration of acetonitrile at which insect defensins eluted from an HPLC column in Cociancich[53] experiments was about 30%. Lowenberger[49] had insect defensins eluting from his HPLC column at an acetonitrile gradient of about 35%. The retention time of the peptide in peak α was 23-25 minutes that occurs when the acetonitrile gradient was at 30-33%. When this value is compared to those seen in the above experiments[39,49,53] it can be seen that the results for peak α correlate strongly with those seen in these other studies.

All of the peptides with a retention time of 23-25 minutes have in relatively small absorbance that range between 200 and 800 mV indicating a low concentration. As discussed earlier the control haemolymph showed an absorbance of 400 mV. It can therefore be concluded that there is little significant difference between the active and control haemolymph at the 22-25 minute retention time peak for fractions in peak α. This suggests that the antibacterial activity seen in peak α has not been significantly increased by the inoculation of the larvae with bacteria.

The HPLC traces for peak α also show that there is a small peak of absorbance at 36 to 40 minutes. Again this smaller peak shows no significant difference in magnitude when compared to results from the control. When this peak was assayed for antibacterial activity against both *S. aureus* and *E. coli* no positive results were seen. It is possible that this small peak contained protein fragments that

where not inducible antibacterial peptides. These fragments bound to the ion exchange column with the same affinity as the antibacterial molecules but were separated from them by the HPLC. This is a normal part of the purification process due to the use different methods to separate the peptides.

Figs 42-49 show the HPLC traces for the peaks separated by ion exchange chromatography and subsequently labelled β. The peptides in peak β had been shown to have antibacterial activity against S. aureus. All of these HPLC traces show a very large peak of absorbance at a retention time of between 20 and 25 minutes. In all cases this peak is a single, discrete peak of great magnitude, over 1500 mV in every case. The HPLC traces for active sample 1 (Figs 42 and 43) show a small peak of TFA after three minutes and then a large peak of absorbance at a retention time of 25 minutes. This large peak of protein absorbance indicates the presence of a pure, highly concentrated sample. After this large peak there are no other significantly resolved peaks of protein absorbance. Thus indicating that there are no other protein products within the sample that could be responsible for the antibacterial activity. These HPLC traces were interpreted as showing a pure sample of the peptide under investigation with a retention time of 25 minutes. When these are compared to the control (Fig 35) it can be seen that there is a significant difference between the absorbance of the active sample (3000 mV) and the control sample (400 mV). These values represent a significant difference between the active sample and the control sample indicating a large synthesis/release of antibacterial peptides post inoculation with bacteria.

The β peaks of active samples 1 and 2 are not aligned. Fig 44 shows that after an initial large TFA peak there is a second peak of absorbance after 25 minutes and a third after 38 minutes. The first of these two peaks is similar to the one seen in active sample one. It was also seen to contain antibacterial activity. Antibacterial activity was not associated with the second of the two large peaks. Thus this protein (38 min) was regarded as being similar to that with a retention time of 25 minutes (in terms of ion exchange chromatography) but unremarkable in terms of antibacterial activity. Thus this protein fraction has been separated by HPLC from the antibacterial peptide. Fig 45 shows large peak of absorbance with a retention time in the mid 20 minutes that has been observed in all of the other samples. This peak is not as well defined as

the others seen with the same retention time as this has a broader outline. There is also another very large peak of absorbance in fig 45, this one having a retention time of 17 minutes. No other active sample examined by HPLC showed this peak. No antibacterial activity was observed for this protein. Therefore the protein present is again thought to be a separation product.

The results from the HPLC of active sample 3 (Figs 46 and 47) are very similar to those seen for active sample 1. There is a large peak of protein absorbance after 21-25 minutes and then a subsequent later peak after 40 minutes. When these peaks were assayed for antibacterial activity it was again seen that the first large peak contained antibacterial activity whereas the peptides in the later peak had none. This indicates that the antibacterial peptides of interest reside in the peak with a retention time of 21-25 minutes.

The final HPLC traces, Figs 48 and 49, show the results for the analysis of peak δ. This small peak of absorbance after ion exchange chromatography was seen to have antibacterial activity against E. coli. Fig 48 shows the HPLC trace for the control sample exhibits a clearly defined peak of absorbance after 15 minutes. There are also many other smaller peaks, the largest of these being a broad based peak occurring with a retention time of around 23 minutes. All of the fractions from this HPLC were assayed for antibacterial activity against both *S. aureus* and *E. coli* and all of the results were negative. Fig 49 shows the HPLC trace for active haemolymph from peak δ, these results show that there was no clearly defined peak of absorbance with many impurities being seen. When assayed for antibacterial activity it was seen that there was slight anti *E. coli* activity in the fractions with retention times of 40-45 minutes. These fractions were thus selected for electrophoresis and peptide sequencing.

The results form HPLC show that there are significant differences between the active samples and the control samples. The HPLC of the control samples revealed that there was a small peak of protein concentration at 23 minutes and another at 40 minutes. These two peaks occur after a similar time period as those seen in the active samples. The peaks are significantly smaller in the control sample than in the active samples. Thus indicating that there has been an increase in the amount of antibacterial peptide post inoculation with bacteria. The peaks seen in the active

samples are discrete, self contained peaks that have no sliding, indicating that the protein contained in the peak is a single protein sample with no impurities. This protein was seen to display the same antibacterial activity that was seen after ion exchange chromatography. Therefore it can be concluded that the peptide with antibacterial activity is present in high concentration in the fraction with approximate retention time of 25 minutes.

In many of the active samples investigated it can be observed that there is a large peak of protein concentration after 25-28 minutes and a secondary peak of protein concentration after 40-42 minutes. This secondary peak of protein concentration is of a smaller magnitude than that seen earlier. Attempts were made to isolate and analyse this second peak. No antibacterial activity was seen for the fractions collected from this peak. Similarly no results were obtained after subjecting these samples to gel electrophoresis and peptide sequencing. This second peak of protein concentration is most likely to be a protein that had the same affinity for the ion exchange column as the peptide showing antibacterial activity but has a different affinity for the HPLC resin. Alternatively this second peak of protein concentration could be a break down product of the original antibacterial peptide that could have degraded over the time spent in storage.

The results of the HPLC of peak α provide some evidence that the peptide purified here has many similarities with peptides isolated in other studies that have been characterised as insect defensins. The retention time on the HPLC column is similar to that seen in studies in insect defensins[39,49,53]. These retention times occur when the concentration of acetonitrile is around 30%. The antibacterial evidence also correlates with these other studies as the peptide isolated from peak α has activity against gram positive bacteria and not against gram negative. Again it has been noted by many studies that insect defensins only have antibacterial activity against gram positive bacteria.

The results of the HPLC of peak β show a peak of protein concentration after 25 min that is significantly larger than that seen in the control. The retention time of this protein again occurred when the concentration of acetonitrile is around 30%. The studies that have identified insect defensins[39,49,53] also showed that the concentration of acetonitrile at which insect defensins eluted from the HPLC column was around

30%. This therefore could again indicate the presence of insect defensins. The evidence of the antibacterial assays again showed antibacterial activity against gram positive bacteria but no activity against gram negative bacteria.

6.3 Analysis of Purification

6.3.1 Gel Electrophoresis and Peptide Sequencing.

The results from the gel electrophoresis were inconclusive, the only positive result obtained was not repeated and therefore its validity must be questioned. It can be seen however that the sample ran in only one band indicating a pure peptide. This peptide ran at around the same rate as the molecular marker of 5 kDa indicating that the peptide could well be a member of one of the groups of inducible antibacterial peptides discussed earlier.

The results of peptide sequencing show that the peptide isolated from peak β is very rich in proline residues. There have already been inducible antibacterial peptides rich in proline identified by other research groups. These proline rich peptides, apidaecin, abaecin, drosocin, lebocin and formaecin, are characterised as having antibacterial activity directed against gram negative bacteria. The peptide purified from peak β was seen to have no antibacterial activity against E. coli (a gram negative bacterium) but to have large antibacterial activity against S. aureus, a gram positive. These two pieces of evidence lead to a conclusion that the peptide identified from peak β is different for the previously identified proline rich, inducible, antibacterial peptides.

The partial sequence of the peptide obtained was compared in a data base (Genbank) to all known peptides to establish if this was a piece of a known peptide. The results were that there are many proteins that contain a 75% sequence match with the amino acid sequence QPP-LEPPY. As this peptide sequence is only a fragment of the full sequence it is hard to establish an identical match for the peptide in peak β. However this peptide is rich in proline residues and as discussed in 3.5 above there have been a number of antibacterial peptides identified that are rich in proline residues [60,61,62]. These peptides were first identified by Casteels in 1990[61] and have shown to have antibacterial activity against gram negative bacteria.

Fig 51:- Amino acid sequence of the proline rich antibacterial peptides

```
Apidaecin Ia        GNNRPVYIPQ PRPPHPRI
Apidaecin Ib        GNNRPVYIPQ PRPPHPRL
Apidaecin II        GNNRPIYIPQ PRPPHPRL
Abaecin             YVPLPNVPQP GRRPFPTFPG QGPFNPKIKW PQGY
Drosocin            GKPRPYSPRP TSHPRPIRV
```

It can be seen from these proline rich peptides that none of them has an identical amino acid sequence to the peptide fragment QPP-LEPPY. There is some homology with the proline, glutamine and glutamic acid residues but not enough to directly correlate one to the other.

Peptide sequencing was attempted on the HPLC fractions for peaks α and δ but no consistent results were obtained. The reasons for this are probably the small sample size utilised for peak α and possible impurities in the sample for peak δ. The second peptide sequence obtained for fractions in peak β (GVATI) was also compared on the Genbank database to other known proteins. The short length of the sample sequence obtained resulted in many possible matches for this brief sequence.

Fig 52 Fragment of the amino acid sequence of the sarcotoxins

```
Sarcotoxin IIA      VATNSKGGQDVSVKLAATNLGNKHVQPIAEVFAEGNTKGGNVLRGATVG
Sarcotoxin IIB      VATNSKGGQDVSVKLAATNLGNKHVQPIAEVFAEGNTKGGNVLRGATVG
Sarcotoxin IIC      VATNSKGGQDVSVKLAATNLGNKHVQPIAEVFAKGNTQGGNVLRGATVG
Sarcotoxin IID      VATNSKGGQDVSVKLAATNLGNKHVQPIAEVFAEGNTKGGNVLRGATVG
```

The sequence fragment obtained from peak β (GVATI) shows some correlation with a section of the sarcotoxin GATV. The sarcotoxins are glycine rich antibacterial peptides that have antibacterial activity against gram negative bacteria[66]. The evidence of antibacterial activity in this study (fig 28-30) show that the antibacterial peptide present in peak β is only active against gram positive bacteria and therefore it is unlikely that the peptide is either a sarcotoxin or any of the known proline rich peptide. The two peptide fragments could well be different parts of the same molecule and more work on the sequencing is needed to establish whether this is the case or not.

The amino acid sequencing showed no cysteine residues that would indicate the presence of insect defensins. The reason for this could be due to one of three reasons:- because there were no cysteine residues in the peptide under investigation; the peptide sequencer finished its run before a cysteine residue was reached; or because there was a difficulty in the identification of cysteine residues by the HPLC step of amino acid sequencing.

There is also an absence of high cationic residues (arginine R, histidine H, or Lysine K) identified by the amino acid sequencing. This is a surprise as this peptide bound strongly to the cation exchange resin and required a large concentration of ammonium acetate to remove from the column.

The process by which the peptide sequencing occurs involves cleaving the N-terminal amino acid from the peptide chain. This resulting fraction then undergoes HPLC to determine the amino acid thus cleaved. As this process is imprecise failures to achieve clear and concise results are common and a maximum of 20 amino acids can be identified as the clarity of the results degrades with each successive cycle. A way of combating this problem is to cleave the peptide under investigation with a known protease and then size separating the fragments thereby establishing a known starting point from which the sequencing can take place on a small fragment. In this way a larger peptide can be sequenced.

7.0 Conclusion

From all of these results it can be seen that a number of molecules with antibacterial activity were induced in the larvae of *Calliphora vomitoria* by inoculation with bacteria. These molecules were seen to have antibacterial activity against both gram positive and some gram-negative bacteria. The molecule that has antibacterial activity against gram-positive bacteria is a peptide that is heat stable, relatively pH stable, and cationic. The molecule that has antibacterial activity against gram-negative bacteria is a peptide that has no heat stability.

The peptides responsible for the antibacterial activity against gram positive bacteria are released into the insect's haemolymph immediately after inoculation with a bacterial species. The concentration of antibacterial peptide reaches a peak after 24 hours. The concentration of antibacterial peptide returns to control levels after 72 hours. There is no heightened response after a second inoculation of bacteria.

Three major peaks of peptide concentration that showed antibacterial activity against gram positive bacteria were isolated by ion exchange chromatography. Further purification of these peptides by HPLC showed there to be a high concentration of antibacterial peptide with activity against gram positive bacteria present in peak β. This peptide had the same retention time on the HPLC column as other peptides identified as insect defensins in studies by various groups. This peptide had antibacterial activity against gram positive bacteria only, a trait that is shared by members of the insect defensin family. When the amino acid sequence was investigated no correlation between the amino acid sequence obtained and a protein library could be found.

Problems with the amino acid sequencer led to being unable to identify cysteine residues therefore no positive identification that the peptide isolated in this study is a member of the insect defensin family could be obtained. There is a strong probability that the peptide present in peak β is an insect defensin.

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9.0 Appendix

Nutrient Broth

Beef extract	10.0g l^{-1}
Balanced peptone No. 1	10.0g l^{-1}
NaCl	5.0g l^{-1}

pH 7.3 +/- 0.2

Nutrient Agar

Beef extract	5.0g l^{-1}
NaCl	3.0g l^{-1}
Balanced peptone No. 1	8.0g l^{-1}
Agar No. 2	12.0g l^{-1}

pH 7.3 +/- 0.2

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