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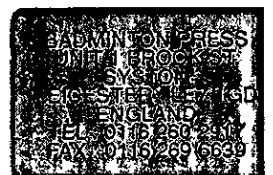
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**RAPID SCREENING OF PROTEOLYTIC
ENZYMES AND THEIR INHIBITORS USING
FLUORESCENCE METHODS**

By Marie Louise Beaumont

A Doctoral Thesis submitted in partial fulfilment of the requirements for the
award of


DOCTOR OF PHILOSOPHY

of Loughborough University

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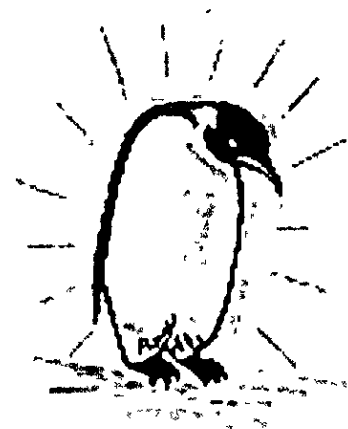
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ABSTRACT

New fluorescence methods for the determination of proteolytic enzymes and their inhibitors have been investigated. Fluorescence polarisation and energy transfer methods have both been examined and of these two approaches energy transfer methods have proven superior. Out of the energy transfer methods, the use of an over-labelled substrate, which is virtually non-fluorescent but becomes highly fluorescent upon proteolytic liberation of dye-labelled peptides, has been demonstrated to be most successful. A substrate utilising α -casein labelled with a long-wavelength BODIPY® dye has been synthesised and shown to be a suitable intramolecularly quenched conjugate for the determination of proteolytic enzymes and their inhibitors in flow-based screening programmes. The assays are rapid, taking at most 2 minutes for the enzyme assays and 5 minutes for the inhibition assays. Aprotinin, a serine protease inhibitor also used as a drug in surgery to prevent blood loss, has been detected at concentrations as low as 0.161 nM, and limits of detection of 2.50 μ M, 0.14 μ M, and 7.20 nM were obtained for 3-nitrophenylboronic acid, leupeptin, and pepstatin A respectively. The adaptation of these assays to a multi-determination format has also been considered.

These fluorogenic assays combine the sensitivity of long-wavelength fluorescence with the ease of automation that can be applied to flow injection analysis and could be incorporated into a simple portable fluorescence instrument. They have many clinical applications, since proteases are involved in virtually all biological functions and dysfunctions and therefore are promising as drug targets. The flow-based procedure described here can be used in the high-throughput screening of candidate drug molecules.

RESEARCH AIMS

The objective of this investigation was to develop new fluorescence methods for the determination of proteolytic enzymes and their inhibitors. Upon finding a sensitive method, this assay was to be transferred to a flow injection analysis system to create a procedure that would be suitable for the high throughput screening of drug candidate molecules. In order for the assay to be used in a high throughput screening function, the assay had the essential requirement of being rapid. With this in mind the progression of this work was to aim towards the adaptation of the already established assay to a set-up that would allow the multi-determination of proteolytic enzymes and their inhibitors.

SCOPE OF WORK

The work presented in this thesis reports the investigations that were carried out to achieve the above aims. Chapter 1 provides an overview of the subject areas that are pertinent to the investigation, while chapter 2 describes the materials, instrumentation, and general procedures that have been adopted for use during the practical work.

Chapter 3 deals with the first investigation of a fluorescence method, fluorescence polarisation, for the determination of proteolytic enzymes. This chapter discusses the work carried out and the conclusions derived from that work.

Chapters 4 and 5 investigate the use of energy transfer fluorescence methods. The use of fluorescence resonance energy transfer involving substrates that are labelled with two different fluorophores is explored as a fluorescence method in

chapter 4. Chapter 5 describes the synthesis of over-labelled single fluorophore substrates resulting in energy transfer between neighbouring fluorophore molecules. The application of this substrate to a flow injection analysis system and subsequent proteolytic inhibitor determinations is demonstrated.

Chapter 6 discusses set-ups appropriate for multi-determination and investigates these set-ups for use in these types of assays.

Finally, chapter 7 provides a discussion on the practical work that has been carried out and the conclusions that can be derived. Ideas on further extensions of the work carried out and future directions are also discussed.

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LIST OF ABBREVIATIONS

3-NPBA	3-nitrophenylboronic acid
ACE	Angiotensin converting enzyme
BCA	Bicinchoninic acid
CPG	Controlled-pore glass
D.P	Dye:protein
DMF	<i>N, N</i> -dimethylformamide
DMSO	Dimethylsulphoxide
FIA	Flow injection analysis
FITC	Fluorescein isothiocyanate
FP	Fluorescence polarisation
FRET	Fluorescence resonance energy transfer
HIV	Human immunodeficiency virus
HTS	High throughput screening
LED	Light emitting diode
LOD	Limit of detection
PMT	Photo-multiplier tube
QY	Quantum yield
TMRITC	Tetramethylrhodamine isothiocyanate
UV	Ultraviolet
ϵ	Molar extinction coefficient

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CHAPTER 1

Introduction

1.1 FLUORESCENCE

Fluorescence spectroscopy is an extremely powerful analytical tool that has seen a remarkable growth in its use over the past 15 years in a wide range of areas from clinical chemistry to environmental monitoring. There are numerous sources of information on the theory of fluorescence and its applications [1, 2, 3, 4] with the consequence that the theory will not be discussed in great detail here. An excellent paper covers the basic principles of fluorescence spectroscopy [5] and a recent book by Lakowicz provides a wealth of knowledge on the basic aspects on fluorescence spectroscopy, as well as covering the many experimental and instrumental advances that have been made during the last 16 years [6]. Reviews covering new developments in the theory and practice of molecular luminescence for chemical analysis are published every two years [7, 8] and yearly reviews on advances in atomic emission, absorption, and fluorescence spectrometry, and related techniques are also published [9, 10].

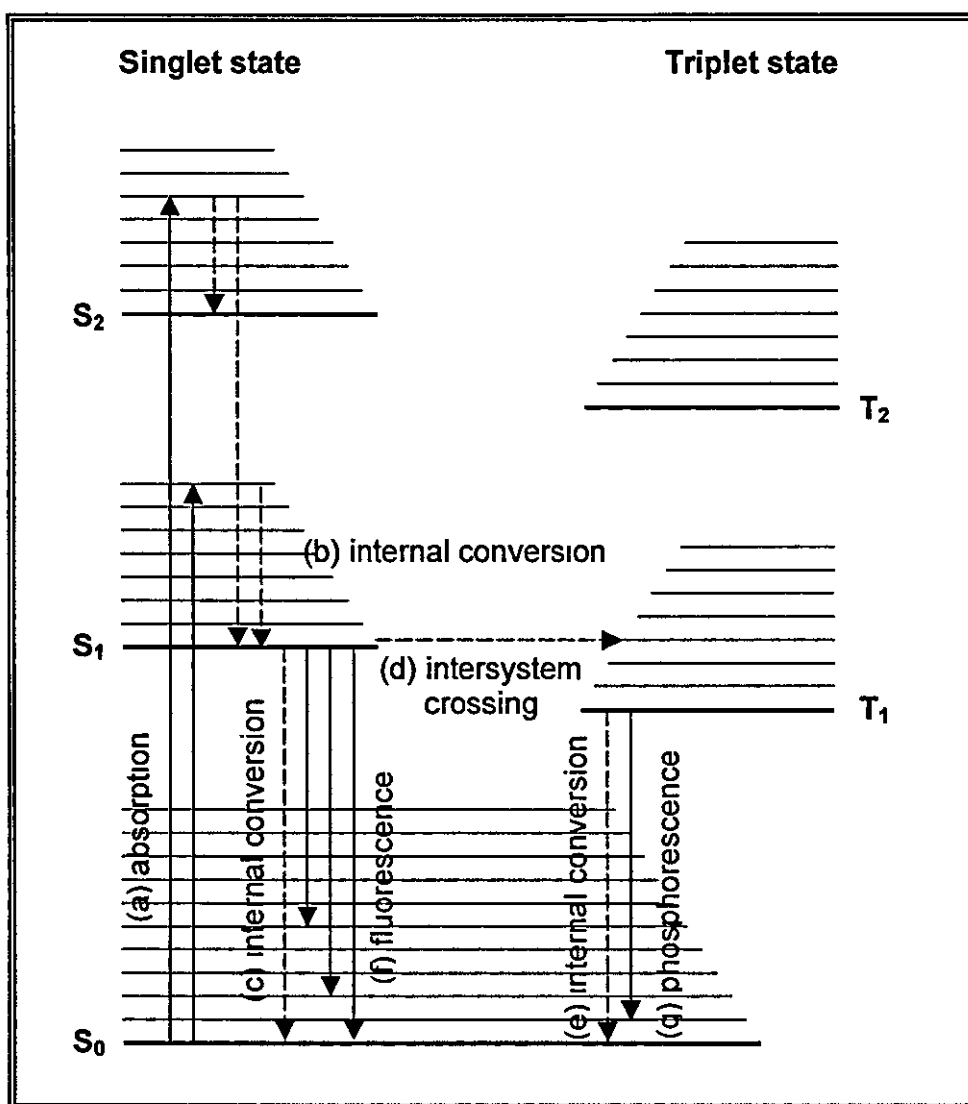
1.1.1 Theory of fluorescence

The sequence of events that occur in fluorescence are shown in figure 1.1 and are as follows:

1. A quantum of light is absorbed by a dipole (a)
 2. Rapid radiationless relaxation (internal conversion) takes the dipole to the thermally relaxed S_1 level (b)
 3. The dipole retains this energy for a certain period of time, generally designated as the lifetime (τ)
-

4. The excess energy is dissipated through radiationless de-excitation (c, d, and e) caused predominantly through vibrational and rotational modes and collisions with solvent molecules, or by photon emission (f, g). When this photon emission is from a singlet electronic state, it is referred to as fluorescence (f). Conversely, when the emission is from a triplet state, phosphorescence is the result (g).

Figure 1.1 – Jablonski diagram showing absorption and emission processes of fluorescence and phosphorescence. S_0 = ground singlet state, S_1 and S_2 = first and second singlet levels respectively, T_1 and T_2 = first and second triplet levels respectively



1.1.2 Advantages of fluorescence

When compared with conventional absorbance-based techniques, fluorescence is several orders of magnitude more sensitive and more selective. Increase in sensitivity arises because the emitted radiation is measured directly and can be increased simply by increasing the incident power. Moreover, as fluorescence is measured at right angles to the incident beam, it is considered a zero background technique. Absorbance however, is a measure of the difference between incident and transmitted intensities i.e., signals are measured as a small change in a large background.

Selectivity in fluorescence-based techniques is also much higher than in absorbance-based approaches. All molecules absorb photons. However, only a few fluoresce and fewer still phosphoresce at ambient temperatures. Thus the rarity of luminescent materials makes fluorescence an attractive technique for the resolution of trace fluorescent components in complex mixtures. In addition, the ability to employ both excitation and emission wavelengths as selectivity parameters gives fluorescence increased selectivity.

The ease of handling fluorophores compared with the expense and difficulties of handling and disposing radioactive material makes fluorescence an attractive alternative to radioactivity.

1.1.3 Long wavelength fluorescence

Fluorescence spectrometry has been accepted for many years as a major technique for trace analysis, but in real samples the sensitivity of fluorescence is not as high as that seen in pure solutions that contain the same fluorophore.

molecules being effectively non-fluorescent. Thus, fluorophores with moderate molar absorptivities and quantum yields can give excellent limits of detection because of the low backgrounds due to so few fluorophores that absorb and emit at these wavelengths.

The development of semiconductor-based detectors, photodiodes, and inexpensive long wavelength laser diodes and LEDs has allowed the reduction of detector noise and an enhancement of the fluorescence signal. There is therefore the opportunity for simple, robust but sensitive instruments based on solid-state light sources and photodiode detectors and cheap and effective fiber optics. Both the simplicity and sensitivity of long wavelength fluorescence thus make it an obvious choice as a detection method.

Further discussions of the advantages of long wavelength fluorescence and its applications can be found [15, 16].

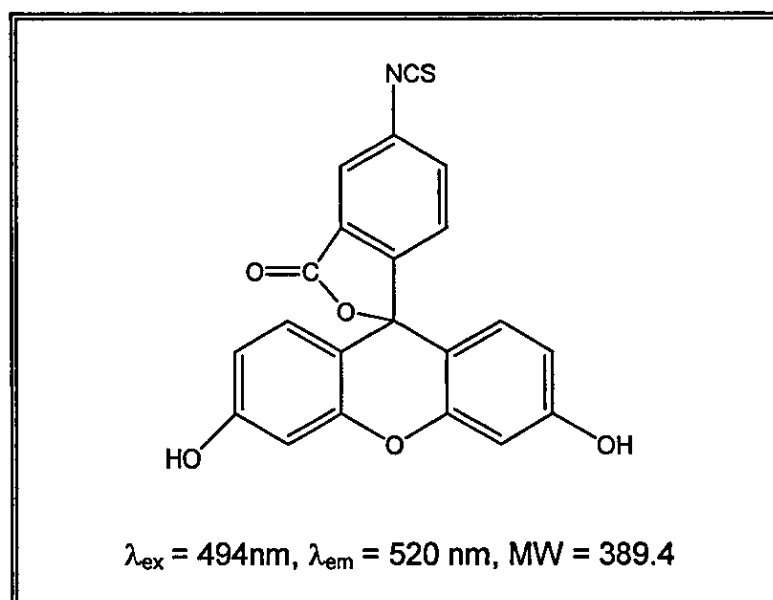
1.1.4 Fluorescent labels

A variety of fluorescent labels have been used in this study, and the structure, advantages, and disadvantages of each fluorophore are discussed below. The energy capture efficiency of a fluorescent dye is expressed as the extinction coefficient, ϵ , and usually ranges from 10 000 – 250 000 $\text{cm}^{-1} \text{M}^{-1}$. The emission efficiency is expressed as the quantum yield, QY, and usually ranges from 0.05 – 1.0. Quantum yield is strongly influenced by the local environment and so is not usually reported as a 'constant' like the extinction coefficient. The best fluorophores will have both a high extinction coefficient and high quantum yield.

1.1.4.1 Fluorescein isothiocyanate (FITC)

A more detailed description of FITC can be found elsewhere [17].

Figure 1.2 – Structure of FITC, isomer I



Advantages:

- high extinction coefficient ($73\ 000\ \text{cm}^{-1}\ \text{M}^{-1}$)
- excellent fluorescence quantum yield (~ 0.9)
- good water solubility

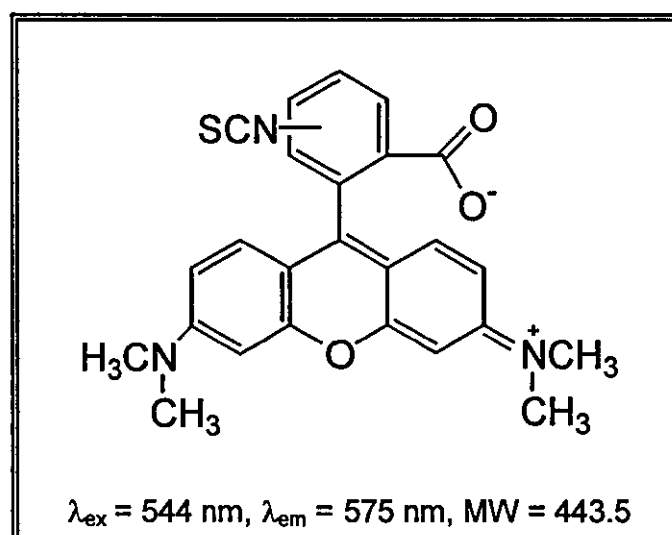
Disadvantages:

- relatively high rate of photobleaching
- pH-sensitive fluorescence that is significantly reduced below pH 7.0 ($\text{pK}_a \sim 6.4$)

1.1.4.2 Tetramethylrhodamine isothiocyanate (TMRITC)

Further reading on this fluorophore can be found [18].

Figure 1.3 – Structure of tetramethylrhodamine, mixed isomers



Advantages:

- good photostability
- insensitive to solvent polarity

Disadvantages:

- prone to aggregation

This is because of background-scattered light and fluorescence signals from endogenous sample components. This problem is particularly prevalent in biological samples where high levels of proteins and other natural polymers cause intense light scattering, and which commonly contain fluorophores whose spectra overlap those of the target fluorophore. Substances found in blood plasma, for example proteins and amino acids, exhibit fluorescence emission at 350 nm, and protein-bound bilirubin shows emission at 520 nm.

A major source of errors in all fluorescence measurements is interferences due to scattered light. Scatter is a property of the solvent or particulate matter in the sample. Some photons are uniformly scattered without change in frequency due to collisions with the solvent molecules. The emitted light has the same wavelength as the exciting light since the absorbed and emitted photons are of the same energy. This scattering is termed Rayleigh scattering and occurs at all wavelengths. Its intensity, however, varies in proportion to λ^{-4} , so its effect can be minimised by working at longer wavelengths [11]. Raman scattering is a source of interference that occurs in aqueous solutions, which are typically used for biological samples. Raman scatter always occurs at a constant wavenumber difference from the incident light. With longer excitation wavelengths, the Raman band decreases in intensity, becomes further displaced from the Rayleigh scatter band, and has a wider bandwidth [12]. At longer wavelengths therefore, both Rayleigh and Raman scatter are much reduced.

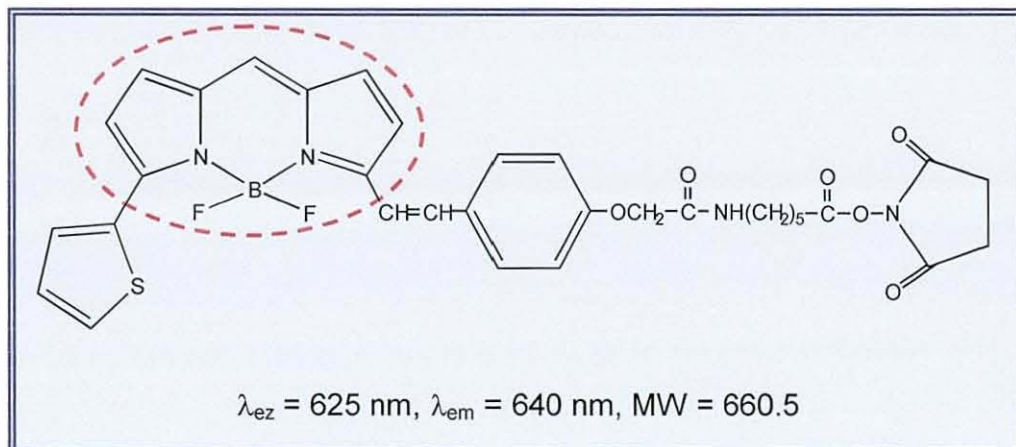
Background fluorescence originating from endogenous fluorescent species and from Raman and Rayleigh scattering can greatly limit the potential sensitivity of the analysis. However, long wavelength fluorescence detection in the region 600 – 1000 nm has proven to reduce interference [13].

Photochemical theory predicts a sharp diminution in the quantum yields of fluorophores as their emission wavelengths increase [14]. In practice the long wavelength region boasts a few fairly intense fluorophores, with almost all other

1.1.4.3 BODIPY® fluorophores

The BODIPY dyes are dibenzopyrrometheneboron difluoride compounds and were synthesised for the first time by Treibs and Kreuzer [19], in 1968. Later an ionic derivative was synthesised by Worries *et al.* [20], and several reactive forms have been prepared recently by Kang and Haugland [21]. All the BODIPY dyes have a difluoro-bora-diaza-indacene structure indicated by the red-circled moiety shown in figure 1.4. Different substituents are then added onto this basic structure to produce different BODIPY dyes with different excitation and emission wavelengths. The particular BODIPY dye that has been used in this study is shown in figure 1.4. Numerous sources of information can be found on these fluorophores [22, 23, 24].

Figure 1.4 – Structure of BODIPY® 630 / 650 –X, succinimidyl ester



Advantages:

- high extinction coefficients (100 300 $\text{cm}^{-1} \text{M}^{-1}$ for BODIPY® 630/650-X, SE))

- high fluorescence quantum yields (often approaching 1.0, even in water)
- spectra that are insensitive to solvent polarity and pH due to lack of ionisable groups (hence no ionic charge)
- narrow emission bandwidths
- greater photostability than fluorescein – they remain intensely fluorescent even when constantly illuminated

Disadvantages:

- expensive (~£153 per 5 mg BODIPY® 630/650-X, SE)
- small Stokes shift (15 nm)

1.1.4.4 Cyanine dyes (Cy™ 5 and Cy™ 5.5 bisfunctional dyes)

Papers are available for more comprehensive reading on the cyanine dyes Cy 5 and Cy 5.5 [25, 26, 27].

Figure 1.5 – Structure of Cy™ 5 bisfunctional dye

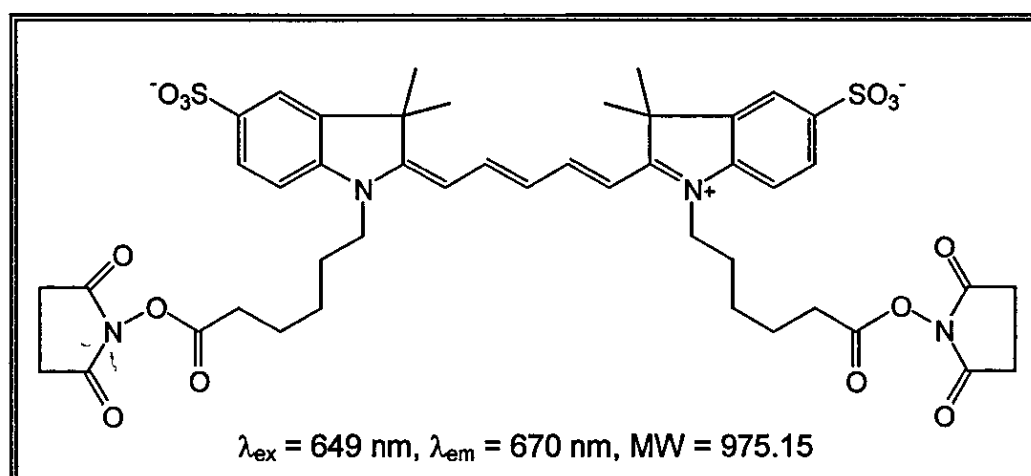
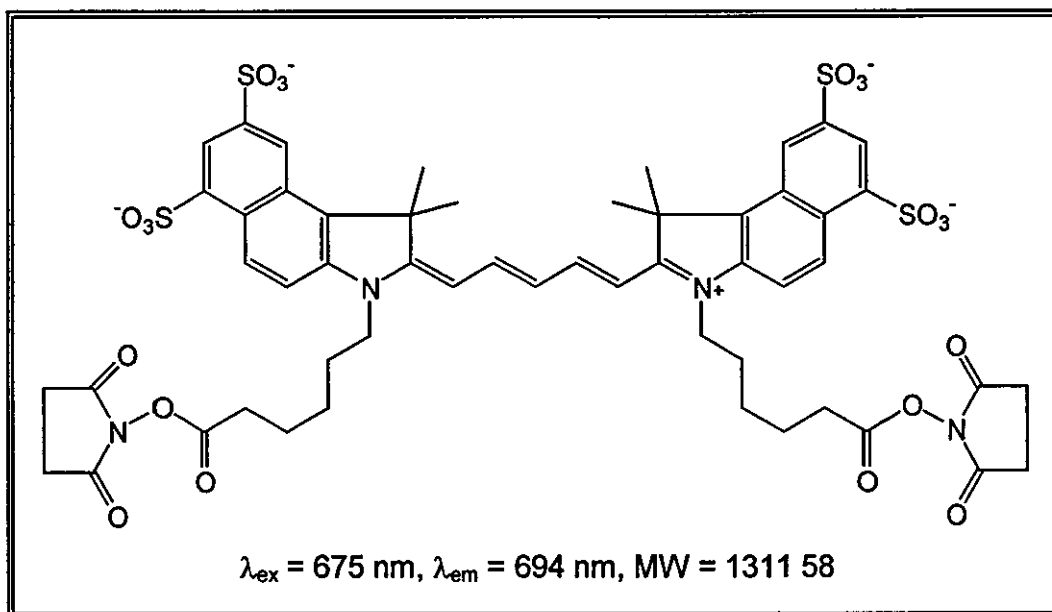


Figure 1.6 – Structure of Cy™ 5.5 bisfunctional dye**Advantages:**

- high extinction coefficients ($250,000 \text{ cm}^{-1} \text{ M}^{-1}$)
- quantum yields of >0.28
- highly water soluble

Disadvantages:

- small Stokes shift (21 nm for Cy 5 and 19 nm for Cy 5.5)
- expensive ($\sim\text{£}25.00$ per mg)

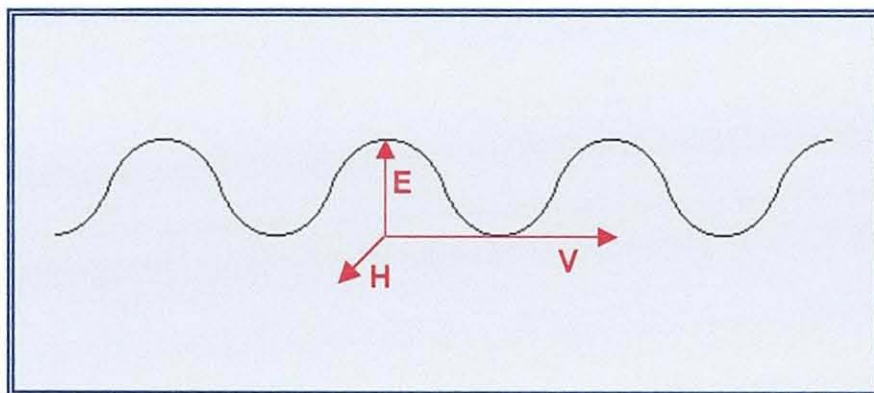
1.2 FLUORESCENCE POLARISATION

The practical application of fluorescence polarisation to the determination of proteolytic enzymes has been carried out in order to evaluate the potential of this detection method for use in protease high throughput screening assays. There are numerous references on the theory of fluorescence polarisation and its applications [28, 29, 30, 31, 32]. The theory of fluorescence polarisation is crucial to the understanding of the idea behind these assays and will therefore be described.

1.2.1 Theory of fluorescence polarisation

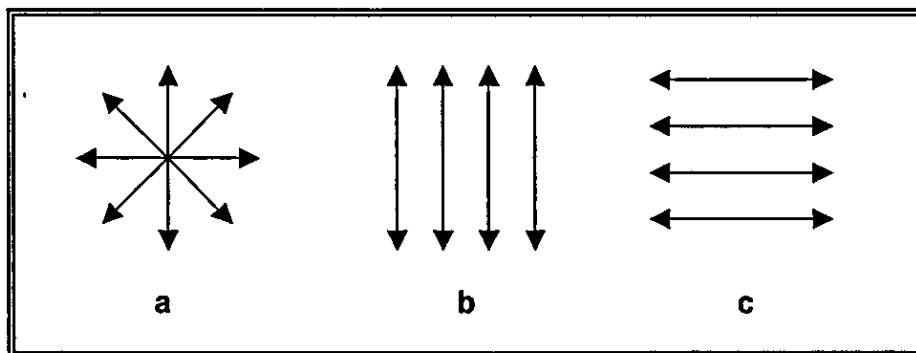
The concept of molecular movement and rotation is the basis of fluorescence polarisation. To fully understand the theory of fluorescence polarisation, consider the wave model of light as shown in figure 1.7.

Figure 1.7 - The wave model of light (V = velocity, E = electric field, H = magnetic field, vectors)



This single ray may be characterised by the orientation of its electric vector within a co-ordinate system. Thus, if one looked directly into an oncoming ray, the electric vector would be at some angle θ . Likewise, a beam composed of a number of rays could be characterised by the angular distribution of its electric vectors. Two extreme situations are possible: the vectors can be evenly (randomly) distributed (figure 1.8a), or they can be parallel (1.8b and 1.8c).

Figure 1.8 – Electric vectors of light randomly distributed (a) and parallel (b and c)



These two situations can be differentiated experimentally by viewing light through a polariser, which allows maximum transmission of light when it is aligned parallel to the electric vector and zero transmission when it is perpendicular. Therefore, as the polariser is turned through 180° in 1.8a, the intensity remains constant. However, in 1.8b the intensity would be a maximum with the polariser vertical and zero with it horizontal, the converse being true for 1.8c.

It is this experimental observation that allows one to define the polarisation of light as: $p = \frac{(V-H)}{(V+H)}$ (equation 1), where p = polarisation, V = vertical component of emitted light, H = horizontal component of emitted light, when excited by vertical plane polarised light.

As can be seen by equation 1, P ("polarisation unit") is a dimensionless entity and is not dependent on the intensity of the emitted light or on the concentration of the fluorophore. This is a fundamental power of fluorescence polarisation. For convenience the term "mP" (pronounced "millipee") is used, where 1 mP equals one thousandth of a P . Applying equation 1 to the situation in figure 1.8a the two components (V and H) are always equal and $p = 0$. In 1.8b, $H = 0$ and $p = 1$, while in 1.8c $V = 0$ and $p = -1$. When $p = 0$, the light is called *unpolarised* or *natural light*. With $p = \pm 1$, it is *completely polarised*, whereas with $1 \geq p \geq -1$, but not equal to 0, it is *partially polarised*.

1.2.1.1 Dipole model of absorption and emission

Light is absorbed by and emitted from molecules via electric dipoles, which have certain orientations relative to the structure of the molecule. Maximum absorption of light occurs when the absorption dipole is parallel to the electric vector of the light. Specifically, the probability of absorption is proportional to $\cos^2 \theta$ where θ is the angle between the dipole and the electric vector. Therefore, absorption is a maximum when $\theta = 0^\circ$ and is zero when $\theta = 90^\circ$.

Emission from a dipole can occur with the electric vector at any angle to the dipole, but the probability is proportional to $\sin^2 E$ where E is the angle between the dipole and the electric vector.

1.2.1.2 Depolarisation

The sequences of events that occur in fluorescence have already been discussed in section 1.1.1. In each of these processes, changes can occur such

that the polarisation of the fluorescent light is different from that of the exciting light.

The absorption process itself is the first source of depolarisation. As discussed in section 1.2.1.1 when undergoing excitation with vertically polarised light the vertical dipoles will be preferentially excited. In reality emission from these dipoles could give only partial polarisation, as non-vertical dipoles do absorb some of the vertically polarised excited light.

If the emission dipole is at an angle to the absorption dipole, a second source of depolarisation is evident which is dependent upon the angle between the two dipoles, the larger the angle the greater the depolarisation.

A third important depolarising factor is the Brownian rotation of the molecule. Rotation of the molecule will result in rotation of the emitting dipole to a new orientation. Therefore, if the excited state lifetime is comparable to the time required for Brownian rotation to occur over an appreciable angle, significant depolarisation results.

Depolarisation can also result from non-radiative energy transfer. Transfer takes place preferentially between parallel dipoles, but nonparallel dipoles can also undergo transfer with resulting depolarisation.

A final mechanism of depolarisation is trivial reabsorption the light emitted from one molecule is absorbed by another molecule, which then reemits it at a different orientation.

The term p_0 is defined as the intrinsic polarisation of a molecule in the absence of any depolarisation due to Brownian rotation or intermolecular processes.

1.2.1.3 Relationship between fluorescence polarisation and molecular weight

Rate of rotation of a molecule is described by the Stokes equation:

$\rho = \frac{3\eta V}{RT}$ (equation 2), where ρ = rotational relaxation time, η = viscosity of the

medium, V = molecular volume, R = gas constant, and T = temperature in degrees Kelvin.

The molecular volume is defined by: $V = vM$ (equation 3), where V = molecular volume, v = partial specific volume, and M = molecular weight in Daltons.

From equations 2 and 3 it can be seen that the higher the molecular weight of a molecule the higher the molecular volume and therefore the higher the rotational relaxation time will be.

The Perrin equation (equation 4), which was first described in 1926 [33], describes the relationship between the observed fluorescence polarisation, the limiting polarisation, the fluorescence lifetime of the fluorophore, and its rotational relaxation time: $\left(\frac{1}{P} - \frac{1}{3}\right) = \left(\frac{1}{P_0} - \frac{1}{3}\right) \left(1 + \frac{3\tau}{\rho}\right)$ (equation 4), where P = polarisation,

P_0 = limiting polarisation, τ = fluorescence lifetime, and ρ = rotational relaxation time. From equation 4 it can be seen that the smaller the fluorescence lifetime, the higher the fluorescence polarisation will be. The smaller the rotational relaxation time, the smaller the fluorescence polarisation will be.

Combining the Stokes equation (equation 2) and the Perrin equation (equation 4), the relationship between the molecular weight of a molecule and its fluorescence polarisation is achieved – the fluorescence polarisation of a fluorophore is proportional to its rotational relaxation time, which in turn depends

upon its molecular volume and hence its molecular weight ($1/P$ is proportional to $1/M$). The smaller the molecule, the faster it rotates, and so the lower the fluorescence polarisation will be. This relationship stands provided that the temperature and solution viscosity remain constant.

1.2.2 Advantages of fluorescence polarisation

- fluorescence polarisation is a homogenous technology requiring no immobilisation, washing or separation
- measurements are very rapid
- reagents are stable
- large batches may be prepared which results in high reproducibility
- fluorescence polarisation has proven to be highly automatable
- in theory fluorescence polarisation is independent of intensity, so is relatively immune to the inner filter effect and so works in coloured solutions and cloudy suspensions
- fluorescence polarisation is relatively insensitive to instrument changes such as drift, gain settings, lamp changes, etc
- fluorescence intensity is obtained in addition to polarisation, if required

1.2.3 Disadvantages of fluorescence polarisation

- fluorescence polarisation is instrument dependent, the quality of the results is directly linked to the quality of the instrument
- the difference between the size of the starting material and the end material should be significant enough to give a reasonable polarisation change

1.3 FLUORESCENCE RESONANCE ENERGY TRANSFER (FRET)

1.3.1 Theory of FRET

Much of the work presented in this thesis relies upon the phenomenon of FRET. As numerous sources of information are available on the theory of FRET [34, 35, 36, 37], it will not be discussed in great detail here. However, as this technique is crucial to understanding the theory behind much of the practical work, a brief description will be given.

FRET is a distance-dependent interaction between the electronic excited states of two dye molecules in which energy from an electronically excited molecule (the donor fluorophore) is shifted to a neighbouring molecule (the acceptor fluorophore), returning the donor molecule to its ground state without the emission of a photon. Once excited, the acceptor can undergo de-excitation by the same emissive and non-emissive processes that occur upon excitation of a fluorophore

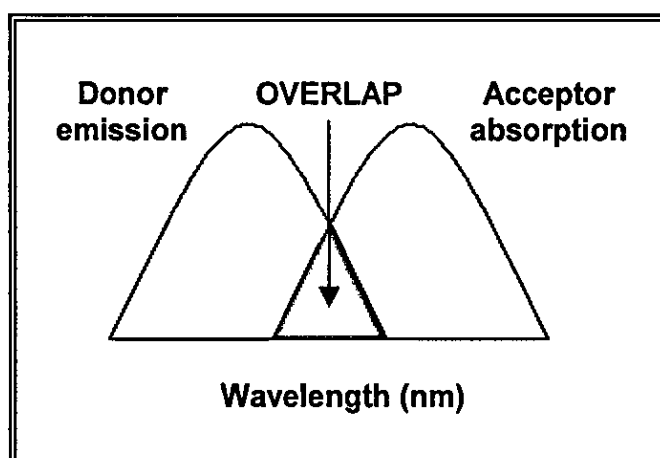
Although the phenomenon of resonance energy transfer was observed by Perrin at the beginning of the 1900's, it was Forster who proposed a theory describing long-range molecular interactions by resonance energy transfer in the late 1940's [38]. Forster derived a transfer rate equation that related the interchromophore distance and the spectroscopic properties of the chromophores. The rate constant for energy transfer is proportional to the inverse sixth power of the intermolecular separation, making it useful over distances comparable with the dimensions of biological macromolecules [39]. Thus, FRET is an important technique for investigating a variety of biological phenomena where markers that track physical proximity are necessary or useful. As the efficiency of FRET is proportional to r^{-6} where r = distance of intermolecular separation, not only does

energy transfer only occur over short ranges, but it definitely does not occur over long ranges. Energy transfer is thus an excellent method for use in protease and protease inhibition assays where proteolytic action completely abolishes the energy transfer between fluorophores that occurs in the intact protein. Digestion of the protein substrate separates the fluorophores, increasing the distance of intermolecular separation and so completely preventing the phenomenon of energy transfer from being able to occur.

1.3.2 Conditions for FRET

A number of conditions are crucial for the mechanism of FRET to occur. The primary requirement for FRET is that the energy lost by de-excitation of the donor molecule be matched by the energy required for excitation of the acceptor. In other words, the absorption spectrum of the acceptor molecule must overlap the emission spectrum of the donor molecule, as shown in figure 1.9.

Figure 1.9 – Schematic representation of spectral overlap required for FRET



The spectral overlap seen in figure 1.9 can involve two chemically different molecules as donor and acceptor (as is the case in the protease substrate

described in chapter 4), or two identical groups due to the common tendency for the excitation and emission spectra of fluorophores to overlap (as is the case in the protease substrate described in chapter 5).

Donor and acceptor molecules must also be in close proximity – typically 10 – 100 Å. FRET is a distance-dependent energy transfer, and the distance at which energy transfer is 50 % efficient (i.e. 50 % of excited donors are deactivated by FRET) is defined by the Förster radius (R_0). The magnitude of R_0 is dependent upon the spectral properties of the donor and acceptor dyes (the magnitude of FRET is dependent upon the quantum yield of the donor), and for good donor-acceptor pairs, R_0 values of 30 to 60 Å are common.

One other fundamental requirement for FRET is that donor and acceptor transition dipole orientations must be approximately parallel.

1.3.3 FRET donor / acceptor pairs

In recent years, numerous donor / acceptor pairs have been proposed; these include 5-(2-aminoethyl)aminonaphthalene-1-sulphonyl (EDANS) / 4-(4-dimethylaminophenylazo)benzoyl (DABCYL) [40], fluorescein / rhodamine [41], pairs based upon the BODIPY® dyes [42], coumarin / fluorescein [43], o-aminobenzoyl (Abz) / 3-nitrotyrosine [44], as well as near-infrared squaraine dyes Sq635 / Sq660 [45]. Different donor / acceptor dye pair combinations have previously been discussed with regard to dye pairs with superior spectroscopic properties [46].

Non-fluorescent acceptors exist which accept energy from a donor without any resulting fluorescence emission. These acceptors as a group are known as “dark quenchers”, for example DABCYL. These dyes have the particular advantage of

eliminating the potential problem of background fluorescence resulting from direct acceptor excitation.

1.3.4 Advantages of FRET

- simultaneous observation of quenching and enhancement effects allows specific effects to be distinguished from non-specific environmental effects on fluorescence intensity
- distances or changes in distances can be measured in a complex of molecules
- structural information can be gained

1.3.5 Disadvantages of FRET

- direct excitation of acceptor causes a high background
- the availability of adequate donor / acceptor pairs is somewhat limited, especially at near infrared wavelengths
- when measuring a change in distance between two probes, the result is scalar and gives no indication of which probe (donor and / or acceptor) moves
- the presence of free unbound labels in solution could mask a change in energy transfer

1.4 FLOW INJECTION ANALYSIS (FIA)

1.4.1 History of flow injection analysis

The term flow injection analysis was coined in the early spring of 1974 at the Technical University of Denmark by Ruzicka and Hansen [47]. FIA was used to describe a type of continuous flow analysis that utilises a flowing stream, unsegmented by air bubbles, into which highly reproducible volumes of sample are injected. Application of this principle to automatic analysis yields a fast, precise, accurate, and extremely versatile system that is simple to operate.

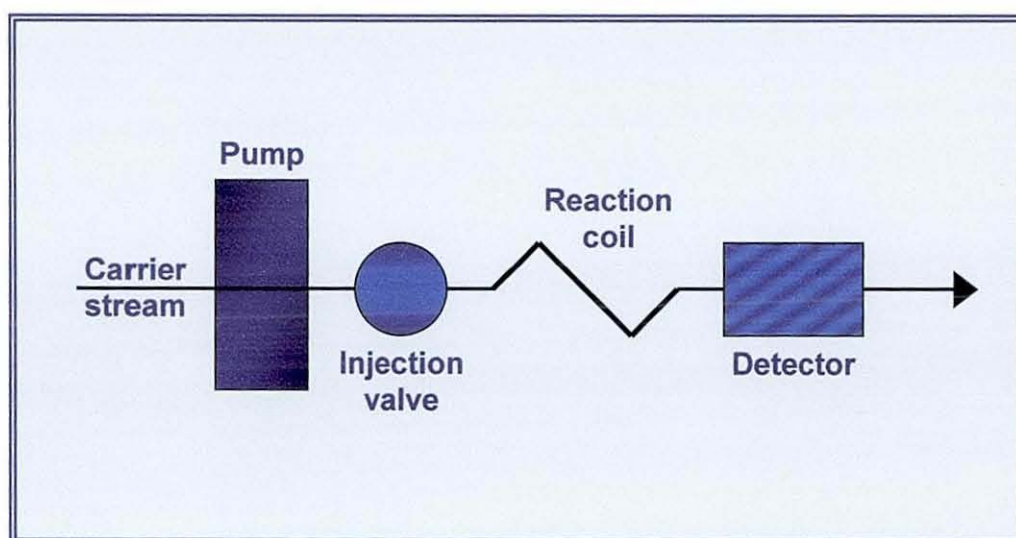
The concept of FIA received earliest attention from the electrochemists. In 1970 Nagy, Feher, and Pungor published their first in a series of papers describing injection of a sample into a flowing stream of electrolyte [48]. Their system involved passage of the sample carrier stream through a magnetically stirred mixing chamber followed by flow past a silicone-rubber-based graphite electrode.

This technique was subsequently modified simultaneously by Stewart, Beecher, and Hare in the United States [49] and by Ruzicka and Hansen in Denmark [47]. Their primary innovation was in the use of flow-induced sample dispersion as the sample carrier stream was pumped through narrow bore tubing. This allowed controlled mixing of the sample as opposed to the gross mixing generated in the mechanically stirred chamber, thus avoiding excessive sample dilution. The Danish group developed the method using primarily instrumentation normally associated with segmented flow analysers (SFA). In contrast, the American group based their initial work on high-performance liquid chromatography (HPLC) components.

1.4.2 Theory of flow injection analysis

Theory, applications, and developments of FIA are covered by many papers [50 - 57] and books [58, 59]. The technique of FIA depends on four primary factors: unsegmented flow, sample injection, reproducible timing, and controllable sample dispersion. A typical FIA system is shown in figure 1.10 and usually consists of the following components: (a) a pump (commonly a peristaltic pump), which is used to propel the carrier stream through a thin tube at a steady pulse-less rate; (b) an injection port, which allows a well-defined volume of sample solution to be injected into a carrier stream in a reproducible manner without stopping the flow; (c) a reaction coil in which the sample zone disperses and reacts with the components of the carrier stream; and (d) a flow-through detector, for example a fluorescence spectrophotometer, which transduces some property of the analyte into a continuous signal which is recorded. A typical detector output has the form of a peak, the height and area of which is related to the concentration of the analyte.

Figure 1.10 – Schematic diagram of a typical simple FIA system



When the sample is first injected, it forms a well-defined sample plug in the stream. As the sample is swept downstream through the narrow bore tubing, the plug disperses into, and thus mixes with, the carrier stream under laminar flow conditions to form a gradient. The magnitude of this dispersion is dependent upon the operating parameters applied to the system, including sample volume, tubing bore size, tubing length, flow rate, the molecular diffusion coefficient of the species concerned, and, possibly, coil diameter. By changing these parameters, the dispersion can be easily manipulated to suit the requirement of a particular analytical procedure so that an optimum response is obtained at minimum time and reagent expense.

For the purpose of FIA, the dispersion (D) is defined as the ratio of concentrations before and after the dispersion process has taken place in the element of fluid that yields the analytical readout. The dispersion or dilution of a sample in an FIA system is given by the following equation: $D = \frac{C_0}{C_{\max}}$ (equation 5), where D is the dispersion coefficient at the peak maximum produced by the ratio between C_0 , the concentration of a pure dye, and C_{\max} , the concentration of that same injected dye as it passes through the detector.

A number of alternative modes of FIA have been developed, illustrating the versatility of this technique. These include merging zone [60], stopped flow [61], sequential injection [62 - 64], bead injection [65], all injection analysis [66], and multi-component [67 - 72] techniques.

Flow injection analysis has also been used on a miniature scale where microfluidic devices commonly have channels 200 μm wide and 100 μm deep with typical channel lengths of 2 – 3 cm long. This is a vast difference to those dimensions used in typical FIA where tubing of around 0.8mm internal diameter with lengths of the order of tens of cm are typically used.

1.5 HIGH THROUGHPUT SCREENING (HTS)

1.5.1 Concept of high throughput screening

Drug discovery is a numbers game. The well-known attrition rate of candidates screened to drugs on the market is dismal (commonly cited to be 5,000 – 10,000 to 1). With increasing pricing pressure on marketed drugs, escalating research costs and competition, and expiring patents on legacy cash cows, the pharmaceutical industry needs to improve its efficiency [73]. One simple, but hopefully effective, strategy is to screen more compounds more efficiently: test more, discover more.

High throughput screening (HTS) is the process by which large numbers of compounds are tested, in an automated fashion, for activity as inhibitors or activators of a particular biological target, such as a cell surface receptor or a metabolic enzyme.

It can be seen from figure 1.16 that since 1995 the number of published articles on high throughput screening has increased from year to year, illustrating the importance that research in this area is gaining.

Nearly all drug discovery research projects in the pharmaceutical industry employ HTS assays as initial steps to discover the chemical leads. The primary role of the technology is to detect lead compounds and supply directions for their optimisation using other techniques. Many properties critical to the development of a successful drug cannot be assessed by HTS, such as specificity, toxicity and pharmacokinetics. The dropout rate during development is still considerable and is associated with high financial losses in the pharmaceutical industry. Only one out of approximately ten potential drug candidates entering phase I of

development will finally reach the market. Insufficient pharmacological and pharmaceutical properties represent the majority of reasons for failure.

1.5.2 History of screening

In the 1970s, lead identification typically involved the manual synthesis of about 2 g of a compound with screening being carried out *in vivo* by injecting about 80 mice. The major pharmaceutical companies screened approximately two compounds per week.

In the 1980s, *in vivo* tests were largely replaced by *in vitro* biological assays which were faster and required less compound to be synthesised – screening increased to tens of compounds per week.

HTS has been developed in the last few years to test several thousands of compounds for biological activity per day. Companies are now looking to screen hundreds of thousands of compounds per day per machine.

Although any assays performed on the bench top can, in theory, be applied to HTS, conversion to an automated format imposes certain constraints that affect the design of the assay in practice. Procedures that are routine at the bench are often extremely difficult to automate. Also, the more steps required for an assay, the more difficult to automate the HTS. The ideal assay is one that can be performed in a single well with no other manipulation other than addition of the sample to be tested.

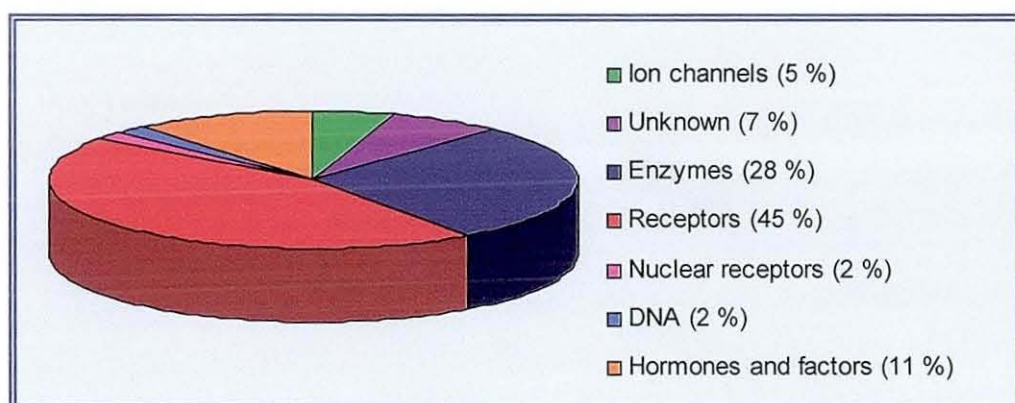
A number of assay formats have been developed or modified over the past few years to conform to the constraints imposed by HTS. Several of the more recently developed or exploited assay protocols for HTS have been the focus of several papers [74, 75].

1.5.3 The drug discovery process

Drug discovery is a complex process, which generally starts with the identification and characterisation of a target like an enzyme or other protein implicated in a disease.

A comprehensive analysis of the drug targets underlying current drug therapy undertaken in 1996 showed that present-day therapy addresses only about 500 molecular targets and as shown in figure 1.11, cell membrane receptors, largely heterotrimeric GTP-binding protein (G protein)-coupled receptors, constitute the largest subgroup with 45 % of all targets, and enzymes account for 28 % of all current drug targets. This analysis was discussed in an excellent review that also covers the evolution of drug discovery and discusses the historical aspects of drug discovery from its infancy to the current day [76].

Figure 1.11 – Biochemical classes of drug targets of current therapies



HTS assays are developed to screen for a desired activity against the target. The introduction of robotic HTS, with its ability to devour up to 100,000 samples per day per machine in mass screening, has given rise to an insatiable demand for new compounds and the flood of targets that will ultimately come from the Human Genome Project will further compound this demand. Continuing

advances in molecular biology, human genetics, genomics [77], and proteomics [78, 79] have also accelerated identification of the mechanisms underlying a growing number of human diseases. This progress has increased the number of novel protein targets available for potential therapeutic intervention by drug treatment. Historically, a medicinal chemist is able to synthesise 25 – 100 compounds per year one-by-one, depending upon complexity. Clearly this classical approach is a totally unsatisfactory way to feed the HTS process.

Combinatorial Chemistry (Combi Chem) is an attempt to increase the productivity of medicinal chemists by synthesising large numbers of compounds with the help of robots using standardised chemistry (dozens to millions of compounds in one run, depending upon technology).

The bottleneck in drug development has shifted from the generation of lead structures to their transformation into orally active drugs with the desired physiological properties and performance results in clinical trials. It will therefore no longer be enough to synthesise large and diverse libraries; rather, it will become more and more important to find ways to integrate demands such as bioavailability at an early stage of the drug search. These challenges can only be met by creating efficient interfaces between combinatorial syntheses and bioassays. Recent developments in this area have been the focus of a recent article [80].

A selection of compounds from combinatorial chemistry is screened using the HTS assays to identify active compounds. Hits identified in this first round must be retested to confirm their activity. By synthesising a series of compounds that are chemically related to the lead compound, lead optimisation then attempts to improve the activity, availability, stability, and specificity while reducing toxicity and potential side effects. Their improved biological activity is reconfirmed and, those few that seem to be promising are then moved forward into pre-clinical animal studies to evaluate biological activity *in vivo*.

1.5.4 Miniaturised high throughput screening

The transition from slow, low throughput screening to industrialised robotic ultra high throughput screening in the last few years has made it possible to screen hundreds of thousands of compounds against a biological target in a short time-frame. The need to minimise the cost of screening has been addressed primarily by reducing the volume of sample to be screened. This, in turn, has resulted in the miniaturisation of HTS technology as a whole.

Miniaturisation of HTS results in three significant benefits that address the pharmaceutical industry's needs: lower costs, faster turnaround, and reduced space requirements. Approximately three-quarters of the costs of screening derive from the costs of both chemical and biological reagents. Miniaturisation reduces these costs in proportion to the reduction in volume (typical flow dimensions were given in section 1.4.2). Miniaturisation also allows for the compression of more samples onto one system, reducing sample-to-sample distance. As sample transport is a significant time factor in screening, the total analysis time per sample is reduced significantly by miniaturising. Due to the picoliter volumes of highly miniaturised systems, and the high-density formats that can be designed, assay throughput can be increased without an associated research and development cost. The concept, scope, and impact of miniaturised methods as applied to HTS have been explored [81, 82]. Trends in the field of ultra high throughput screening and the technological developments that are necessary to enable the routine application of miniaturised systems within an industrial environment have been summarised [83].

In a recent paper, the parallel development, adaptation, and integration of different microelectronic sensors into miniaturised biochips was demonstrated [84]. This was carried out for a multiparametric, functional on-line analysis of living cells in physiological environments, which would be applied to HTS

1.6 ENZYMES

1.6.1 Introduction to enzymes

Enzymes are the reaction catalysts of biological systems. They have high molecular weights ranging from around 5,000 to over 1 million. They increase the rate of chemical reactions taking place within living cells without themselves suffering any overall change. The reactants of enzyme-catalysed reactions are termed *substrates* and each enzyme is quite specific in character, acting on a particular substrate or substrates to produce a particular product or products via an enzyme-substrate intermediate.

With the exception of a small group of catalytic RNA molecules, all enzymes are proteins. Their catalytic activity depends upon the integrity of their native protein conformation. If an enzyme is denatured or dissociated into subunits, catalytic activity is usually lost. If an enzyme is broken down into its component amino acids, its catalytic activity is always destroyed. Thus the primary, secondary, tertiary, and quaternary structures of enzymes are essential to their catalytic activity. The part of the tertiary structure that is responsible for the catalytic activity is called the 'active site' of the enzyme, and often makes up only 10 – 20 % of the total volume of the enzyme. The active site contains the residues that directly participate in the making and breaking of bonds. These residues are called the catalytic groups.

Water is normally excluded from the active site unless it is a reactant. The nonpolar character of much of the active site enhances the binding of substrate. However, the active site may also contain polar residues, creating a microenvironment in which certain of these residues acquire special properties essential for catalysis. In addition to hydrophobic interactions, electrostatic

bonds, hydrogen bonds, and van der Waals forces mediate reversible interactions between enzyme and substrates. These highly specific non-covalent enzyme-substrate binding interactions confer upon the enzyme high substrate specificity.

Many sources of literature covering the structure, function and basic concepts of enzymes are available for a more detailed discussion [85 - 87].

1.6.2 Factors affecting enzyme activity

Several factors affect the rate at which enzymatic reactions proceed – temperature, pH, and the presence of any inhibitors or activators.

Like most chemical reactions, the rate of an enzyme-catalysed reaction increases as the temperature is raised to a maximum level, known as the optimum temperature. Activity then abruptly declines with further increase of temperature, normally due to denaturation of the enzyme.

Enzymes are affected by changes in pH. The most favourable pH – the point where the enzyme is most active – is known as the optimum pH. Extremes of pH generally result in complete loss of activity for most enzymes where activity may not be restored even after re-adjustment of the pH.

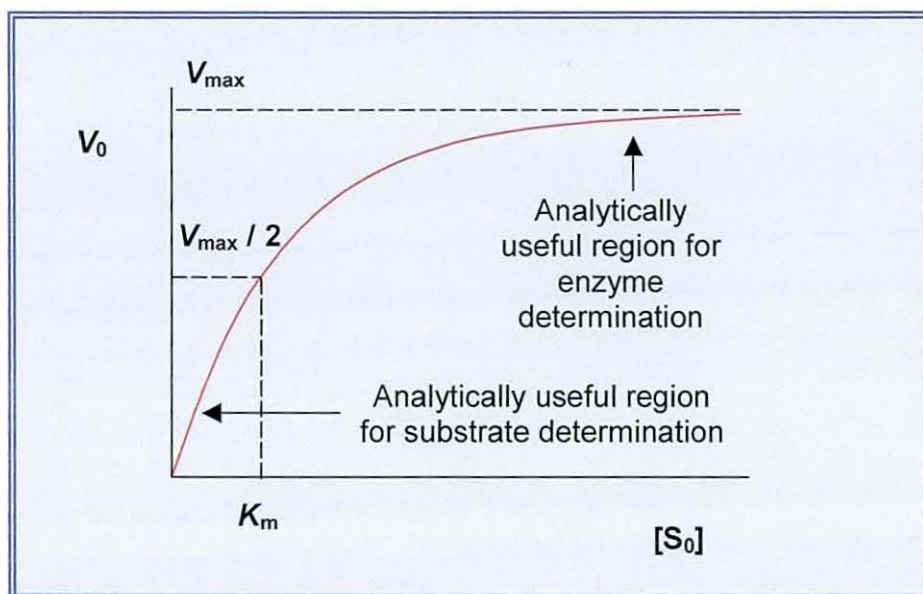
Enzyme inhibitors are substances that alter the catalytic action of the enzyme and consequently slow down, or in some cases, stop catalysis. Enzyme inhibition is discussed in more detail in section 1 9.

Activators are substances that increase the catalytic activity of an enzyme, are used in very small amounts and which, unlike coenzymes, do not participate in the enzyme reaction.

1.6.3 Enzyme kinetics

Experimental evidence shows that for many single-substrate enzyme-catalysed reactions a hyperbolic relationship exists between initial velocity v_0 and initial substrate concentration $[S_0]$ as shown in figure 1.12.

Figure 1.12 - Dependence of initial velocity on substrate concentration for a single-substrate enzyme-catalysed reaction

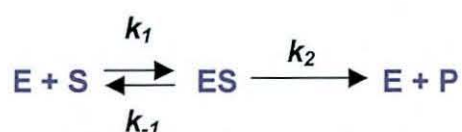


The graph V_0 versus $[S_0]$ (figure 1.12) shows three distinct regions:

1. At low substrate concentration the velocity of the reaction is directly related to the substrate concentration; this is the region of first order kinetics.
2. At intermediate substrate concentration there is a big curve, where the velocity of the reaction increases more slowly with increase in the substrate concentration.

3. At high substrate concentration V_{max} is attained and the observed velocity is independent of further addition of substrate; this is the region of zero order kinetics.

Kinetic models to explain these findings were proposed by Henri (1903) and Michaelis and Menten (1913). These were essentially similar, but Michaelis and Menton did a great deal of experimental work to give their work a sounder basis. The critical feature in their treatment is that a specific enzyme – substrate (ES) complex is a necessary intermediate in catalysis. The model proposed, which is the simplest one that accounts for the kinetic properties of many enzymes, is:



where E = enzyme, S = substrate, ES = enzyme – substrate complex, and P = product. E combines with S to form ES with a rate constant of k_1 . ES then has two fates. It can dissociate to E and S with a rate constant of k_{-1} or proceed to form P with a rate constant of k_2 .

$V_0 = \frac{V_{max} [S]}{K_M + [S]}$ (equation 6) is the *Michaelis-Menten equation*, the rate equation for a single-substrate, enzyme-catalysed reaction. It is a statement of the quantitative relationship between the initial velocity V_0 , the maximum initial velocity V_{max} , and the initial substrate concentration [S], all related through the Michaelis-Menten constant K_M .

The meaning of K_M is evident from equation 6. When $[S] = K_M$, then $V = V_{max} / 2$. Thus K_M is equal to a substrate concentration at which the reaction rate is half its maximal value.

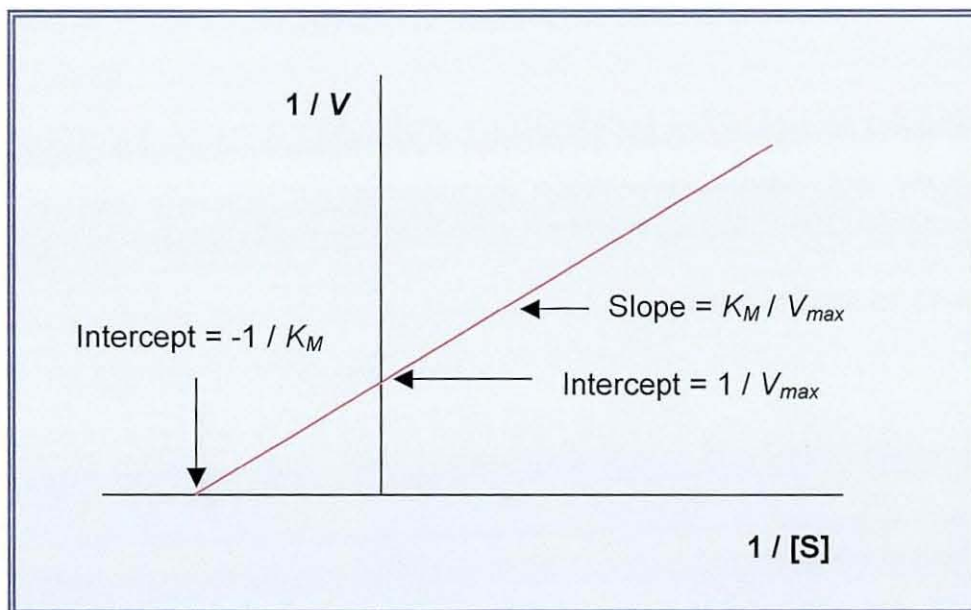
The Michaelis-Menten equation gives a hyperbolic curve as shown in figure 1.12. This is not entirely satisfactory for the determination of V_{max} and K_M . Unless, after a series of experiments, there are at least three consistent points on the plateau of the curve at different $[S]$ values, then an accurate value of V_{max} , and hence of K_M cannot be obtained.

This problem was overcome by Lineweaver and Burk (1934) without making any fresh assumptions. They simply inverted the Michaelis-Menten equation thereby

conveniently transforming it into a straight-line plot: $\frac{1}{V} = \frac{K_M}{V_{max}} \cdot \frac{1}{[S]} + \frac{1}{V_{max}}$

(equation 7). This is of the form $y = mx + c$, which is the equation of a straight line graph; a plot of y against x has a slope of m and intercept of c on the y -axis. As shown in figure 1.13, K_M and V_{max} can be read directly from the Lineweaver-Burk plot.

Figure 1.13 – Lineweaver-Burk plot



Numerous sources of literature are available that discuss enzyme kinetics in greater detail [88 - 90].

1.6.4 Proteolytic enzymes

Proteases are enzymes that catalyse the hydrolysis of peptide bonds. They are widely distributed in cells, and are either soluble, or associated with plasma membranes and subcellular organelles, or secreted. This extensive distribution of proteases in both organs and subcellular compartments suggests that they play a key biological role in both physiological and pathophysiological situations. In 1960 the biochemist Hartley [91] proposed that proteases should be classified according to their catalytic mechanism: serine proteases, cysteine proteases, aspartate proteases, and metallo proteases.

The serine proteases are characterised by the presence of a catalytic triad composed of aspartic acid (Asp), serine (ser) and histidine (His). Serine acts, in this case, as a very reactive residue, which forms a covalent bond with the substrate. Hydrolysis of the substrate occurs in two successive stages: acylation and deacylation of the enzyme. The carbonyl group of the peptide bond to be cleaved undergoes nucleophilic attack by the unusually reactive OH group of the active site serine, resulting in the formation of a covalent tetrahedral intermediate. Decomposition of the tetrahedral adduct results in the release of the amino portion of the substrate and the formation of an acyl serine derivative. The departing amino group receives a proton from the imidazole ring of histidine. Subsequent hydrolysis of the acyl enzyme to active enzyme and the carboxylic acid product occurs through a reaction sequence analogous to that involved in the formation of the acyl enzyme. This catalytic process is facilitated by the proximity at the enzyme's active site of the histidine and aspartate residues, which thus actively participate in the catalysis.

The cysteine proteases are characterised by the catalytic triad cysteine (Cys), histidine (His), and aspartic acid (Asp). Cysteine is a very reactive residue, which is involved in the formation of the intermediate complex with substrates.

The aspartate proteases are also called acid proteases due to their low pH optima. Their catalytic site consists of two aspartic acid residues. The enzyme-substrate intermediate is of the general acid-base type rather than the covalent type. The two aspartic acid molecules are linked through a hydrogen bond. Aspartate proteases facilitate nucleophilic attack by two simultaneous proton transfers: the first from a water molecule to the carboxylate ion of one aspartic acid residue, and the second from the carboxyl group of the second aspartic acid to the oxygen atom of the carbonyl group of the substrate. These transfers lead to the formation of a neutral tetrahedral intermediate. The same dual transfer mechanism destroys this intermediate, which donates a proton to the carboxylate ion of the first aspartic acid, during which the proton of the carboxyl group of the second aspartic acid is transferred to the nitrogen atom, thus hydrolysing the C-N bond of the substrate.

The metallo proteases have an active site that contains a metal ion, usually zinc (Zn^{2+}), which is an integral part of the protein structure. They do not form covalent enzyme-substrate intermediates. In addition to a metal ion, the catalytic site always contains a glutamic acid (Glu) residue, sometimes associated with tryptophan (Trp) or histidine (His).

Serine and cysteine proteases therefore form a covalent intermediate complex with their substrates because the amino acids at the catalytic site are very strong nucleophiles. Aspartate and metallo proteases, on the other hand, usually act by an acid-base catalytic mechanism, using a water molecule.

A variety of sources can be found in the general area of proteolytic enzymes [92 - 95].

The proteases play a key role in the digestion of food proteins, zymogen activation, activation of the complement system, and in blood coagulation and

fibrinolysis. They are also involved in the regulation of blood pressure, tissue regeneration, and in reproduction both during ovulation and the fusion of gametes. Proteases are also involved in immune response, processing of peptides and proteins, and growth and development. When the balance of proteases in these biological functions becomes upset, pathological conditions occur. As discussed in section 1.8.1, as the proteases are involved in virtually all biological functions and dysfunctions they represent an important drug target.

Five different proteolytic enzymes have been investigated in this work and thus a brief description of each is given below. An extremely comprehensive book covers databanks, name and history, activity and specificity, structural chemistry, preparation, biological aspects, and distinguishing features of proteolytic enzymes known to date [96].

1.6.4.1 Alkaline protease

Alkaline proteases are covered in detail elsewhere [97]. Enzyme commission number: unknown, type: serine, MW: 50,000, activity: ~ 26 Units / mg solid, specificity: broad, pH: maximal activity at pH 11.0, additional information: type XXI protease.

1.6.4.2 Proteinase K

Specific proteinase K information is available [98, 99]. Enzyme commission number: 3.4.21.64, type: serine, MW: 28,930, activity: 10 – 20 Units / mg protein, specificity: peptide bond adjacent to the carboxyl group of aliphatic and aromatic amino acids with blocked alpha-amino groups, pH: active over the range 7.5 to 12.0, but most often used in pH range 7.5 – 9.0, additional information: remains active in the presence of sodium dodecyl sulfate and urea.

1.6.4.3 Chymotrypsin

Enzyme commission number: 3.4.21.1, type: serine, MW: 25,000, activity: 40 – 60 Units / mg protein, specificity: hydrolyses peptide bonds with aromatic or large hydrophobic side chains (Tyr, Trp, Phe, Met) on the carboxyl end of the bond, pH: maximal activity around pH 8.0, additional information: α - and γ -chymotrypsins have identical primary structures, but different enzymatic properties, stabilities and tertiary structures - during experimental work α -chymotrypsin has been used.

1.6.4.4 Trypsin

Enzyme commission number: 3.4.21.4, type: serine, MW: 23,800, activity: 1,000 – 2,000 Units / mg protein, specificity: peptide bonds with arginine or lysine on the carboxyl end of the bond with a preference for arginine over lysine that is 2- to 10-fold, pH: maximal activity around pH 8.0, additional information: first described and named in 1876 by Kühne as the proteolytic activity in pancreatic secretions.

1.6.4.5 Pepsin

Enzyme commission number: 3.4.23.1, type: aspartic acid, MW: 35,000, activity: 3200 – 4500 Units / mg solid, specificity: preferentially cleaves C-terminal to phenylalanine, leucine, and glutamate, does not cleave at valine, alanine, or glycine, pH: pH optimum from 2 – 4 (irreversibly inactivated at pH > 6), additional information: active in 4 M urea and 3 M guanidine HCl.

1.7 ENZYME IMMOBILISATION

The term 'immobilised enzyme' describes an enzyme that has been chemically or physically attached to a water-insoluble matrix, polymerised into a water-insoluble gel, or entrapped within a water-insoluble gel matrix or water-insoluble microcapsule resulting in reduced or complete loss of enzyme mobility. These immobilised enzymes retain their catalytic activity and can be used repeatedly and continuously. The usage of binding enzymes onto solid materials goes back to 1916 when Nelson and Griffin [100] first immobilised invertase on charcoal and observed that the enzyme retained most of its activity over a long period of time. However, this work was largely ignored until the 1950s. Since then immobilisation of proteins in general, and enzymes in particular, has been widely studied and the number of applications of immobilised enzymes is increasing. Many sources of information can be found on the preparation, characteristics, and application of immobilised enzymes for more detailed reading [101 - 105].

1.7.1 Why immobilise enzymes?

There are several reasons for the preparation and use of immobilised enzymes. In addition to a more convenient handling of enzyme preparations, the two main benefits are (1) easy separation of the enzyme from the product, and (2) reuse of the enzyme.

Easy separation of the enzyme from the product simplifies enzyme applications and supports a reliable and efficient reaction technology. Reuse of enzymes provides cost advantages, which are often an essential prerequisite for establishing an enzyme-catalysed process in the first place.

Immobilised enzymes offer many more advantages over their soluble analogues than the two main benefits mentioned above [106]. These include:

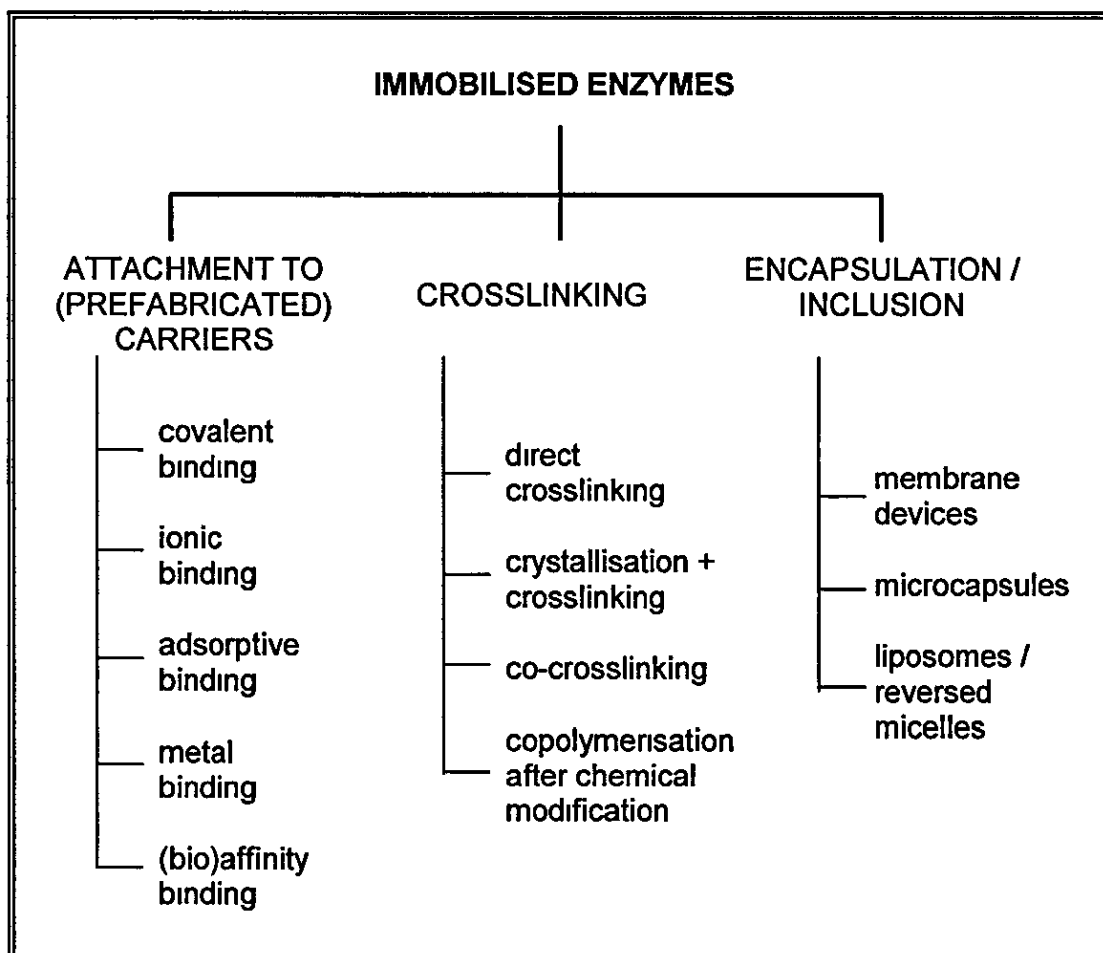
- increased stability against denaturing conditions such as high temperature and extreme pH during storage and use
- ease of use as no enzyme solution preparation is required
- greater applicability to multi-determination due to the ease of combining immobilised enzymes in a variety of designs in flow systems
- they do not contaminate the sample and insoluble enzymes can be easily recovered from batch experiments, while in the case of continuous flow systems the sample is passed through enzyme reactors

1.7.2 Immobilisation methods

Immobilised enzymes can be classified on the basis of the methods adopted for their immobilisation, and the classification of immobilisation methods according to different chemical and physical properties is shown in figure 1.14. Chemical methods of immobilisation include any that involve the formation of at least one covalent bond between residues of an enzyme and a functionalised water-insoluble support or between two or more enzyme molecules. Physical methods include any that involve localising an enzyme in any manner, which is not dependent upon covalent bond formation.

The immobilisation procedure should be conducted in a way that allows the enzyme to maintain its active conformation and necessary catalytic flexibility. Furthermore, catalytically essential residues of the enzyme should be undisturbed and conserved.

Figure 1.14 – Classification of immobilisation methods



1.7.2.1 Covalent binding method

The method of enzyme immobilisation that has been adopted for use in the practical work described in this thesis has been the use of the bi-functional reagent glutaraldehyde to covalently immobilise proteases onto the support controlled-pore glass (CPG). This method has been described elsewhere [107] and the reaction scheme of the coupling of enzyme to CPG is shown in figure 6.3.

Glutaraldehyde is one of the most extensively used linking reagents and has been discussed in detail elsewhere [108]. For this study, aminopropyl-CPG has been used. Glutaraldehyde has been used to covalently link proteolytic enzymes to the CPG as it has a high reactivity with an amino group. The reaction of an amino-terminal support with glutaraldehyde leads to the introduction of aldehyde groups onto the support that can bind enzymes. This reaction has been termed activation.

Controlled pore glass has gained much acceptance as a support for immobilisation and has been widely used over the last couple of decades [109 - 114].

CPG has many advantages as a support:

- high surface area giving high coupling of ligands and high yield
- high mechanical strength
- thermostable and autoclavable
- high flow rate giving higher throughput
- rigid glass structure which is immune to biological degradation and inert to solvents or changing conditions

1.7.3 Modification of enzyme properties after immobilisation

Immobilisation of an enzyme results in a change in many of the physical and kinetic properties of the corresponding soluble counterpart. One of the main concerns is the reduction in the biological activity due to immobilisation. However, immobilisation can also induce novel characteristics to make enzymes tolerant to even harsh environments. Some attempts to intentionally modify the catalytic behaviour have been reviewed [115]. These changes in enzyme

properties that occur upon immobilisation may be due to changes in the protein conformation after immobilisation, structural modification of the protein during immobilisation, or changes in the protein microenvironment resulting from the interaction between the support and the protein. Goldstein *et al.* made one of the earliest demonstrations that immobilisation can markedly affect the properties of enzymes [116]. It was shown that the pH-activity curves of immobilised trypsin, when compared with those of soluble trypsin, were displaced 2 – 3 pH units towards the alkaline region. This effect was attributed to a pH difference between the microenvironment of the polymer matrix and the bulk fluid.

Numerous enzymes have been reported with an increase in long-term storage and thermal stability upon immobilisation [117, 118]. This increase in stability may be due to the stabilisation of the native structure of the enzyme.

1.7.4 Immobilised enzymes in FIA

A variety of flow injection systems using immobilised enzymes have been described [119 - 129].

The application of immobilised enzymes to flow injection analysis systems allows the combination of immobilised enzymes in different arrangements to permit multi-determination. An excellent review discusses the determination of two or more species in the same sample with a single flow injection analysis system [70] and the simultaneous determination of several components by flow injection analysis using one or more detectors has also been discussed [71]. Analysis of more than one analyte is made possible by using immobilised enzyme reactors in parallel [130 - 132] or in series [133]. Striking examples of the advances that have been made in this area are the determination of a single analyte by using a single immobilised column and progressing towards the determination of five or six analytes [69, 134 - 135].

1.8 PROTEASE ASSAYS

1.8.1 Importance of protease assays

Since the discovery of pepsin in the late eighteenth century there has been continuing investigation into the chemistries and activities of the proteolytic enzymes. Recent years have seen a remarkable acceleration of the pace of this research, fuelled by numerous applications in biotechnology, and the realisation that the proteases are major therapeutic targets. As a result of worldwide media attention, the term "protease inhibitor" has been closely associated with HIV – so much so, that the publicity has eclipsed the fact that proteases are involved in virtually all biological functions and dysfunctions. Protease inhibitors for HIV work by blocking a key enzyme involved in viral replication, the HIV protease. First introduced in late 1995, protease inhibitors are widely used for the treatment of HIV infection, in combination with other antiretroviral drugs. Approximately 215,000 of the estimated 350,000 patients receiving treatment for HIV infection in the United States take at least one protease inhibitor. The worldwide market for protease inhibitors was nearly \$2 billion in 1999. However, the proteases are involved in many other conditions such as lung disease [136 - 138], osteoporosis [139], microbial infections [140, 141], viral infections [142 - 144], malaria [145], and the common cold [146]. As such, protease inhibitors have broad potential as a class of drugs [147].

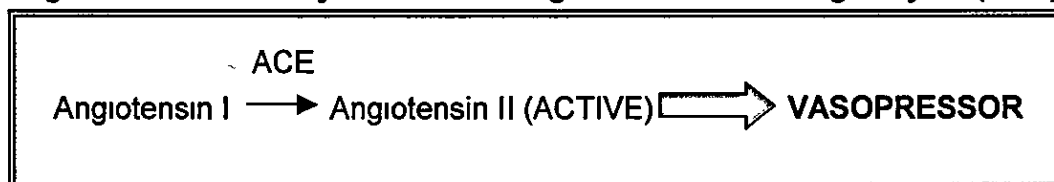
There are around 400 known human proteases and of these about 14% are currently under investigation as drug targets [148]. In addition to the involvement of these enzymes in so many disease states their popularity as drug targets also stems from the accumulated data on their enzymology, high throughput assays, biochemistry, physiology, pathology, 3D structures, small-molecule inhibitors, and endogenous inhibitory proteins. The Human Genome Project, by revealing all

potential proteins, will also have a major effect on both academic and pharmaceutical protease research.

Proteases as drug targets can be broken down into groups – the serine proteases, for example thrombin as a target for cardiovascular disease [149 - 152], cysteine proteases, e.g. the cathepsins as targets for inflammatory disease, the aspartic proteases, for example renin which has had tremendous effort expended on it for the treatment of cardiovascular disease [153], and also HIV inhibitors for the treatment of HIV [154], and the metalloproteases, for example angiotensin converting enzyme inhibitors for the treatment of cardiovascular diseases and more recently the matrix metallo protease inhibitors which are being looked at for inflammation and cancer [155 - 157].

One of the most successful groups of protease inhibitors from the pharmaceutical industry point of view over the last 20 years has been the discovery of the angiotensin converting enzyme (ACE) inhibitors. These have seen tremendous sales in the treatment of high blood pressure (hypertension) and heart failure. As shown in figure 1.15, the proteolytic angiotensin converting enzyme converts angiotensin I into an active form called angiotensin II. Angiotensin II is a vasopressor and so stimulates the contraction of the smooth muscle of blood vessels causing a rise in blood pressure. If the formation of the vasopressor angiotensin II could be prevented there would be a drop in blood pressure. A protease inhibitor that would stop angiotensin converting enzyme from working, hence preventing the formation of angiotensin II, would therefore be a potential drug for high blood pressure.

Figure 1.15 – Proteolytic action of angiotensin converting enzyme (ACE)



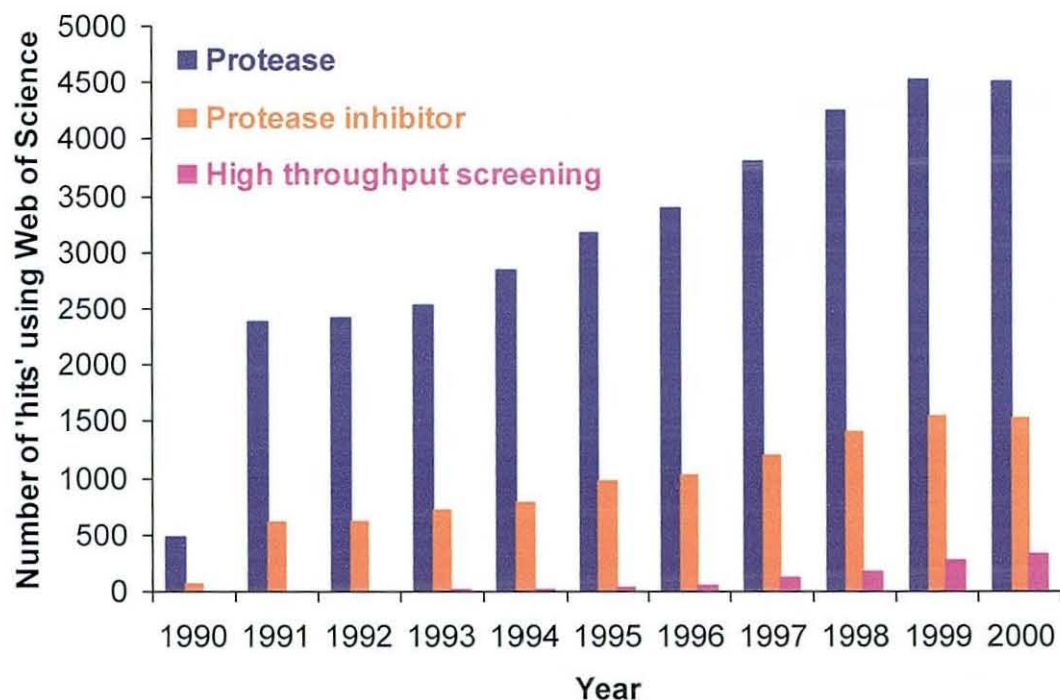
GENERIC NAME OF DRUG	TRADE NAME OF DRUG	SALES FOR FINANCIAL YEAR ENDING 2000
Lisinopril	Zestril + others	\$ 1, 800, 000, 000
Enalapril	Renitec + others	\$ 1, 800, 000, 000
Captopril	Capoten + others	\$ 400, 000, 000

Table 1.1 – Worldwide sales figures for selected angiotensin converting enzyme inhibitors as drugs

Table 1.1 shows just three compounds that are angiotensin converting enzyme inhibitors. Their “generic” names are given and one example each of a Trade Name (drugs are marketed under different Trade Names usually depending on the country of sale or whether the compound is licensed to additional companies for marketing). The worldwide sales for these three inhibitors alone were more than 4 billion US dollars. This is a very significant amount in drug sales terms and illustrates just how profitable protease inhibitors can be to pharmaceutical companies.

During screening for promising drug candidates, hundreds of thousands of compounds need to be assayed. Figure 1.16 shows the number of ‘hits’ i.e. the number of published articles, obtained on Web of Science when the terms protease, protease inhibitor, and high throughput screening are put as keywords. A trend can be seen over the last ten years of an increase in published papers in these three fields illustrating the importance of the application of high throughput screening assays for protease inhibitors. The development of a simple, sensitive, and efficient method for assaying protease activity, which would facilitate the testing of a large number of samples using HTS, was therefore investigated in this work.

Figure 1.16 – Trend in number of published articles in the areas of proteases, protease inhibitors and high throughput screening over the last decade



Detection of protease activity is also important for biochemical applications such as quantifying protease activity in cell or tissue extracts, during enzyme purification, and for quality control testing. Contaminating protease activity directly impacts product or reagent stability and biological efficacy in the synthesis, production, and purification of naturally occurring or genetically engineered recombinant proteins. Some protease assays may require detection of specific protease activity; for example certain pathogens use proteolytic activity as a virulence mechanism and differ from their non-pathogenic counterparts in this characteristic. Conversely, assay for contamination of biological preparations by unknown proteases requires a substrate that can detect a variety of proteases.

1.8.2 General protease assay methods

The concept of assaying proteolytic activity is not a new one and this type of assay has been around for many years. Gel electrophoresis [158 - 160], radioactivity [161], capillary electrophoresis [162], a dot-blotting method [163], immuno-quantification [164], and detection through an indicator enzyme [165] have all been used in the past to detect proteolytic enzymes. However, such methods are either time consuming or utilise relatively large quantities of substrates.

Several protease assays have been described that use HPLC to analyse the products of peptidase hydrolysis of peptide or protein substrates [166 - 169]. There are, however, several drawbacks to HPLC procedures, aside from the high cost of the equipment. Only one sample can be analysed at a time, turn-around times for injections may be long and will vary with the type of column and solvents used, contamination of the column and loss of resolution can occur, and analytical columns need to be replaced at regular intervals.

Luminogenic substrates have also been applied to the assay of proteolytic enzymes [170 - 174], as well as chromogenic substrates [175 - 178]. However, due to fewer luminescent species than fluorescent ones and the increase in sensitivity of fluorescence over absorbance-based methods, fluorescence spectroscopy seems more versatile as a method.

Different methods for assaying proteases have been reviewed elsewhere [179], and so discussions here will concentrate solely on fluorimetric protease assays as these are relevant to this particular work. Comprehensive reading on fluorimetric assays of proteolytic enzymes can also be found [180 - 183].

1.8.3 Fluorescent protease assay methods

Protease assays using a variety of fluorescently labelled substrates have been in use for many years [184 - 187]. These assays have frequently employed proteins such as haemoglobin [187], and fluorescent dye-labelled proteins such as fluorescein thiocarbamoyl casein (FTC-casein) [188 - 190]. These methods include precipitation assays where the undigested substrate is insoluble, such as fibrin [186], or is made insoluble by treatment with trichloroacetic acid or hydrochloric acid [187, 191]. However, such assays require sampling at time intervals, careful control of sample volume, and quantitative separation of the labelled peptides from the unhydrolysed protein.

1.8.3.1 Fluorescence polarisation protease assays

Fluorescence polarisation was first described in 1926 by Perrin [33]. This was expanded to cover biological applications by Weber [192 - 194]. The first instrumentation for the measurement of fluorescence polarisation was also developed by Weber [195]. Several groups have used this technique for the study of antigen-antibody and hormone-receptor interactions [196 - 199], as well as its use commercially for the monitoring of therapeutic drug levels [200]. Recently this technique has been introduced for high throughput screening in the drug discovery process [201 - 205].

However, despite the fact that there is a solid theoretical foundation with regards to fluorescence polarisation, it has proven to be a very under-utilised tool and only recently has it become recognised as a valuable tool in the area of high throughput screening. The main reason for this is lack of high-performance and low-cost instrumentation.

The principle of how fluorescence polarisation is used to detect protease activity using a fluorescent substrate is shown in figure 1.17 a and b.

A relatively large fluorescent molecule such as a fluorophore-labelled protein has a significant fluorescence polarisation value. If a protease is introduced, the labelled fragments produced have a lower fluorescence polarisation value which drops with time, the decrease in fluorescence polarisation being a measure of proteolytic activity.

Figure 1.17a – Principle of the use of fluorescence polarisation for the detection of a large lightly labelled fluorescent protein

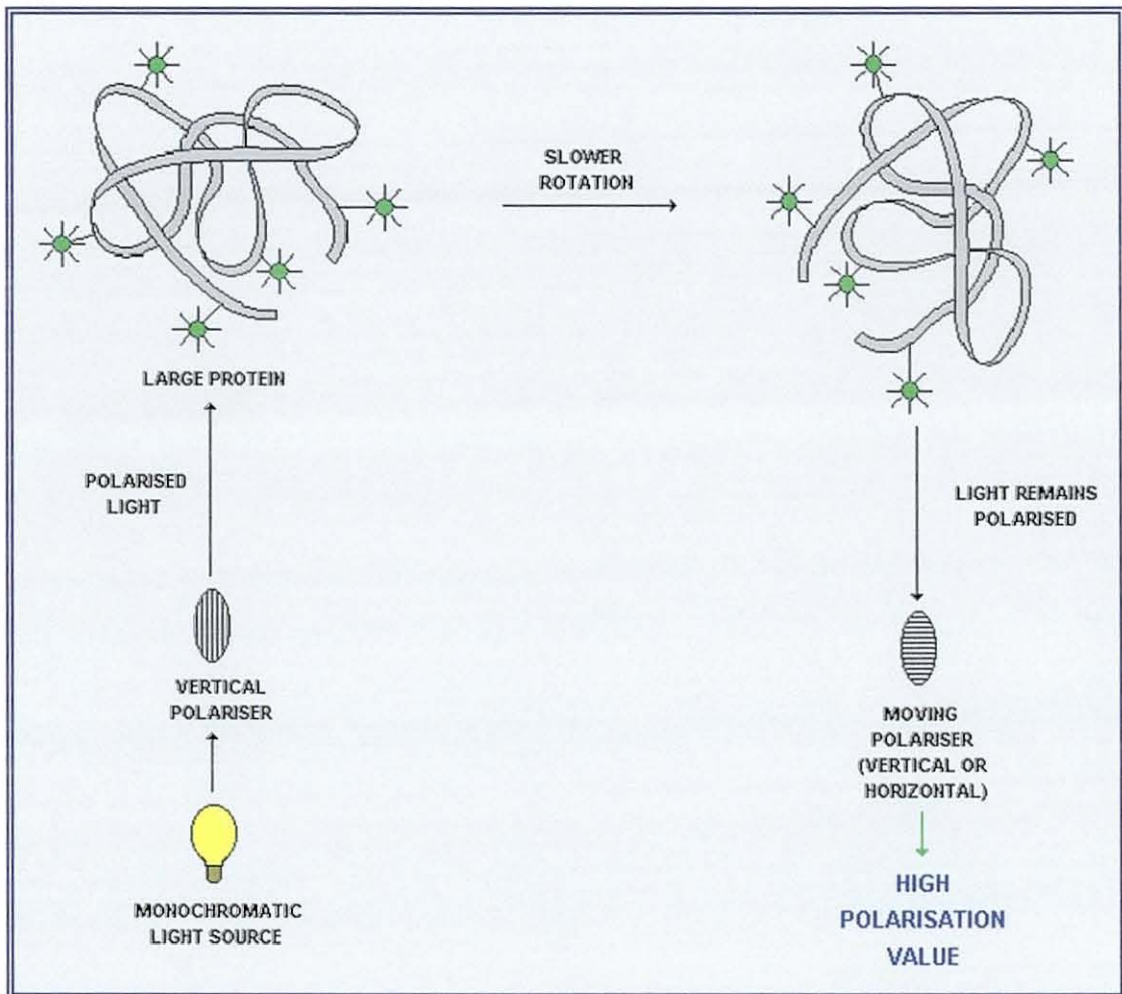
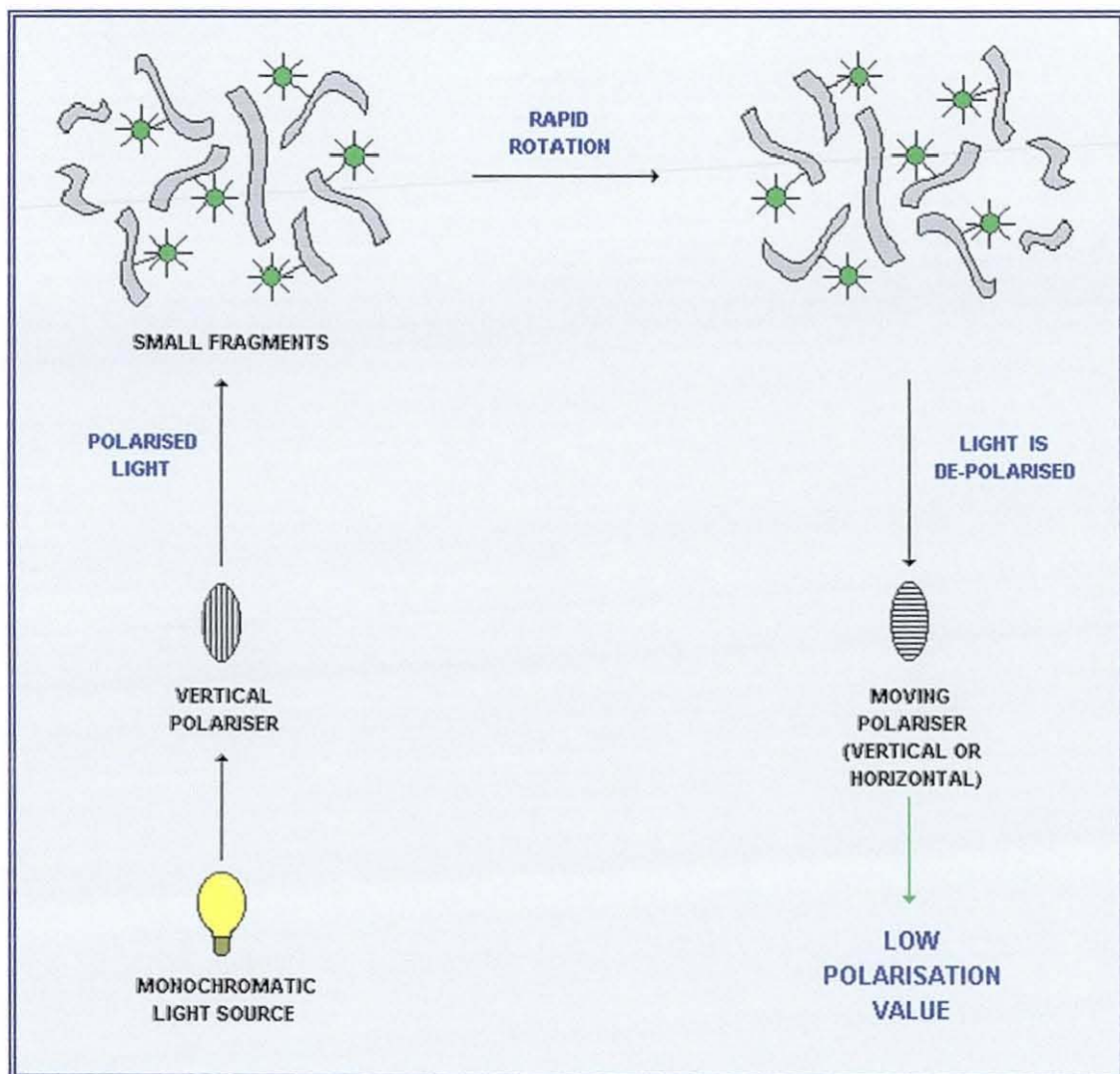


Figure 1.17b – Principle of the use of fluorescence polarisation for the detection of protease digested fluorescence peptides



The application of fluorescence polarisation to protease assays has long been established [189,190, 203, 207, 208].

An early paper by Maeda [189] described a detailed study of proteolytic enzyme assay development in which various fluorescein isothiocyanate conjugated proteins were used as substrates. These reactions were carried out in a static

cuvette and enzyme reactions that required a pH that was different to that of the pH of the measurement (pH 7 to 9) were adjusted back prior to detection. The results showed a near linear decrease in polarisation with time due to the apparent decrease in molecular weight of the substrates with hydrolysis. This method was shown to be simple and sensitive with trypsin activity being detected as low as 0.05 $\mu\text{g} / \text{ml}$. This was comparable at the time to the most sensitive methods using synthetic chromogenic substrates where trypsin was used at 0.01 to 0.1 $\mu\text{g} / \text{ml}$ [206] or to those utilising radiolabeled substrates, which allowed the determination of trypsin at 0.065 $\mu\text{g} / \text{ml}$ [161]. Maeda also demonstrated the proteolytic inhibition of papain or trypsin using various protease inhibitors (leupeptin, soybean trypsin inhibitor or antipain).

More recently Bolger and Checovich [190] compared the sensitivity of a fluorescence polarisation-based protease assay that used fluorescein thiocarbamoyl (FTC)-casein as a substrate with a non-fluorescence polarisation acid precipitation technique, as described by Twining [188]. Twining had previously estimated that the FTC-casein assay, which requires separation of the intact casein from the cleaved peptides by differential precipitation with trichloroacetic acid (TCA) and subsequent quantification of cleaved peptide by fluorimetry, was several orders of magnitude more sensitive than the measurement of cleaved peptides at A_{280} . In general it was found that the fluorescence polarisation assays resulted in an increase in sensitivity of 2- to 20-fold compared with the non-fluorescence polarisation assay of Twining, depending on the protease tested. In addition, the fluorescence polarisation assay also eliminates the centrifugation and two transfer steps required for the TCA assay and therefore is much simpler in design. These fluorescence polarisation assays took 1 hour to be carried out.

Due to the pH sensitivity of FITC, these methods cannot be utilised at low pH values without the necessity of adjusting the solution pH prior to detection. In

more recent years a new pH-independent fluorescent protein substrate has been developed based upon BODIPY-FL: α -casein [202, 207].

Since many proteases of interest in drug discovery do have optimum pH values below pH 6, where fluorescein is essentially non fluorescent, and because of a general desire to move into the red portion of the spectrum to minimise potential interferences from fluorescent library members, the potential of BODIPY fluorophores was explored as labels in fluorescence polarization protease assays [202]. The BODIPY fluorophores used were BODIPY FL (ex = 502 nm, em = 510 nm) and BODIPY TRX (ex = 588, em = 616). The assays were highly sensitive and could detect less than 1 ng / ml pepsin and less than 10 ng / ml trypsin, with a 5 minute incubation time. Soybean trypsin inhibitor (STI) could be readily detected at less than 50 ng / ml with a 5 minute incubation time.

BODIPY FL (ex = 504 nm, em = 511 nm) conjugated to α -casein was also shown to be successful as a substrate for proteases over the pH range 2 to 11 [207]. The molar ratio of BODIPY to protein was 0.14 and it was suggested that a molar ratio of 0.1 to 0.2 is optimum for fluorescence polarisation work. Addition of trypsin at a final concentration of 0.5 μ g / ml gave a decrease in fluorescence polarisation of about 150 mP in 3 minutes at 37°C. This large decrease in the fluorescence polarisation of BODIPY: α -casein showed that this conjugate was excellent for use as a substrate for protease assays. *S. griseus* alkaline protease activity on the BODIPY: α -casein substrate produced a decrease in polarisation of 137 mP units in the first minute and proteinase K produced a 128 mP units decrease in the first minute.

Molecular Probes Inc. currently market a polarisation kit for proteases using BODIPY FL and BODIPY TR-X conjugates of casein [208].

A fluorescence polarisation assay was designed to measure proteolytic cleavage of a specific peptide substrate for human cytomegalovirus protease in a 96 well

format [209]. The peptide substrate was derivatised by biotinylation of the amino-terminus and labelled with a fluorophore at the carboxy-terminal end. Incubation of the substrate with the protease and addition of avidin produced a polarisation signal that was proportional to the relative amounts of cleaved and uncleaved substrate. Due to the specificity of the substrate for the protease of interest, this protease assay is more useful than those previously described assays and can be further utilised by designing specific substrates. The effects of absorptive interferants were evaluated by including increasing concentrations of dye interferants absorbing in the wavelength region of either the fluorophore absorption or emission. The assay was found to be insensitive to a wide range of absorptive interferants that may be present in complex mixtures such as blood, when screening for protease inhibitors. An absorptive dye interferant resulted in greater than 90% loss in total fluorescence, while the fluorescence polarisation varied less than 10%. The results showed that while absorptive interferants dramatically affected total fluorescence, they had little effect on fluorescence polarisation. The ability of this assay to study the inhibitory activity of 3, 4 – dichloroisocoumarin (a cytomegalovirus protease inhibitor) suggested the versatility of this technique for the study of protease inhibitors.

BODIPY: α -casein has also been used to measure the proteases in subgingival plaque [210], implicated in the pathogenesis of adult periodontitis. This assay was rapid, sensitive and quantitative.

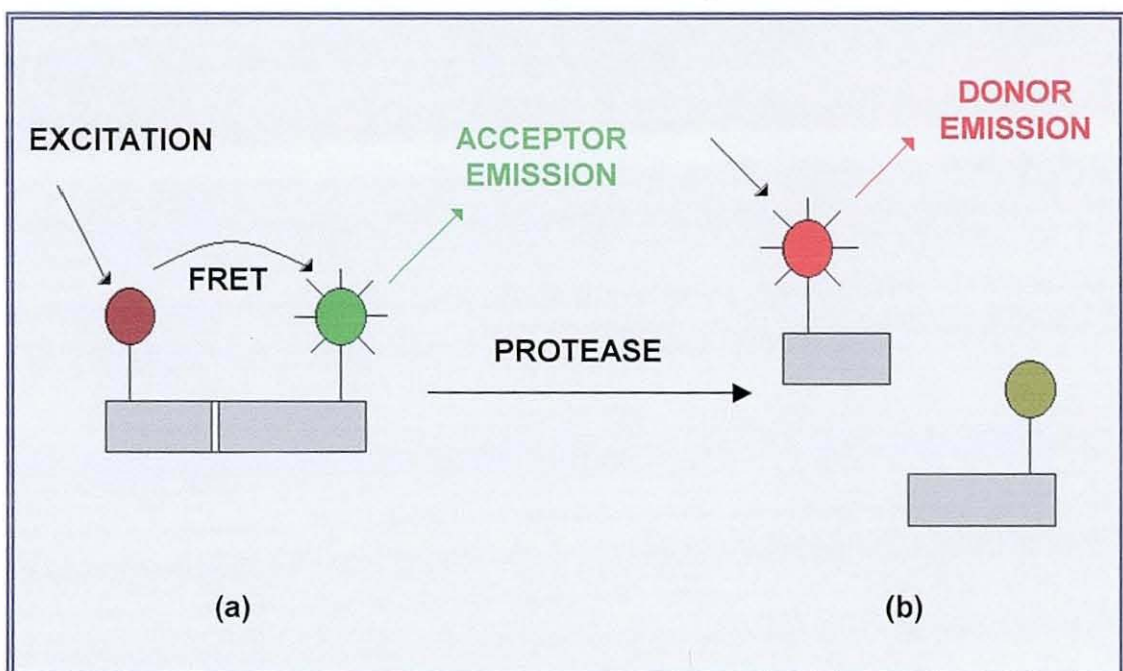
1.8.3.2 Fluorogenic protease assays

The use of fluorogenic substrates for proteases is widespread [211 - 214]. These substrates are non- or weakly fluorescent but are converted by an appropriate enzyme to a highly fluorescent product. Such methods combine the amplification effects of the catalytic enzyme action with the high sensitivity of fluorescence spectrometry alongside low background fluorescence.

1.8.3.2.1 Fluorogenic FRET protease assays

Specific proteases have commonly been assayed using fluorogenic substrates involving Fluorescence Resonance Energy Transfer (FRET). Substrates in which a peptide sequence is constructed to match the specificity of a particular enzyme are labelled at one end by a donor fluorophore and at the other by an acceptor fluorophore. FRET from donor to acceptor quenches the donor emission, an effect reversed upon proteolysis due to separation of donor and acceptor moieties. The increase in fluorescence intensity is proportional to enzyme activity. This method of detecting proteases is represented schematically in figure 1.18.

Figure 1.18 – Schematic representation of (a) a peptide sequence labelled at each end by a donor fluorophore (red) and an acceptor fluorophore (green) and (b) cleavage of this peptide by a protease causing separation of donor and acceptor fluorophores



FRET is one of the most sensitive methods for the detection of proteolytic activity as first shown by Latt *et al.* [215] for carboxypeptidase A. This method has been applied to the determination of a variety of specific proteases including Alzheimer's disease-associated proteases [216, 217], hepatitis C virus [218, 219], human and feline immunodeficiency virus proteases [40, 217, 220, 221], *Escherichia coli* leader peptidase [222], renin [217, 223, 224], tail-specific protease (which represents a new class of proteases that have no sequence homology to any well-characterised proteases) [225], and angiotensin-converting enzyme [226] amongst others [227 - 246]. One paper describes a general method for the preparation of these FRET protease substrates using solid-phase synthesis. The paper uses the donor and acceptor pair of 5-[(2'-aminoethyl)-amino]naphthalenesulfonic acid (EDANS) and 4-[[4-(dimethylamino)phenyl]azo]benzoic acid (DABCYL) and synthesises substrates for rennin and HIV proteinase as examples [247]. These assays are sensitive, easy to perform, and ideal for the detection of specific proteases with known sequences of proteolytic cleavage. However, these assays are not useful as general proteolysis detection tools and require synthetic expertise. Several papers describe the synthesis of some of these FRET substrates [248, 249]. Another potential problem with this type of substrate is that peptides in aqueous solutions can take conformations such that the donor and acceptor moieties are in close proximity, causing quenching of the emissions of the fluorophores. In general, if the fluorophores have hydrophobic characteristics then they will form dye-to-dye close contact in an aqueous environment.

A FRET protease assay designed for interstitial collagenase and gelatinase [250] has been used as a test system in an investigation into the feasibility of assay miniaturisation [251]. The investigation described precise and rapid measurement of the progress of an enzymatic reaction and its inhibition, which was carried out by mixing at the 200 μ l level and detecting in 2 μ l volumes with less than 6 minutes of total assay time. For comparison to a standard format, the

same assay was performed in a 96-well microtiter plate in 200 μ l using 30 minutes of incubation. Median inhibitory concentrations (IC_{50}) for actinonin, an analog inhibitor of collagenase, of 73 ± 16 and 100 ± 14 nM were obtained in the 2- and 200- μ l assays, respectively.

The use of these FRET protease substrates was further expanded by using homogeneous time-resolved fluorescence [252]. This assay relied upon the cleavage of a peptide substrate, which was labelled at the N terminus with biotin and had a phosphotyrosine residue downstream of the cleavage site. In the presence of streptavidin conjugated XL665, a cross-linked conjugate of allophycocyanin, and a europium-labelled antiphosphotyrosine antibody FRET occurs and a signal was generated. Upon cleavage, FRET is abolished and the signal decreased. This assay was developed for human immunodeficiency viral (HIV) protease and combined the sensitivity of lanthanide-based techniques with a time-resolved measurement.

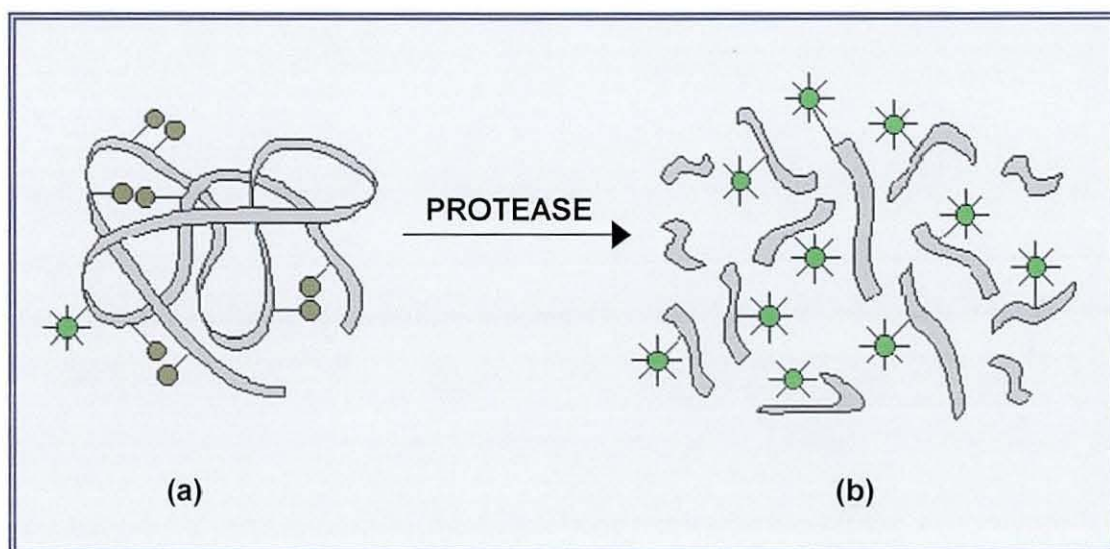
These substrates have also been used in a solid-phase assay for the complete subsite mapping of the active site of endoproteases [253]. Using a variation of this approach, a solid-phase combinatorial assay has been demonstrated which allows the rapid discovery of potent fluorogenic FRET substrates for use in the assay of novel proteolytic enzymes [254]. This technology has the potential to greatly facilitate the drug discovery process by speeding up the development of high throughput screening assays. This exploits more effectively the ever-increasing information gained from genomics and combinatorial chemistry.

1.8.3.2.2 Quenched over-labelled fluorogenic protease assays

Assays based on fluorogenic protein substrates, which are over-labelled by a suitable fluorophore to such an extent that the dye is efficiently quenched by internal energy transfer in the protein conjugate, have also been employed [255 -

259]. As shown in figure 1.19, upon protease digestion this internal energy transfer is abolished and the virtually non-fluorescent substrates release highly fluorescent dye-labelled peptides, the fluorescence intensity increases being proportional to enzyme activity. This type of protease assay removes the necessity for the separation of substrate from degradation products.

Figure 1.19 – Schematic representing the principle of an intramolecularly quenched protein substrate (a) and cleavage of this substrate by a suitable protease to release fluorescent peptides (b)



Fluorescein-labelled bovine serum albumin (BSA) or casein have commonly been used in this type of assay [256, 257]. *Porphyromonas gingivalis* was used in a study using FITC-labelled proteins as it has been implicated as one of the causative organisms of periodontal disease in humans [256].

FITC labelled bovine serum albumin (BSA) has been shown to serve as a general protease fluorescent enhancement substrate [257]. However digestion with trypsin yielded a lower fluorescence increase than that seen with pronase, α -chymotrypsin and proteinase K. This reduced fluorescence enhancement was consistent with the specificity of FITC conjugation for the ϵ -amino groups of lysine

residues, which along with arginine residues are required for trypsin activity. This effect is serious, as it is hard to achieve an adequate degree of fluorescence quenching in fluorescein conjugates unless the degree of labelling is very high. These heavily labelled conjugates may no longer be suitable as substrates for the assay of enzymes that depend upon the availability of the lysine residues to which the dyes are typically conjugated. The substitution of a large number of positive lysine residues with the negative charges of fluorescein may also cause conformational changes such that the interaction of the protein with the active site of the enzyme becomes problematic. Moreover fluorescein is only weakly fluorescent below pH 6, so fluorescein conjugates are not suitable for studies of acid proteases.

For these reasons the BODIPY® fluorophores have been investigated [255]. The BODIPY dyes, discussed in section 1.1.4.3, have no charge and it was found that conjugation of equivalent amounts of fluorescein and BODIPY dyes to both casein and BSA gave substrates that were 89 – 93% and 98 – 99% quenched, respectively. Conjugation of 4.5 to 6 BODIPY FL or even fewer BODIPY TR-X dyes to each casein molecule was optimal. As less BODIPY dye is needed for quenching this should minimise the effects of dye conjugation on the protein substrate conformation and enzyme-active site interactions. A dramatic increase in detection sensitivity of various enzymes with the BODIPY-casein substrates was seen (2- to 30-fold depending on which enzyme), compared to the FTC-casein fluorescence polarisation assay as reported by Bolger & Checovich, 1994 [190]. Hydrolysis of the BODIPY FL casein substrate by chymotrypsin, protease XIV, and proteinase K, generated over a twofold increase in fluorescence intensity when compared with hydrolysis of BODIPY FL BSA, indicating that BODIPY FL casein may have advantages for use as a general protease substrate relative to BODIPY FL BSA. The BODIPY dye labelled substrates are insensitive to pH, making it possible to measure the activity of various enzymes such as pepsin and cathepsin D, which are most active at low pH. The inhibition of trypsin proteolytic activity by pre-incubation

with aprotinin was demonstrated. Trypsin was pre-incubated with aprotinin for 20 minutes and then incubated with a casein conjugate of the fluorescein analogue BODIPY-FL for 1 hour at room temperature, followed by fluorescent measurement. Full inhibition was observed at $4 \mu\text{g ml}^{-1}$ aprotinin and 44 % inhibition was observed with $2 \mu\text{g ml}^{-1}$ aprotinin.

The BODIPY FL dye has also been used in these over-labelled substrates for the determination of the protease calpain in a microtiter plate format [258] and as a probe for monitoring of protein degradation in intact cells [259].

Molecular Probes market two protease assay kits using intramolecularly quenched substrates of BODIPY FL:casein and BODIPY TR-X:casein which emit at 513 nm and 617 nm, respectively. These assays are carried out in 1 hour [260].

A new assay for O-Sialoglycoprotein endopeptidase was demonstrated which was based on the increased fluorescence which resulted from proteolytic cleavage of a fluorescence-quenched micellar substrate, BODIPY-FL:glycophorin A [261]. Micellar association of glycophorin A molecules resulted in 97% fluorescence quenching despite a low molar ratio of BODIPY-FL:glycophorin A. This assay was different to those that use hyperconjugated dye protein substrates in that this substrate has only five lysines available for conjugation. However, it readily aggregates, and the resultant intermolecular interactions between fluorophores contribute to fluorescence quenching within the micelle. Glycoprotease was incubated with substrate for 10 – 15 minutes and as little as 1 ng of glycoprotease could be detected.

1.9 ENZYME INHIBITION

1.9.1 Types of inhibition

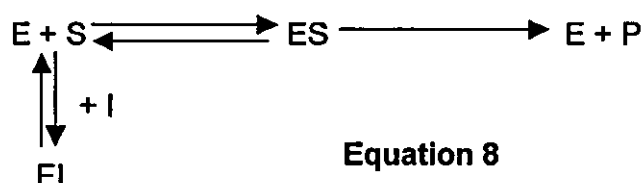
Inhibitors are substances that decrease the rate of an enzyme-catalysed reaction. There are two broad classes of enzyme inhibitors: reversible and irreversible. These types of inhibition will be described briefly, but more detailed accounts can be found for further reading [262, 263]. Information covering the use of enzyme inhibitors as drugs can be found elsewhere [264, 265].

1.9.1.1 Reversible inhibition

In this type of inhibition the inhibitor binds to the enzyme in a reversible fashion and can be removed by dialysis (or simply dilution) to restore full enzymatic activity. Three distinct mechanisms of reversible inhibition are known, which are recognised by the effect of substrate concentration on the inhibition.

(i) *Competitive inhibition*

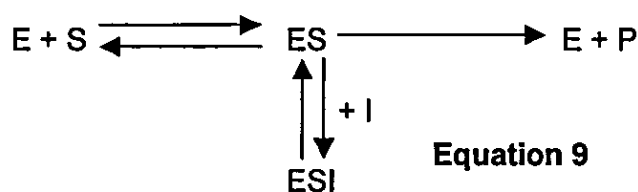
Competitive inhibitors often closely resemble the substrates whose reactions they inhibit, and because of this structural similarity they compete with the substrate (S) for the active site of the enzyme (E). Inhibitor (I) binds to the enzyme to form an enzyme-inhibitor (EI) complex. While the inhibitor occupies the active site it prevents binding from the substrate.



It can be seen from equation 8 that EI and ES complexes are formed and no ESI complex appears in this type of inhibition. As the inhibitor binds reversibly to the enzyme, the competition can be biased to favour the substrate simply by adding more substrate and hence making the reaction proceed towards ES and then product (P) formation. Inhibitor can only bind with free enzyme and not with the ES complex. By increasing the substrate concentration, percentage inhibition decreases

(ii) *Uncompetitive inhibition*

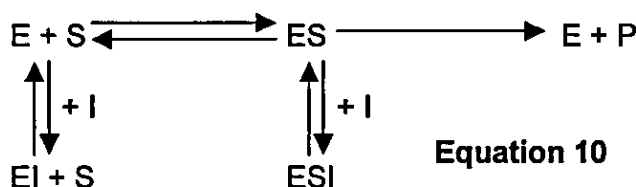
An uncompetitive inhibitor binds to the enzyme at a site distinct from the substrate. As shown in equation 9, in uncompetitive inhibition the inhibitor can bind only with the ES complex and not with the free enzyme. In this case therefore, a high concentration of substrate is unable to reverse the inhibition, rather an increased substrate concentration actually increases the percentage inhibition



(iii) *Noncompetitive inhibition*

A non-competitive inhibitor is one that binds to a site distinct from that that binds the substrate. Inhibitor binding does not block substrate binding (or vice versa). This means that inhibitor and substrate can bind at the same time to an enzyme

to form ES, EI, and ESI complexes (equation 10). The enzyme is inactivated when inhibitor is bound, whether or not substrate is also present. As such, non-competitive inhibition completely depends upon the inhibitor concentration and is unaffected by the substrate concentration.



1.9.1.2 Irreversible inhibition

Irreversible inhibitors are those that combine with or destroy a functional group on the enzyme that is essential for its activity. Formation of a covalent link between an irreversible inhibitor and an enzyme is common. In this type of inhibition the enzymatic activity cannot be restored by dilution or dialysis.

1.9.1.3 Product inhibition

The final step in an enzyme-catalysed reaction, the release of product, is often neglected, but is of course an essential component of the process. The product is bound to the active site by the same bonds that bind the substrate. As a result, product molecules are capable of binding to free enzyme to form enzyme-product complexes. In a single-substrate enzyme this is really a specialised form of competitive inhibition as substrate and product are mutually exclusive and compete for the enzyme active site as both cannot bind together.

1.9.2 Protease inhibition

In this work a variety of inhibitors have been investigated and thus a brief description of each is given. Comprehensive accounts of different protease inhibitors can be found in an excellent book [266].

1.9.2.1 Aprotinin

Aprotinin (also known as basic pancreatic trypsin inhibitor (BPTI), Kunitz protease inhibitor, and trasylol) is a competitive reversible serine protease inhibitor with an approximate molecular weight of 6,500. Most aprotinin-protease complexes dissociate at $\text{pH} > 10$ or < 3 . It is water-soluble and due to its compact (pear-shaped) tertiary structure is relatively stable against denaturation due to high temperature, acid, alkalis, organic solvents, or proteolytic degradation. Numerous papers cover the biochemistry and kinetics of aprotinin [267 - 272].

Aprotinin has been used in numerous clinical settings, including open heart surgery and coronary artery bypass surgery [273], acute pancreatitis, adult respiratory distress syndrome, trauma, and septic shock. In the 1960s, aprotinin was used in adult cardiac surgery in an attempt to reduce excessive blood loss thought to be due to increased fibrinolysis. By 1984, the benefits of aprotinin in paediatric cardiopulmonary bypass surgery were being studied, and decreases in postoperative blood loss and fibrin split products were demonstrated [274]. The ability of aprotinin to blunt the inflammatory response is, in fact, equally as effective as corticosteroid treatment. Aprotinin has also been investigated as an anti-coagulant [275].

1.9.2.2 Leupeptin

Leupeptin is a serine and cysteine competitive reversible inhibitor. It has a molecular weight of 475.6 and is a transition-state analogue, acting by mimicking the tetrahedral intermediate formed during peptide bond hydrolysis. Leupeptin is a tripeptide containing an aldehyde moiety in place of the usual carboxylate. It is water-soluble and solutions are stable for just a few hours. More information on leupeptin can be found elsewhere [276, 277].

1.9.2.3 3-Nitrophenylboronic acid

3-nitrophenylboronic acid is a reversible competitive serine protease inhibitor with a molecular weight of 166.93 and acts as a transition state analogue. Saccharides have been shown to act as a 'co-inhibitor' in the boronic acid inhibition system [278]. Investigation into the mechanism of binding of peptide boronic acids to proteases have been carried out [279, 280].

1.9.2.4 Pepstatin A

Pepstatin A is a potent uncompetitive reversible inhibitor of pepsin and other aspartate proteases. It has a molecular weight of 685.9, is a pentapeptide-like compound, and is soluble in methanol and DMSO but insoluble in water. Pepstatin A was discovered in 1970 by Umezawa and co-workers [281] in the course of searching for inhibitors of therapeutically important enzymes. The crystal structure of human pepsin and its complex with pepstatin A has been determined [282].

CHAPTER 2

Materials, instrumentation and general procedures

2.1 MATERIALS AND INSTRUMENTATION

2.1.1 Reagents

REAGENT	SUPPLIER
Buffer reagents	
Citric acid, dihydrate	Sigma
Glycine	Sigma
Sodium carbonate, anhydrous	Sigma
Sodium dihydrogen orthophosphate	Fisher Scientific UK
Sodium hydrogen carbonate	Fisher Scientific UK
Sodium phosphate, dibasic anhydrous	Sigma
Enzymes	
α -chymotrypsin Type II: from bovine pancreas	Sigma
Pepsin: from Porcine stomach mucosa	Sigma
Protease Type XXI: from <i>Streptomyces griseus</i>	Sigma
Proteinase K: from <i>Tritirachium album</i>	Sigma
Trypsin Type II-S: from porcine pancreas	Sigma
Fluorescent labels	
BODIPY® 630 / 650-X, SE	Molecular Probes
Cy™ 5 bisfunctional dye	Amersham Pharmacia Biotech UK
Cy™ 5.5 bisfunctional dye	Amersham Pharmacia Biotech UK
Fluorescein isothiocyanate isomer I	Sigma

Naphthofluorescein	In-house [283]
Tetramethylrhodamine isothiocyanate mixed isomers	Sigma
Immobilisation supports	
Controlled pore glass, aminopropyl, 972 Å mean pore diameter, 200 / 400 mesh	Cambio
Controlled pore glass, aminopropyl, 182 Å mean pore diameter, 200 / 400 mesh	Cambio
UltraLink™ biosupport medium	Pierce
Inhibitors	
Aprotinin: from bovine lung	Sigma
4-hydroxycoumarin	Sigma
Leupeptin hemisulfate salt	Sigma
3-nitrophenylboronic acid	Sigma
Pepstatin A	Molecular Probes
Warfarin (3-(α -acetylbenzyl)-4-hydroxycoumarin)	Sigma
Other reagents	
Bicinchoninic acid	Sigma
CHAPS (3-[3-cholamidopropyl]-dimethylamino)-1-propane sulfonate	Sigma
Copper (II) sulfate pentahydrate 98 %	Sigma
α -cyclodextrin	Sigma
β -cyclodextrin	Sigma
<i>N, N</i> -dimethylformamide (DMF)	Sigma
Dimethyl sulphoxide (DMSO)	Sigma
Ethanolamine	Fisher Scientific UK
Glutaraldehyde grade II	Sigma

2-hydroxypropyl- β -cyclodextrin	Sigma
Methanol, spectrophotometric grade	Sigma
Sodium cyanoborohydride	Sigma
Urea	Fisher Scientific UK
Proteins	
α -casein* from bovine milk	Sigma
Albumin, bovine	Sigma
Albumin, bovine – fluorescein isothiocyanate	Sigma

Table 2.1 – Reagents used and their respective supplier

2.1.2 Cells

Quartz fluorescence cell (3500 μ l volume, 1 cm light path)	Hellma
Quartz UV cell (3500 μ l volume, 1 cm light path)	Hellma
Flow cell (UV and fluorescence, 25 μ l volume, 1.5 mm X 1.5 mm light path)	Hellma

2.1.3 Chromatographic columns

PD-10 column	Amersham Pharmacia Biotech UK
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2.1.4 Flow system equipment

Enzyme reactors (3.0 mm microbore chromatography column, 25 mm length with 0.17 ml bed volume, and 50 mm length with 0.35 ml bed volume), frits (stainless steel 2 μ m, and PTFE 25 μ m), injection valves

(manual with 25 μl sample loop), Teflon® flow tubing (1.6 mm OD X 0.8 mm ID), 'T' 3-way flow connectors, and switching 4-port manual rotary valve Omnifit
 Peristaltic pump (Gilson minipuls 3) AD Instruments
 Pump tubing Elkay

2.1.5 Pipettes

Automatic adjustable pipettes: P20, 100, 200, 1000 and 5000 .. Anachem
 Volumes 0.5 μl – 10 μl Eppendorf
 Volumes 0.5 μl – 10 μl Elkay

2.1.6 Spectrometers

Model F-4500 Fluorescence Spectrophotometer Hitachi
 LS 50B Fluorescence Spectrophotometer Perkin Elmer
 Unicam 8700 Series
 UV / Visible spectrometer Unicam Analytical Systems

2.1.7 Supplier names and addresses

A list of supplier names and addresses is given in appendix A.

2.1.8 Temperature control

Water bath (0 – 99 °C) Grant

2.2 GENERAL PROCEDURES

2.2.1 Buffer preparation

Buffers were prepared in triply deionised water throughout. Carbonate, citrate, glycine, and phosphate buffers have been used during experimental work.

2.2.2 pH measurement

pH measurements were carried out using a Whatman PHA 230 bench pH meter, with a pH range of 0.00 to 14.00 and an accuracy of ± 0.01 pH. The instrument was calibrated with high-resolution buffer solutions at pH 4.000, 7.000, and 10.000, ± 0.005 , before use.

2.2.3 Water purification

Solutions were prepared using triply deionised water purified by a Maxima ultra pure water system (USF Elga Ltd.).

2.2.4 Mass determination

Weighing measurements were carried out on two balances depending on the weighing range needed:

- Precisa 300 series electronic balance, weighing range from 41 g to 12 400 g
 - Precisa 40SM-200A four decimal place electronic balance
-

2.2.5 Pipetting

Pipetting was carried out using Gilson (supplied by Anachem), Eppendorf, and Elkay variable volume automatic pipettes.

2.2.6 Fluorescence measurement

Two fluorimeters were used in this study – the Hitachi F-4500 Fluorescence Spectrophotometer, and the Perkin-Elmer LS 50B.

2.2.6.1 F-4500 Spectrophotometer

The appearance of the F-4500 spectrophotometer is shown in appendix B. The block diagram (taken from [284]) of the signal processing and control systems of the F-4500 can be seen in appendix C. The spectrophotometer mainframe has a xenon lamp light source, the beam of which is incident on the excitation monochromator. The beam after wavelength selection with the excitation monochromator (λ_{ex}) is irradiated onto the sample cell. Light emitted from the sample in the form of fluorescence (λ_{em}) then passes through the emission monochromator (which is at right angles to the incident light) to remove any light that is not at the emission wavelength. The intensity of fluorescence is detected via a red-sensitive photomultiplier tube. Instrument parameters are shown in appendix D.

2.2.6.2 LS 50B Spectrophotometer

The appearance of the LS 50B Spectrophotometer is shown in appendix E. The optical layout of the LS 50B (taken from [285]) can be seen in appendix F. As

with the F-4500, the LS 50B is composed of a pulsed xenon excitation source, a scanning excitation monochromator, a scanning emission monochromator, and a red-sensitive R928 photomultiplier tube. The LS 50B is fitted with FL WinLab version 2.01 software and parameters for the LS 50B are shown in appendix D. The LS 50B is also fitted with a Fast Filter Accessory for the measurement of fluorescence polarisation (amongst other things), described in 2.2.7.

2.2.7 Fluorescence polarisation measurement

Fluorescence polarisation measurements were carried out on a Perkin Elmer LS 50B spectrophotometer using the Fast Filter Accessory. With this accessory, filters appropriate for the application are rotated into the excitation or emission beam. The rotation of the wheel is synchronised to the mains frequency so that when a filter is momentarily in the correct position in the beam, the source is triggered and energy passes through that filter. The wheel then rotates 90° before the next flash occurs. When the Fast Filter Application is not being used, the fast filter wheels are parked in the clear beam position with none of the filters in either beam. Each measurement takes into account a G-factor measurement to correct for instrumental bias.

2.2.8 Absorbance measurement

Absorbance measurements were carried out on an Unicam 8700 Series UV/Visible Spectrometer using distilled water or buffer solution to obtain a baseline.

2.2.9 Flow system measurements

Flow system measurements were taken using the basic set-up shown in figure 5.19, and variations thereof. Buffer solution was pumped using a peristaltic pump and injections of substrate, enzyme, and inhibitor into the flowing stream were made using manual sample injection valves fitted with 25 μ l sample loops. A manual 4-port rotary valve was used to direct the main flow stream into an incubation loop where assay components were captured. The incubation loop was situated in a thermostatted water bath to enable the reactions to occur at a defined temperature. The rotary valve was then again switched to direct the assay components back into the main stream. This flowed through a fluorescence flow cell situated in the Hitachi F-4500 fluorescence detector to measure the fluorescence intensity and then through to waste.

2.2.10 Preparation of dye:protein conjugates

Dye:protein conjugates were prepared according to the following method. FITC:albumin was prepared for fluorescence polarisation experiments, BODIPY: α -casein for single-labelled experiments, and FITC:TMRITC: α -casein and Cy 5: Cy 5.5: α -casein for double-labelled experiments. The preparation will be illustrated by the conjugation of FITC to albumin (in reaction mixture ratios of 50:1 and 100:1), but the general method is applicable to all conjugates prepared.

1. 2.5 mg / ml albumin was prepared by dissolving 2.5 mg albumin in 1 ml 0.1 M carbonate buffer pH 9.0.
2. Amount of FITC required for 50:1 and 100:1 reaction mixture ratios of FITC:albumin were calculated by multiplying the number of moles of

albumin by the molecular weight of FITC followed by multiplying by the ratio.

3. FITC solutions for each reaction mixture ratio were made up by dissolving the required amount of FITC in DMF.
4. For each reaction mixture ratio conjugate, the standard FITC solution in DMF was added to a 2.5 mg / ml albumin solution. The total volume was made up to 2.5 ml with 0.1 M carbonate buffer pH 9.0.
5. The solutions were incubated for 2 hours in the dark at room temperature.
6. Two PD-10 Sephadex columns were equilibrated each with 25 ml 0.1 M phosphate buffer pH 7.2.
7. Each conjugate was passed through a PD-10 column in order to remove unconjugated dye from the conjugate.
8. 3.5 ml 0.1 M phosphate buffer pH 7.2 was added to each PD-10 column in order to elute the conjugate. Aliquots of conjugate were stored in black microcentrifuge tubes in the freezer.

2.2.11 Final dye:protein ratio calculations

Final dye:protein (D:P) ratios were calculated using the Beer Lambert Law, which states that absorbance is related to the molar extinction coefficient ϵ (in $\text{cm}^{-1} \text{M}^{-1}$), concentration c (in M), and path length l (in cm) by $A = \epsilon cl$. Absorbance values were taken for the conjugate at protein and dye wavelengths, which were corrected for dye absorbance at the protein wavelength and *vice versa*. Using the Beer Lambert Law, the concentration of both dye and protein was calculated

using the actual protein and dye absorbance values and their respective molar extinction coefficients. The dye concentration divided by the protein concentration gave a final dye:protein ratio.

2.2.12 Immobilisation of enzyme onto controlled pore glass (CPG)

Proteolytic enzymes were immobilised onto CPG using a method previously described by Masoom and Townshend [120]. 30 ml 25 % aqueous glutaraldehyde (grade II) was added to 0.5 g aminopropyl CPG in a well-stoppered glass vial in the fume cupboard and left for 1.5 hours at room temperature on a roller bed. Nitrogen was passed through the solution to remove oxygen every 10 minutes for the first 40 minutes. The activated glass was then washed with distilled water.

0.5 g activated CPG was added to a cold (4 °C) solution of enzyme in 2.5 ml 0.1 M phosphate buffer pH 6.0 and placed on a roller bed. Nitrogen was bubbled through the solution every 10 minutes for 2.5 hours. The immobilised enzyme was washed first with cold 0.1 M phosphate buffer pH 6.0, then with cold distilled water to remove free enzyme and then stored moist at 4 °C.

To block unreacted groups 10 ml 3.0 M ethanolamine pH 9.0 was added to the beads. 10 mM sodium cyanoborohydride was also added to the beads to make the linking permanent. Initial mixing by shaking was carried out and then the beads were left standing overnight. The beads were then washed and stored in 0.1 M phosphate buffer pH 6.0 at 4 °C.

The reaction scheme for immobilisation of enzyme onto CPG is given in figure 6.3.

2.2.13 Immobilisation of enzyme onto Pierce UltraLink biosupport medium

Proteolytic enzymes were immobilised onto Pierce UltraLink biosupport medium according to the manufacturer's instructions. 0.125 g of UltraLink were weighed out. A solution of enzyme in 2 ml cold (4 °C) 0.1 M carbonate buffer pH 8.5 was added to the dry beads and the solution vortexed, followed by gentle rotation on a roller bed for 2 hours at room temperature. The solution was then filtered to remove unbound enzyme from the beads and this filtrate was kept in order to determine the amount of enzyme not coupled to the beads using the BCA assay. A quench solution of 2.5 ml 3.0 M ethanolamine pH 9.0 was then added to the beads to block unreacted azlactone sites, vortexed and gently rotated on a roller bed for 2.5 hours at room temperature. The beads were then separated from the quench solution by filtration and resuspended in 2.5 ml 0.1 M phosphate pH 8.0 followed by vortexing and gentle rotation for 15 minutes. Buffer was removed by filtration and the beads were resuspended in 2.5 ml 1.0 M NaCl to remove non-specifically attached protein. The solution was vortexed and rotated for 15 minutes after which the NaCl was removed by filtration and the beads resuspended in 0.1 M phosphate pH 8.0, the solution vortexed and gently rotated for 15 minutes followed by filtration to remove the buffer. This last step was repeated and the beads stored in 0.1 M phosphate buffer pH 8.0 at 4 °C. Figure 6.15 depicts the reaction scheme for immobilisation of enzymes onto UltraLink.

2.2.14 Bicinchoninic Acid (BCA) assay for determination of protein concentration

The BCA assay [286] was used to determine the amount of enzyme immobilised onto UltraLink biosupport medium. This method was adopted as the use of Triton® X-100 surfactant in the bead production may have interfered with an assay using absorbance at 280 nm for the measurement of uncoupled enzyme.

CHAPTER 3

Experimental: fluorescence polarisation protease assays

3.1 INTRODUCTION

The principle of how fluorescence polarisation is used to detect proteolytic activity using a fluorescent substrate was shown in figure 1.17 (a) and (b), and previous work that has been carried out in this area was discussed in section 1.8.3.1.

Lightly labelled substrates for use in fluorescence polarisation studies could offer an advantage over highly quenched fluorogenic substrates. It seems likely that highly derivatised molecules might hinder the proteolytic action of some proteases. The substrate may possess fluorescent ligands at a strategic position(s) that keeps the protein from being cleaved by some proteases due to steric hindrance.

Fluorescence polarisation is a very elegant technique with numerous advantages (discussed in section 1.2.2), the most prominent advantage over other fluorescence techniques being its relative immunity to the inner filter effect.

These obvious benefits gained from using fluorescence polarisation therefore lead to an interest in whether this technique could be suitably applied to the study of proteases

The aim of these experiments was to determine whether fluorescence polarisation is an appropriate method for the sensitive and rapid detection of protease enzymes.

3.2 PREPARATION OF FLUORESCCEIN CONJUGATES

3.2.1 Procedures

Fluorescein:albumin conjugates were prepared according to the method described in section 2.2.10. A concentration of 2.5 mg / ml albumin was used and reaction mixture ratios of 7:1, 25:1, 50:1, 75:1, and 100:1 dye:protein were prepared.

All absorbance measurements were taken using an Unicam 8700 UV / visible spectrometer with a path length of 1 cm. A standard 3 ml quartz absorbance cuvette was used and baselines were taken with buffer prior to measurements.

3.2.2 Results

FITC solution was used to obtain an UV spectrum to calculate absorbance at 280 nm as a percentage of that at 494 nm. This was found to be 40 %, (appendix G), and this absorbance of the dye at the protein wavelength was taken into account during final dye:protein (D:P) ratio calculations (calculated as described in section 2.2.11).

Initial reaction mixture ratios and calculated final dye:protein molar ratios are given for each conjugate in table 3.1.

REACTION MIXTURE RATIO	CALCULATED DYE:PROTEIN RATIO
7:1	1.70:1
25:1	2.14:1
50:1	1.80:1
75:1	1.78:1
100:1	1.61:1

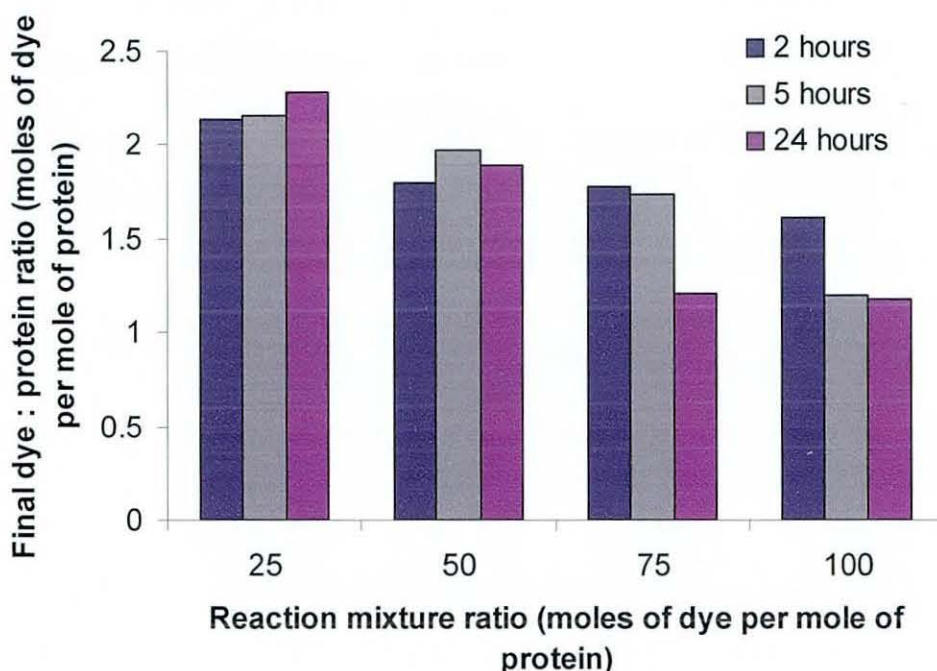
Table 3.1 – Initial reaction mixture and calculated final dye:protein ratios for fluorescein:albumin conjugates

From table 3.1 it can be seen that the calculated final D.P ratios did not appear to truly reflect the number of dye molecules per protein molecule. It would be expected that as the amount of dye in the reaction mixture was increased, a concomitant increase would be seen in the final D.P ratio. However, the calculated final D:P ratios increased with the first increase in reaction mixture ratio and then decreased with increasing amounts of FITC in the reaction mixture, the opposite of what might be expected. For an unknown reason, the rules for the final D:P ratio calculations appeared to have broken down. One possibility was that the absorbance measurements were inaccurate. As the absorbance at 280 nm was much smaller than that at 494 nm, the error associated with the protein concentration was much larger. All protein absorbance values used for the calculation of protein concentration were below 0.4 absorbance units, and the most accurate readings are between 0.5 and 1.0 absorbance units. In addition, above reaction mixture ratios of 50:1, the peak at 494 nm was above 1.5 absorbance units, again outside the accurate range. Unfortunately due to the difference between the absorbance values of the 280

nm and 494 nm peaks, it was impossible to strike a balance and obtain accurate absorbance values. These inaccuracies could very well explain the apparent error in final D:P ratios. All ratios henceforth will therefore be given in terms of reaction mixture ratios.

The protocol for conjugate preparation (section 2.2.10) required an incubation time of 2 hours for the conjugation of dye to the protein before separation of free dye from the conjugate. This incubation time was increased for 4 different reaction mixture ratio conjugates to see if this had an effect upon the final dye:protein ratio. Incubation times of 2 hours, 5 hours, and 24 hours were investigated.

Figure 3.1 – Effect of incubation time on the final dye:protein ratio for different reaction mixture ratio conjugates



It can be seen from figure 3.1 that incubation time did not greatly affect the final dye:protein ratio. An incubation time of 2 hours was therefore kept for all preparations of conjugates.

3.3 FLUORESCENCE POLARISATION PROTEASE ASSAYS

3.3.1 Procedures

As mentioned previously, reaction mixture ratios of 7:1, 25:1, 50:1, 75:1, and 100:1 fluorescein:albumin were prepared. When subjected to polarisation measurements, only the 7:1 reaction mixture ratio conjugate resulted in a decrease in fluorescence polarisation upon incubation with protease. This conjugate was therefore used for further polarisation experiments. This was in line with findings by Jolley [202], who found that at high labelling ratios the initial fluorescence polarisation and assay span were reduced. At these ratios the fluorescence became highly quenched and the fluorescence polarisation dropped to very low values, probably due to a mechanism of depolarisation resulting from non-radiative energy transfer. A low initial substrate fluorescence polarisation value will make a poor substrate for these types of experiments, which monitor decreases in polarisation.

When substrate concentration was varied from 0.5 – 2.3 $\mu\text{g} / \text{ml}$, no polarisation change was seen upon incubation with proteinase K except for at a concentration of 1.19 $\mu\text{g} / \text{ml}$. This substrate concentration was therefore used in all experiments.

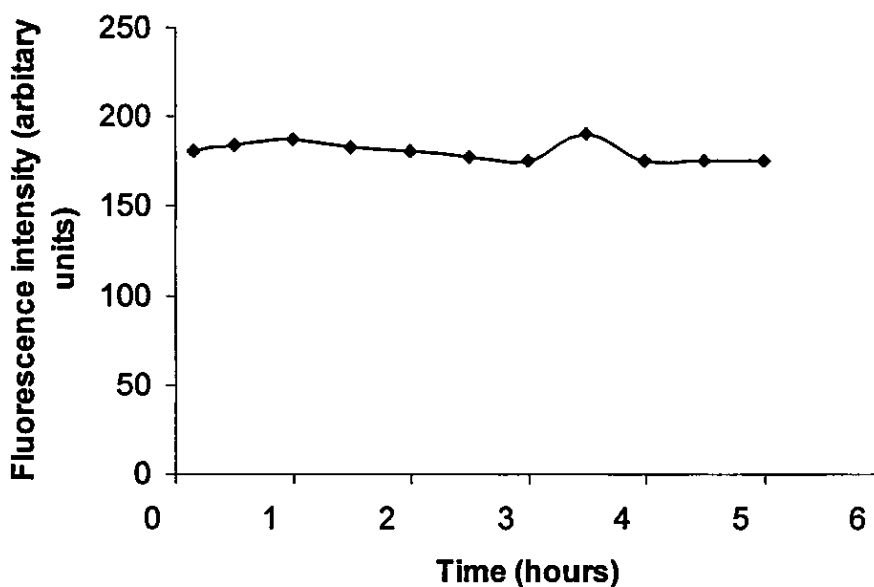
Fluorescence polarisation measurements were carried out on a Perkin Elmer LS 50B spectrophotometer using the Fast Filter Accessory as described in section 2.2.7. A G-factor (grating factor) of 0.916 was used to counteract the instrument polarisation response. An excitation wavelength of 488 nm and emission wavelength of 520 nm was used with excitation and emission bandwidths of 10 nm. A substrate concentration of 1.19 $\mu\text{g} / \text{ml}$ was added to buffer in a standard 3 ml quartz fluorescence cuvette and incubated with enzyme

in a total volume of 3 ml in a thermostatted water bath. The cuvette was then transferred to the LS 50B and fluorescence polarisation readings taken.

To determine the precision of the fluorescence polarisation measurements taken on the LS 50B, 20 replicate readings were taken of a single fluorescein:albumin sample in buffer. Between each reading, the cuvette was removed from the holder and inverted three times. Measurements of 15, 0, 10, 0, 10, 5, 15, 0, 5, 10, 10, 5, 10, 5, 10, 15, 5, 20, 15, and 15 mP units were obtained corresponding to an average of 9 mP units with a standard deviation of 5.8 mP units.

The stability of the Perkin Elmer LS 50B spectrophotometer xenon lamp was tested using a fluorescence reference block of anthracene and naphthalene and an excitation wavelength of 296 nm. As can be seen from figure 3.2, the output of the xenon lamp was relatively stable over a period of 5 hours, which was suitable for carrying out experimental readings.

Figure 3.2 – Fluorescence intensity of a reference block against time after switching on the LS 50B spectrophotometer



Three proteolytic enzymes – alkaline protease, proteinase K, and α -chymotrypsin – were investigated in these experiments (descriptions in section 1.6.4).

3.3.2 Results

The effect of the following parameters on each enzyme were studied and optimised:

- [enzyme]
- incubation time
- temperature
- pH
- buffer system
- [buffer]

Results showing range of values studied and corresponding optimised values for each parameter are shown in table 3.2 for all three enzymes.

KEY: CA = Carbonate, PH = Phosphate

	[ENZYME] ($\mu\text{g} / \text{ml}$)	TIME (minutes)	TEMPERATURE ($^{\circ}\text{C}$)	pH	BUFFER	[BUFFER] (mM)
ALKALINE PROTEASE						
RANGE STUDIED	0 – 14	0 – 20	20 – 50	7 – 11	PH / CA	6 – 100
OPTIMUM	2.67	15	40	11.0	CA	10
PROTEINASE K						
RANGE STUDIED	0 – 14	0 – 20	20 – 60	7 – 11	PH / CA	5 – 100
OPTIMUM	6.67	10	40	9.5	CA	10
α-CHYMOTRYPSIN						
RANGE STUDIED	0 – 14	0 – 20	20 – 55	7 – 11	PH / CA	5 – 100
OPTIMUM	13.33	10	40	7.5	PH	10

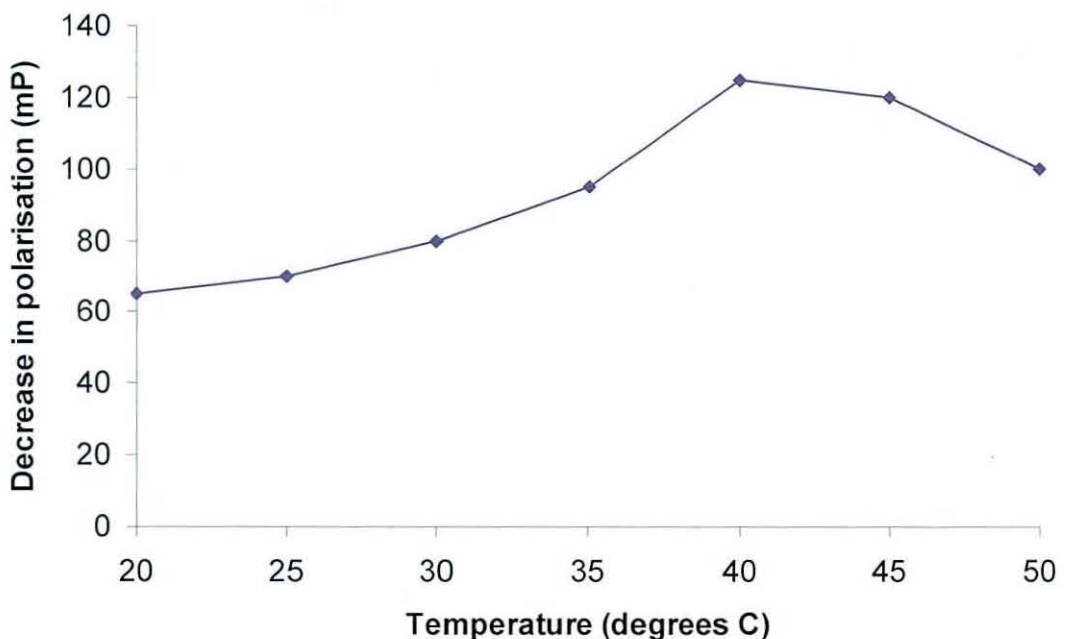
Table 3.2 – Assay parameters and optimum conditions for three protease enzymes using the substrate fluorescein:albumin

As there were multiple enzymes and each behaved similarly with changes in assay parameters, the effect of temperature, pH, buffer system, and buffer concentration will be discussed only for alkaline protease.

3.3.2.1 Effect of temperature

The effect of temperature on the activity of alkaline protease was examined over the range 20 – 50 °C using a thermostatted water bath. The polarisation results were corrected for the changes in polarisation of the blank conjugate with temperature thereby giving specific results for enzyme activity. From figure 3.3 it can be seen that as temperature is increased from 20 – 40 °C there is an accompanying increase in enzyme activity. However, after 40 °C there is a decrease in enzymatic activity. This is probably due to the disruption of the quaternary structure of the enzyme at high temperature and hence a disruption of the catalytic site resulting in loss of activity.

Figure 3.3 – Influence of temperature on the digestion of fluorescein:albumin by alkaline protease

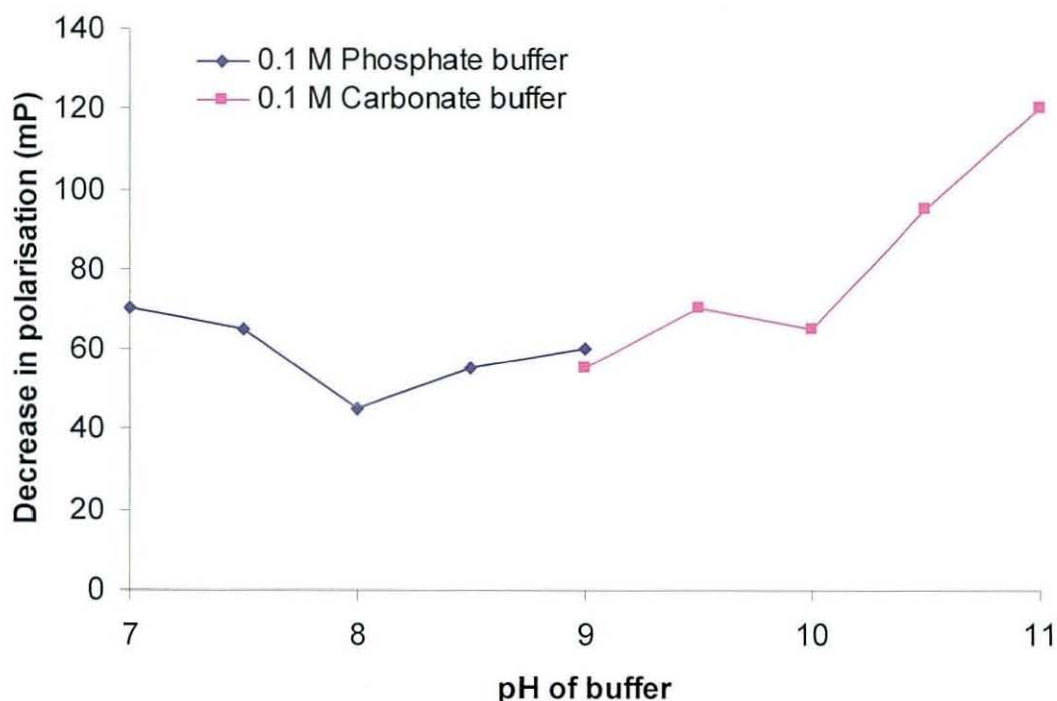


Maximal enzyme activity, signified by the greatest decrease in fluorescence polarisation, occurred at 40 °C and this optimal temperature was therefore used in further experiments. However, as polarisation is temperature-dependent, the effects seen are a combination of temperature effects on the enzyme and polarisation of the fluorophore. As values were corrected for substrate polarisation, these effects are not dependent on the effect of temperature on the substrate.

3.3.2.2 Effect of pH and buffer system

The influence of pH and buffer system on the activity of alkaline protease was also investigated using a pH range of pH 7.0 – 11.0, and two buffer systems – carbonate and phosphate. The buffer concentrations were kept constant at 0.1 M.

Figure 3.4 – Influence of pH and buffer system on the activity of alkaline protease



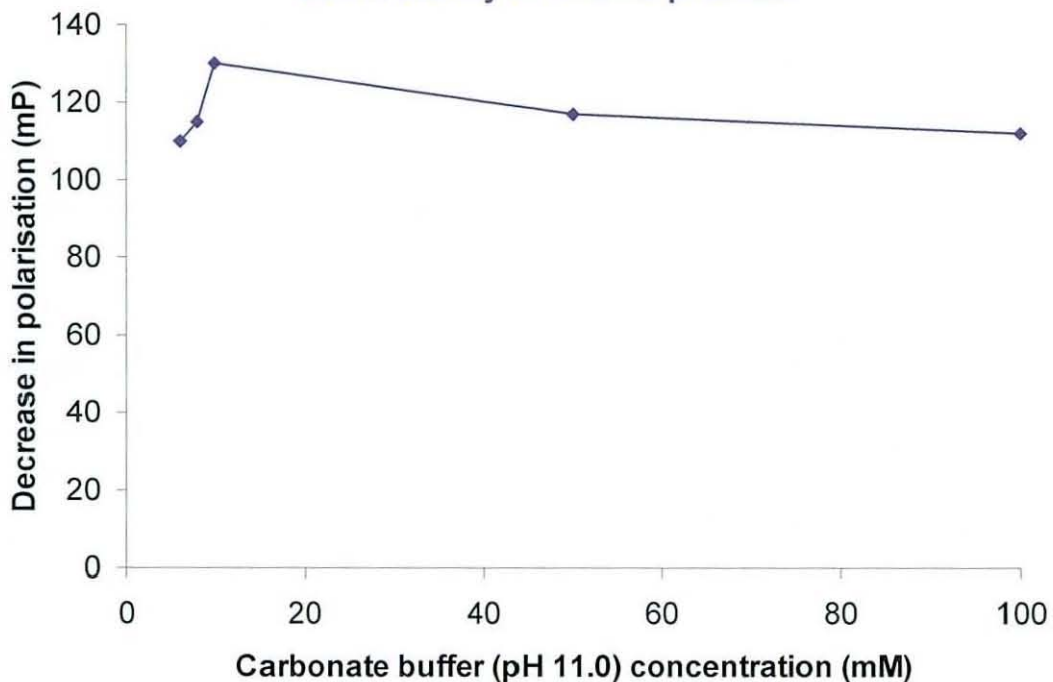
As shown by figure 3.4, a relatively steady polarisation value was seen from pH 7.0 – 10.0 irrespective of buffer system. From pH 10.0 – 11.0 there was a sharp decrease in polarisation value with pH 11.0 showing a maximum decrease. This was expected, as the description of alkaline protease in section 1.6.4.1 states that this enzyme shows maximal activity at pH 11.0.

As optimal enzyme activity occurred at pH 11.0 (carbonate buffer), this pH and buffer system was used in subsequent experiments.

3.3.2.3 Effect of buffer concentration

The influence of carbonate buffer pH 11.0 concentration between the values of 6 – 100 mM on the activity of alkaline protease was studied. As shown in figure 3.5, a maximum peak in activity was seen with a buffer concentration of 10 mM, with a reduction in activity either side of this value.

Figure 3.5 – Influence of carbonate buffer pH 11.0 concentration on the activity of alkaline protease

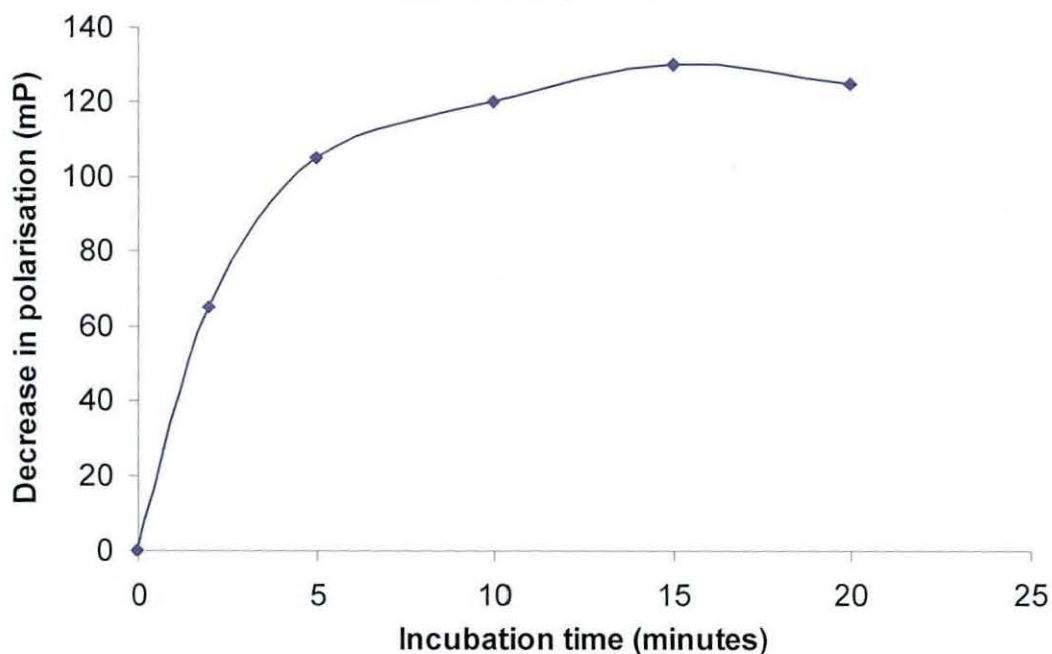


An optimised carbonate buffer pH 11.0 concentration of 10 mM was selected for further experiments as this concentration yields a maximum enzyme activity whilst maintaining sufficient buffering capacity.

3.3.2.4 Effect of incubation time

The influence of incubation time on the enzymatic reaction was evaluated between 2 to 20 minutes. It can be seen from figure 3.6 that as incubation time was increased the amount of proteolytic activity on the substrate also increased, represented by a drop in polarisation indicating the digestion of the protein substrate into smaller peptides. As incubation time was increased from 2 minutes to 15 minutes there was more time for the enzyme to act on the substrate and hence more product was formed. At 15 minutes a maximum was seen and further increases in incubation time produced no further increases in the enzymatic reaction. At this point another factor has become limiting, for example there is no substrate left due to the complete conversion to product.

Figure 3.6 – Influence of incubation time on the conversion of substrate to product

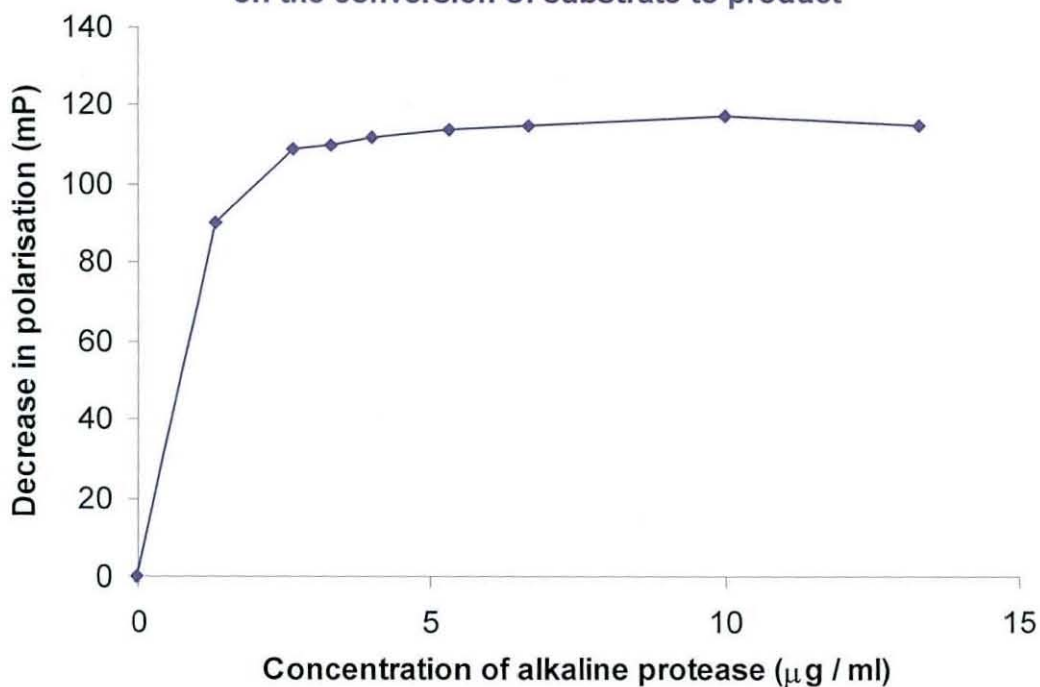


Since a maximum in the enzymatic reaction occurred at 15 minutes of incubation, this was used in all subsequent experiments.

3.3.2.5 Effect of enzyme concentration

Using the optimum conditions found for pH, buffer system, buffer concentration, temperature and time, it was possible to follow the effect of changing the concentration of alkaline protease on the hydrolysis of the substrate. Figure 3.7 shows that at a constant concentration of substrate, the reaction rate increased with increasing enzyme concentration until a maximal velocity was reached. This saturation effect was due to more substrate being converted to product by adding more enzyme to carry out the reaction. However, at an enzyme concentration of $2.67 \mu\text{g} / \text{ml}$ the enzyme was in excess and the substrate became limiting. Further increase in reaction rate was impossible without increasing the concentration of substrate because at an enzyme concentration of $2.67 \mu\text{g} / \text{ml}$ all the substrate was converted to product efficiently by this amount of enzyme.

Figure 3.7 – Influence of alkaline protease concentration on the conversion of substrate to product

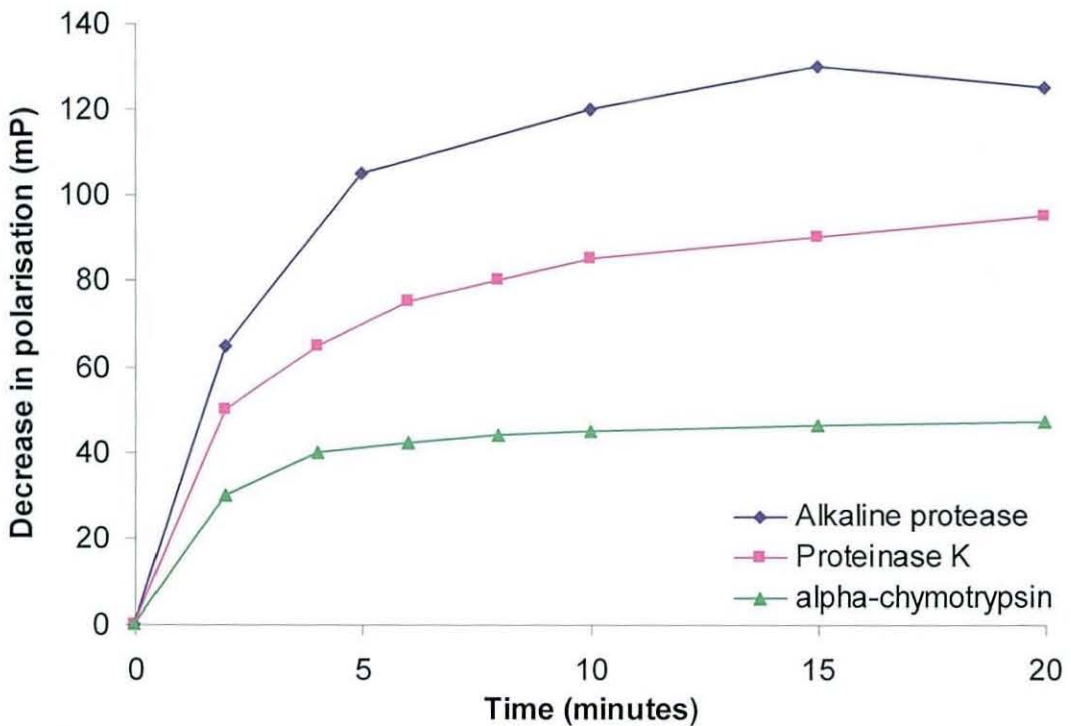


A maximum reaction rate was seen at an alkaline protease concentration of 2.67 $\mu\text{g} / \text{ml}$.

3.3.2.6 Comparison of the activity of three proteolytic enzymes using fluorescence polarisation

Using the optimised conditions shown in table 3.2 for each enzyme, the time dependent proteolysis of fluorescein:albumin by all three enzymes was compared. These results are shown in figure 3.8. From this comparison it can be seen that α -chymotrypsin exhibited poor proteolytic activity on fluorescein:albumin when compared with proteinase K and alkaline protease. Alkaline protease had the greatest proteolytic activity, and proteinase K also has good proteolytic activity although lower than alkaline protease. This method has therefore proved to be applicable to the determination of proteolytic enzymes.

Figure 3.8 – Time-dependent comparison of the activity of three protease enzymes – alkaline protease, proteinase K, and α -chymotrypsin – on the substrate fluorescein:albumin using fluorescence polarisation



3.4 DISCUSSION

The use of covalently linked dye:protein conjugates for fluorescence polarisation studies assumes that the dye:protein conjugate is a rigid particle because then the rotational properties of the dye are identical to those of the macromolecule. The linkage between the fluorophore and the protein must be as short and as rigid as possible to avoid the "propeller effect" i.e. the fluorophore moving independently of the protein. The fluorophore must have the appropriate lifetime and as high a quantum yield and extinction coefficient as possible. Fluorescein fulfils these criteria.

It was suggested in section 3.1 that lightly labelled substrates for use in fluorescence polarisation studies might offer an advantage over highly quenched fluorogenic substrates. The use of fluorescein:albumin as a fluorescent substrate for the determination of protease enzymes using fluorescence polarisation has been investigated. It has been demonstrated that fluorescence polarisation has indeed successfully been used to analyse different proteases with broad specificity. This has enabled the comparison of the catalytic activities of alkaline protease, proteinase K, and α -chymotrypsin with time.

However, polarisation changes are small, and in such cases, the accuracy of the assay suffers. Polarisation changes could be detected down to 5 mP, at which point noise prevented accurate readings from being taken.

The use of fluorescein as a label rather limited the choice of enzymes that could be looked at due to its pH sensitive fluorescence that is significantly reduced below pH 7 ($pK_a \sim 6.4$). Longer wavelength fluorophores would be preferred due to the reasons discussed in section 1.1.3 of increased sensitivity. The BODIPY fluorophores would overcome both of these limitations found with fluorescein.

These dyes are pH insensitive and can be obtained with a variety of excitation and emission wavelengths including the long-wavelength region of the spectrum. Experiments with BODIPY 630/650-X, SE as a label fluorophore were carried out without success using the substrate 5:1 BODIPY: α -casein. It has been observed that fewer BODIPY dye molecules are required to produce quenching effects than with fluorescein [255] and indeed 5:1 BODIPY: α -casein worked extremely well as an intramolecularly quenched protein substrate as discussed in chapter 5. It is apparent that only lightly labelled substrates are needed for this type of assay in comparison to the highly derivatised substrates in fluorescence enhancement experiments. This can be attributed to a high rate of depolarisation through non-radiative energy transfer in over-labelled substrates, as discussed in section 1.2.1.2. This was in line with findings by Jolley [202] who found that at high labelling ratios the initial fluorescence polarisation and span were reduced.

To further the studies of the application of fluorescence polarisation to the assay of proteolytic enzymes an investigation into the use of long-wavelength dyes needs to be carried out. This would constitute future work. Preferably a pH insensitive long-wavelength dye should be used due to advantages of a pH-insensitive dye and of long-wavelength fluorescence. However, such a dye would require a suitable fluorescence lifetime for appreciable Brownian rotation to occur. There are worries about the extent of polarisation effects with short-lifetime long-wavelength fluorophore molecules (section 1.2.1.3). This may be one of the few areas where long-wavelength fluorophores are not very useful. However, if a suitable long-wavelength fluorophore could be found this could prove valuable.

The usefulness of this technique is also limited by the requirement of a cuvette-based approach, with the associated low throughput and high reagent usage. A flow-based system would improve this throughput significantly. To do this an evaluation of the application of fluorescence polarisation to this type of system would be needed in order to determine the related sensitivity.

It seems reasonable to presume that polarisation methods would be less sensitive than straight fluorescence methods. Inherent problems in polarisation methods include (a) polarisation filters reducing exciting and emitted light intensities; (b) polarised light pre-selecting only a fraction of the potentially excitable molecules; and (c) polarisation changes being small and temperature sensitive. One disadvantage of fluorescence polarisation is that measurements are instrument dependent and the quality of the results is directly related to the quality of the instrument. Unfortunately the LS 50B spectrophotometer is not particularly sensitive and the availability of cheap sensitive fluorescence polarisation instruments is somewhat limited. Whilst fluorescence polarisation does possess attractive features in that it is simple, homogeneous, and relatively immune to the inner filter effect that is found in other homogeneous fluorescence techniques and so works in coloured solutions and cloudy suspensions (an obvious advantage in HTS), energy transfer techniques seem more promising. The decision was therefore taken to concentrate on the use of highly quenched fluorogenic substrates in protease assays.

CHAPTER 4

Experimental: double labelled FRET based protease assays

4.1 INTRODUCTION

The aim of this experimental section was to investigate the use of donor – acceptor fluorophore pairs for use in Fluorescence Resonance Energy Transfer (FRET) based protease assays.

The principle behind this work was similar to that described in section 1.8.3.2.1 and depicted in figure 1.18. However, in this work the principle described in section 1.8.3.2.1 has been modified such that instead of using a specific peptide sequence, the protein α -casein has been used as the substrate. This protein was labelled with many donor and acceptor fluorophore molecules (unlike a single donor and acceptor molecule used with specific peptide sequences) with the idea that upon donor excitation, FRET from donor to acceptor would quench donor emission whilst enhancing acceptor emission. Upon proteolytic cleavage this effect is reversed and the increase in donor fluorescence and decrease in acceptor fluorescence is proportional to enzyme activity. The ability to measure both donor enhancement and acceptor quenching offers an advantage over other fluorescence techniques as the simultaneous observation of quenching and enhancement effects allows proteolytic cleavage effects to be distinguished from non-specific environmental effects on fluorescence intensity. As intensity ratio measurements are in effect being taken, instrument fluctuations will largely cancel out, which is a significant advantage over other energy transfer methods.

However, one disadvantage of this method is that, as shown in figure 4.1, several competing events can occur.

Figure 4.1 (a, b, and c) – Alternative scenarios following excitation of the conjugate at the donor excitation wavelength

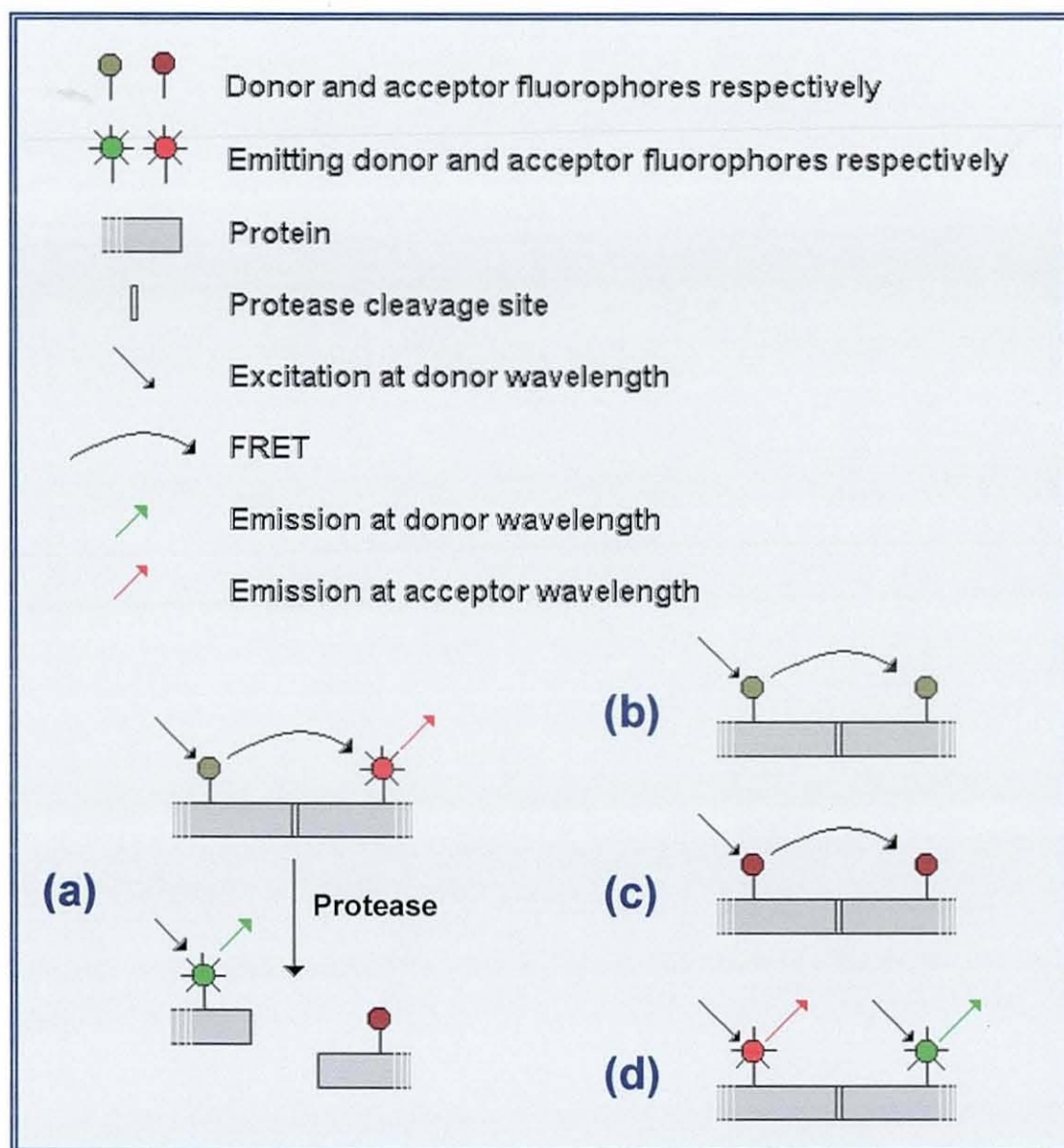


Figure 4.1 (a) shows excitation of the donor fluorophore followed by energy transfer to the acceptor in the protein conjugate, and subsequent disruption of this energy transfer caused by proteolytic cleavage of the substrate accompanied by donor enhancement and acceptor quenching. This sequence of events represents those that are needed for the protease assays. However, (b), (c), and (d) represent competing events that could also occur. Figure 4.1 (b) shows excitation of the donor and internal energy transfer between donor fluorophores, with the consequence that the substrate is virtually non-fluorescent. Figure 4.1 (c) depicts direct excitation of acceptor fluorophore by the donor wavelength and internal energy transfer between acceptor molecules causing quenching. Finally, figure 4.1 (d) shows direct excitation of either or both donor and acceptor molecules followed by emission with no energy transfer.

In reality, any of the situations shown in figure 4.1 may exist, as well as any combination of those situations. As a number of fates of the exciting light can occur, this makes interpretation of the results more complex and also requires much optimisation of the concentrations of donor and acceptor fluorophores needed to favour FRET from donor to acceptor, a requirement of this assay.

The donor-acceptor pairs of fluorescein isothiocyanate (FITC) / tetramethylrhodamine isothiocyanate (TMRITC), and Cyanine™ 5 (Cy 5) / Cyanine™ 5.5 (Cy 5.5) and the conditions required to synthesise a suitable substrate using these pairs for use in protease assays were investigated.

This work was carried out in conjunction with James E. Allard as a research project submitted in partial fulfilment of the requirements for the award of Master of Science.

4.2 INVESTIGATION INTO THE USE OF FLUORESCHEIN AND TETRAMETHYLRHODAMINE FOR FRET PROTEASE ASSAYS

4.2.1^c Procedures

Fluorescence measurements were carried out using a Perkin Elmer LS 50B fluorescence spectrophotometer with an excitation wavelength of 480 nm for FRET conjugate experiments. Excitation and emission wavelengths of 480 nm and 520 nm and 551 nm and 575 nm were used for fluorescein and tetramethylrhodamine free dyes respectively. Excitation and emission bandwidths of 10 nm were used throughout. Measurements were made statically in a standard 3 ml quartz fluorescence cuvette

All absorbance measurements were taken using an Unicam 8700 UV / Visible Spectrometer. A standard 3 ml quartz absorbance cuvette was used and baselines were taken with buffer prior to measurements

Fluorescein:tetramethylrhodamine: α -casein conjugates were prepared as described in section 2.2.10 using 2.5 mg / ml α -casein with reaction mixture ratios of 10:40:1, 25:25:1, 1:50:1, 40:10:1, and 5:75:1. Final molar dye:protein ratios were not calculated due to the complex absorbance spectra obtained with these conjugates.

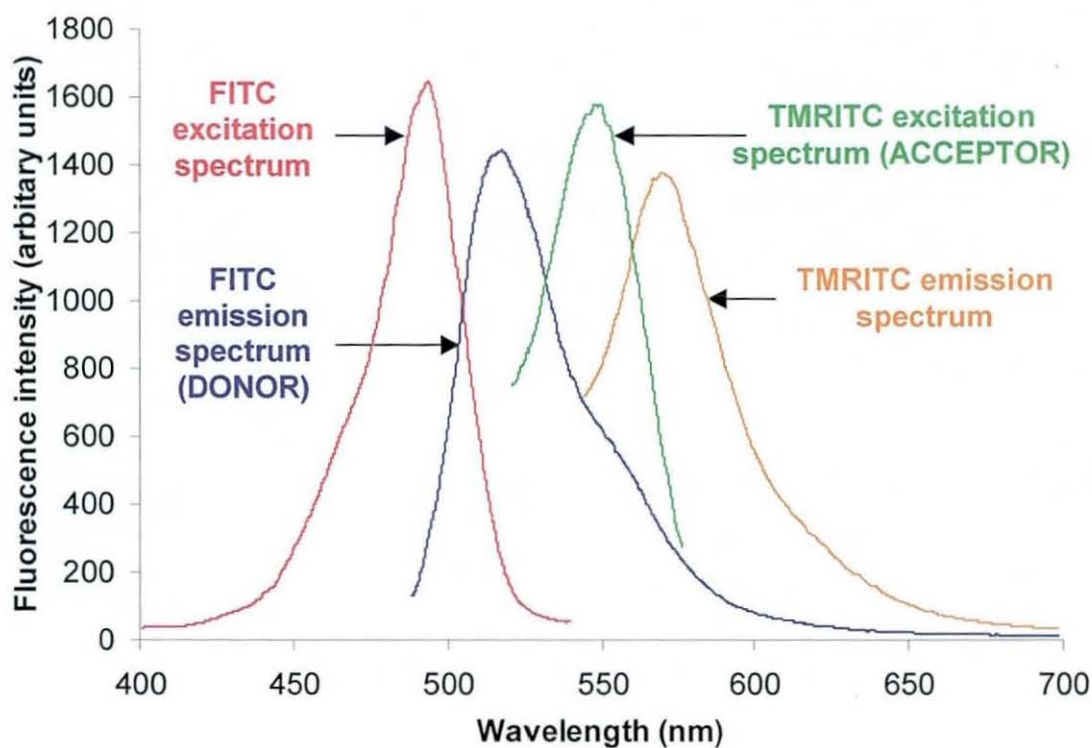
Fluorescein and tetramethylrhodamine were chosen for initial FRET studies as these dyes have promising spectral characteristics and are cheap.

4.2.2 Results

4.2.2.1 Analysis of the overlapping of fluorescein (donor) and tetramethylrhodamine (acceptor) excitation and emission spectra

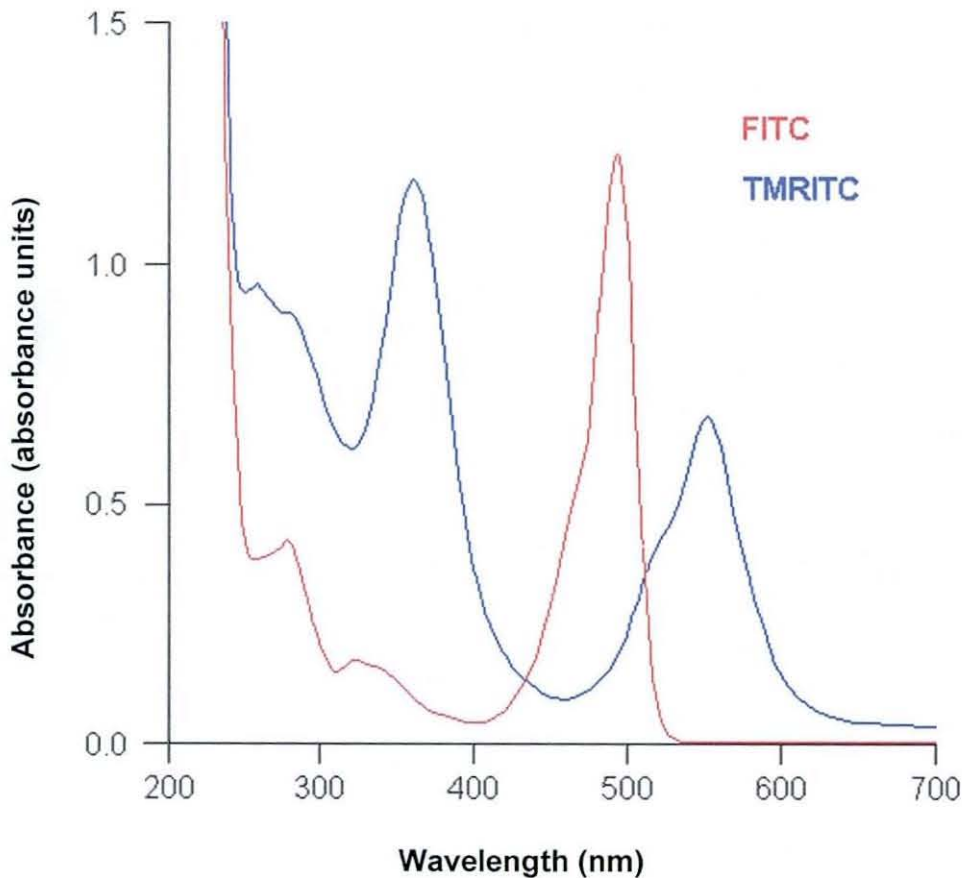
Solutions of FITC and TMRITC were used to obtain the excitation and emission spectra for these dyes. As shown in figure 4.2, the emission spectrum of the donor fluorophore (fluorescein) overlapped the excitation spectrum of the acceptor (tetramethylrhodamine), a primary condition for FRET. This suggested that these dyes were suitable, in theory, for use in FRET protease assays.

Figure 4.2 – Spectral overlap of FITC (donor) and TMRITC (acceptor)



From figure 4.2 it can be seen that the excitation wavelength of fluorescein was 494 nm. However, using this excitation wavelength the influence of the scatter peak on the emission peak was too great and the two were in danger of being inadequately resolved. In addition to this it is evident from figure 4.3 that the absorbance spectra of fluorescein and tetramethylrhodamine strongly overlap, with the consequence that a fluorescein excitation wavelength would also directly excite the tetramethylrhodamine fluorophore. Direct acceptor excitation needed to be kept to a minimum, as excitation by FRET from the donor is required for the protease assays. For these reasons a sub-optimal excitation wavelength of 480 nm was used in all subsequent experiments.

Figure 4.3 – Absorbance spectra of fluorescein and tetramethylrhodamine free dyes



4.2.2.2 Determination of optimum donor:acceptor:protein ratios for use in FRET protease assays

Conjugates were prepared with reaction mixture ratios of 1:50:1, 5:75:1, 10:40:1, 25:25:1, and 40:10:1 fluorescein:rhodamine: α -casein to represent conjugates containing different ratios of dyes with tetramethylrhodamine in excess, fluorescein in excess, and equal quantities of donor and acceptor. As shown in figure 4.4, the 10:40:1, 25:25:1, and 40:10:1 conjugates all showed an overwhelming fluorescein emission peak. The tetramethylrhodamine emission peak at 575 nm was not evident because the fluorescein dye was so fluorescent that its peak completely hid any tetramethylrhodamine fluorescence.

Figure 4.4 – Emission spectra of various fluorescein:tetramethylrhodamine: α -casein conjugates

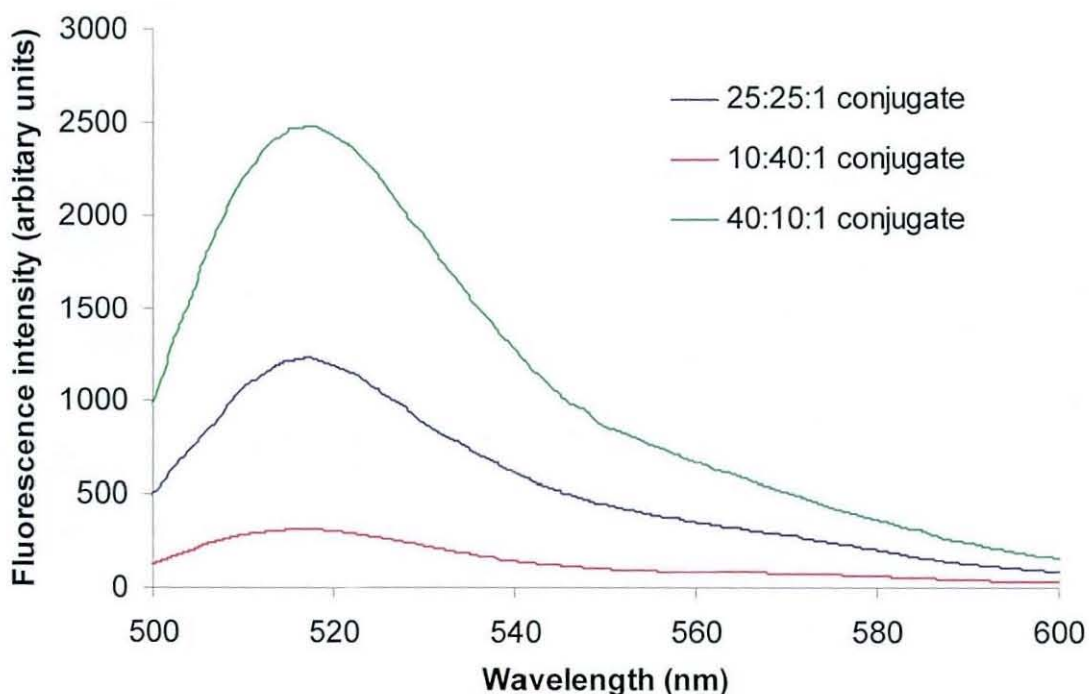
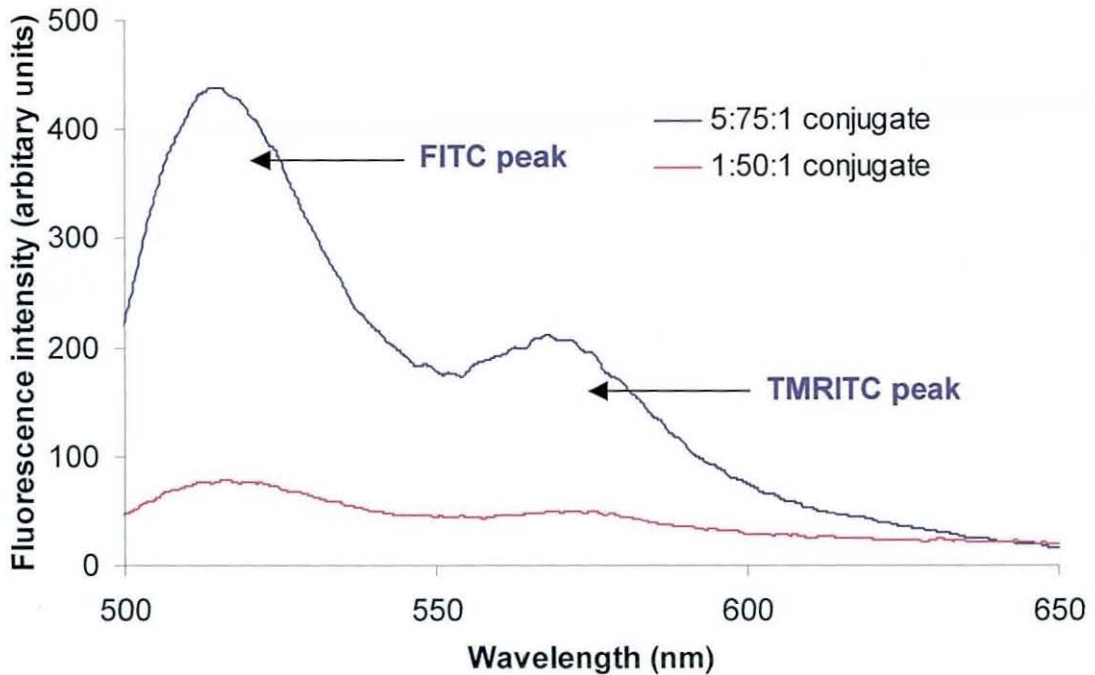


Figure 4.5 – Emission spectra of fluorescein:tetramethylrhodamine: α -casein conjugates exhibiting an emission peak at the TMRITC wavelength



It was found that tetramethylrhodamine emission was only observed in conjugates containing an excess of tetramethylrhodamine, as demonstrated by figure 4.5 which shows the fluorescence of the 5:75:1 and 1:50:1 conjugates.

These results suggest that optimum conditions require lightly labelled donor and heavily labelled acceptor conjugates for use in FRET protease assays. This was likely to be due to the fact that high donor ratios are more susceptible to quenching by the energy transfer mechanism. In these heavily donor labelled conjugates, fluorescein-to-fluorescein energy transfer competes with fluorescein-to-tetramethylrhodamine energy transfer. As shown by figure 4.2, the excitation and emission spectra of fluorescein overlap to a considerable degree. The fluorescein-to-fluorescein energy transfer is thus likely to be quite efficient. To favour fluorescein-to-tetramethylrhodamine energy transfer therefore, an excess

of acceptor is required. As the amount of acceptor increases, the probability of one or more TMRITC groups being attached close to the donor also increases: energy transfer is thus more efficient because of the lower mean distance between the fluorescein and tetramethylrhodamine groups.

A more defined tetramethylrhodamine peak was observed in the 5:75:1 conjugate when compared with the 1:50:1 conjugate. This was probably due to more transferred light from a higher concentration of fluorescein in the 5:75:1 conjugate being able to excite the tetramethylrhodamine fluorophore. As this conjugate looked to be more promising as a FRET substrate further experiments were carried out using the 5:75:1 conjugate.

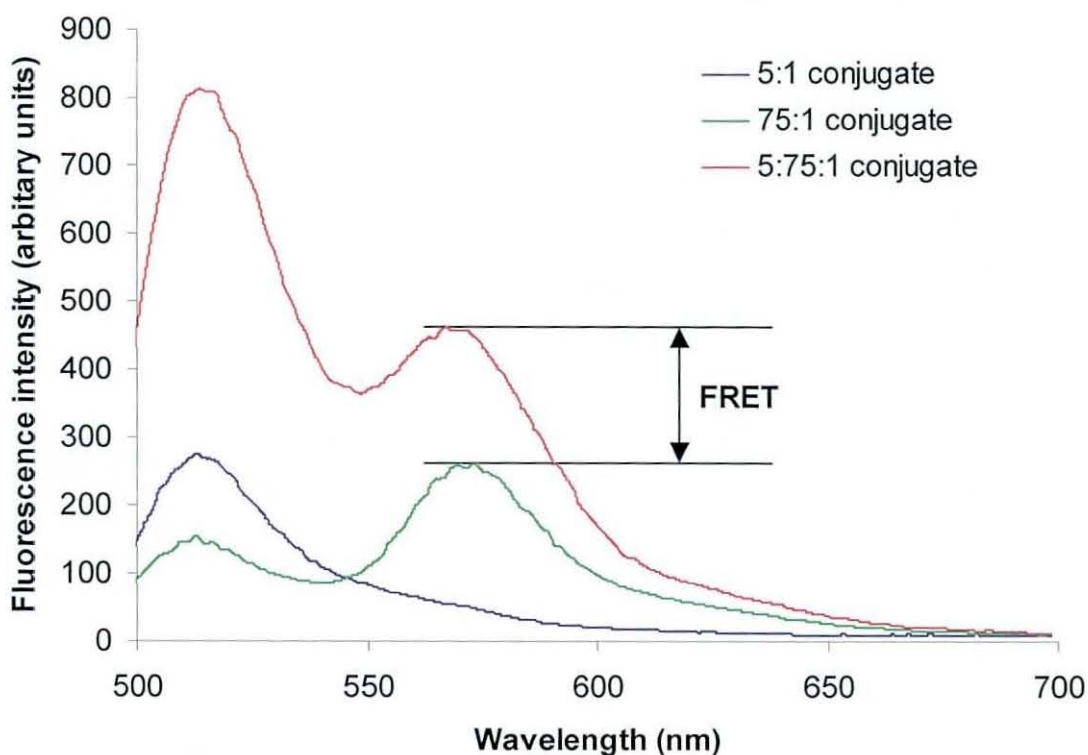
4.2.2.3 Analysis of the 5:75:1 FITC:TMRITC: α -casein conjugate

5:1 FITC: α -casein and 75:1 TMRITC: α -casein conjugates were also prepared with the assumption that in theory these conjugates should contain roughly the same quantity of dye as the doubly labelled conjugate. This would obviously only be a crude estimate because in reality a great excess of the label to protein is normally used in labelling experiments, and possibly even an excess of label to linking (lysine) residues. It is thus not easy to argue that the degree of labelling in the single conjugate is going to be the same as in the double conjugate. If the linking reactions and their rates are assumed to be the same, there is going to be competition between fluorescein and rhodamine for a limited number of lysines, hence in this particular case rhodamine would be more likely to win and less fluorescein would be bound. Nevertheless, this experiment was carried out with a view to considering the rough rather than exact extent of direct excitation of FITC and TMRITC and any donor-to-donor or acceptor-to-acceptor energy transfer.

Tetramethylrhodamine would emit fluorescence only if excited. There were just two ways that this could occur: by direct excitation and by FRET from fluorescein emission.

The 75:1 TMRITC: α -casein conjugate was excited at 480 nm and as shown in figure 4.6 upon excitation at this wavelength an emission peak appeared. Unfortunately direct excitation of TMRITC by the fluorescein wavelength was expected due to the considerable overlap in their absorbance spectra (figure 4.3). However, it was assumed that this peak, and therefore its height, was solely due to the direct excitation of TMRITC. The 5:75:1 conjugate was excited at 480 nm and it could be concluded that the peak that resulted at the tetramethylrhodamine wavelength was an additive combination of direct excitation of TMRITC and FRET from FITC. It therefore followed that the difference in peak heights, in theory, could be attributed to the phenomenon of FRET between donor and acceptor.

Figure 4.6 – Fluorescence emission spectra demonstrating FRET between fluorescein and tetramethylrhodamine



To confirm these results, a 5:1 FITC: α -casein conjugate was prepared with the idea that the fluorescence emission spectrum of this conjugate should have a peak height above the 5:75:1 conjugate. The difference between these two heights would in theory be equivalent to that previously found with 75:1 conjugate as shown in figure 4.6. This was under the assumption that energy gained by TMRITC equals energy lost by FITC.

However, as demonstrated in figure 4.6, this was not observed experimentally. The 5:1 FITC: α -casein peak was much lower than the peak at the fluorescein wavelength found with the 5:75:1 FITC:TMRITC: α -casein conjugate. The most plausible reason for this observation was due to competition for binding sites on the protein molecules during conjugation. In the doubly labelled conjugate fluorescein and tetramethylrhodamine compete for the same sites to bind to whereas in the fluorescein: α -casein conjugate only fluorescein can bind. It would seem likely that more fluorescein is able to bind to the protein in the absence of competition i.e. in the fluorescein: α -casein conjugate. A higher fluorescein concentration in the single labelled conjugate may have produced a quenched substrate due to internal energy transfer between fluorescein molecules, accounting for the low fluorescence intensity found in this conjugate.

It was evident from figure 4.6 that the 75:1 TMRITC: α -casein conjugate displayed a peak in the fluorescein region, which was unexpected. An emission spectrum was taken of the TMRITC free dye obtained from Sigma with extreme care to prevent contamination. It was seen that the Sigma sample also contained a peak at the fluorescein wavelength and it was therefore concluded that the dye supplied by Sigma contained a contaminant. This emission at the fluorescein wavelength may have also contributed to the peak height found in the 5:75:1 conjugate at the fluorescein wavelength which would have further complicated the situation described above where 5:1 FITC: α -casein was synthesised to determine energy transfer.

4.3 INVESTIGATION INTO THE USE OF CY 5 AND CY 5.5 FOR FRET PROTEASE ASSAYS

4.3.1 Procedures

Fluorescence measurements were carried out using a Perkin Elmer LS 50B fluorescence spectrophotometer with an excitation wavelength of 649 nm for FRET conjugate experiments. Excitation and emission wavelengths of 649 nm and 670 nm and 675 nm and 694 nm were used for Cy 5 and Cy 5.5 free dyes respectively. Excitation and emission bandwidths of 10 nm were used and measurements were made statically in a standard 3 ml quartz fluorescence cuvette.

Cy 5: Cy 5.5: α -casein conjugates were prepared as described in section 2.2.10 using 1.0 mg / ml α -casein and reaction mixture ratios of 2:30:1 and 5:20:1. As Cy 5 and Cy 5.5 were more expensive than either FITC or TMRITC, the amount of protein was reduced in order that the reaction mixture ratios could be scaled down to use lower concentrations of Cy dyes. It was decided that 2:30:1 and 5:20:1 Cy 5: Cy 5.5: α -casein conjugates should be synthesised, as it had previously been shown (section 4.2.2.2) that optimum conditions for use in FRET protease assays required lightly labelled donor and heavily labelled acceptor conjugates.

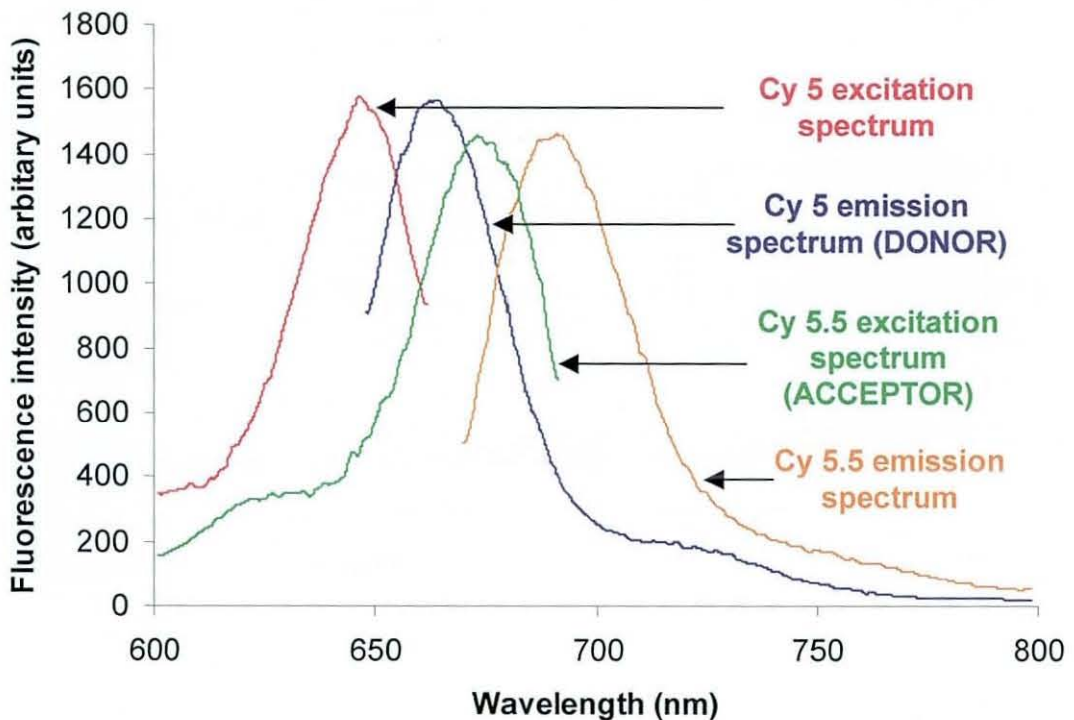
This FRET pair was chosen because these Cy dyes have suitable spectral characteristics, are pH-insensitive, and emit in the long-wavelength region (ca. 600 – 800 nm). Cy 5 and 5.5 have also previously been shown to act as an energy transfer pair [287, 288].

4.3.2 Results

4.3.2.1 Analysis of the overlapping of Cy 5 (donor) and Cy 5.5 (acceptor) excitation and emission spectra

Excitation and emission spectra were taken of the Cy 5 and Cy 5.5 free dyes. From figure 4.7 it can be seen that these dyes showed significant spectral overlap between the Cy 5 (donor) emission and Cy 5.5 (acceptor) excitation. This suggested that these dyes were ideal as a donor-acceptor pair for use in FRET protease assays.

Figure 4.7 – Spectral overlap of Cy 5 (donor) and Cy 5.5 (acceptor)



4.3.2.2 Analysis of the Cy 5: Cy 5.5: α -casein conjugates

Fluorescence emission spectra were taken of the 2:30:1 and 5:20:1 conjugates using an excitation wavelength of 649 nm. However, a broad emission peak was obtained with both conjugates over the spectral region of around 665 nm to 720 nm with no distinct Cy 5 or Cy 5.5 peaks. As the emission wavelengths of Cy 5 and Cy 5.5 were so close (670 nm and 694 nm respectively), it was likely that the broad peak was due to the merging of both the Cy 5 and Cy 5.5 peaks.

It was therefore concluded that the Cy 5 / Cy 5.5 dye pair was not ideal as an energy transfer pair. Unfortunately due to lack of time, the use of Cy 5 and Cy 5.5 was not pursued for use in protease assays. However, from these initial results it would appear that a great deal of work would need to be carried out in order to establish the optimum ratio of donor and acceptor fluorophores for energy transfer to occur and to be able to monitor both the donor enhancement and acceptor quenching effects that would be seen upon proteolytic cleavage of the substrate. In addition, the use of multivariate statistical analysis using a software package such as Unscrambler to resolve overlapping peaks may be needed.

4.4 DISCUSSION

Experiments were conducted to establish whether fluorescein / tetramethylrhodamine and Cy 5 / Cy 5.5 could be used as fluorophore pairs for use in protease assays based on the phenomenon of FRET.

Results suggested that optimum conditions required lightly labelled donor and heavily labelled acceptor conjugates for use in FRET protease assays. This was in agreement with findings by Lim *et al.*, who similarly found that these conditions were necessary for an energy transfer immunoassay for human serum albumin [41]. It was shown that the phenomenon of energy transfer may have occurred in the 5:75:1 FITC:TMRITC: α -casein conjugate. However, these results were extremely difficult to interpret due to the competing events shown in figure 4.1 and therefore not concrete evidence of energy transfer. As the synthesis of these substrates was not successful, the application of these substrates to protease assays was not carried out.

The initial results obtained with Cy 5 and Cy 5.5 were not promising and suggested that this would be a very complicated dye pair to work with.

One area that was not investigated, which would improve these assays and perhaps simplify the analysis of results, would be the effect of altering the fluorescence spectrophotometer bandwidth. Direct excitation of the acceptor fluorophore was a significant obstacle, as it occurred in addition to and in competition with the donor excitation. This happened because of the overlapping of the donor excitation and acceptor excitation spectra. Thus, at larger bandwidths the likelihood of direct acceptor excitation is much greater. The use of smaller bandwidths may have therefore improved the process of FRET between donor and acceptor and would be well worth investigating in any further

studies. However, although narrower bandwidths may show up more energy transfer, it would presumably be at the expense of sensitivity. Laser excitation may therefore be more helpful, which may allow less direct excitation of the acceptor at the same time as increasing the sensitivity.

It was obvious from experiments with both FITC / TMRITC and Cy 5 / Cy 5.5 that optimum conditions required for FRET to occur would need to be investigated to a much greater depth, which time did not permit. The synthesis of these doubly labelled conjugates was evidently not as simple or quick as that for the single labelled conjugates used in chapter 5 and so single labelled conjugates were pursued in this study for the determination of proteolytic enzymes and their inhibitors. However, although making doubly labelled conjugates of this kind is obviously more complex both to investigate and then to achieve in routine practice, if it can be done this kind of method may give advantages over other energy transfer methods.

CHAPTER 5

Experimental: single labelled substrate protease and protease inhibition assays

5.1 INTRODUCTION

During screening for promising drug candidates, hundreds of thousands of compounds need to be assayed. The aim of these experiments was therefore to develop a simple, sensitive, and efficient method for assaying protease activity that would facilitate the testing of a large number of samples using HTS.

The application of a long-wavelength pH-insensitive intramolecularly quenched substrate to the determination of proteolytic enzymes and their inhibitors is demonstrated using the principle that was shown in figure 1.19. A substrate utilising α -casein labelled with a long-wavelength BODIPY® dye has been synthesised and these assays have been incorporated into a flow injection analysis system that allows the rapid determination of protease inhibitors.

Fluorescein:albumin has previously been used in this type of assay [256, 257], but the substitution of a large number of positive lysine residues with the negative charges of fluorescein may cause conformational changes such that the interaction of the protein with the active site of the enzyme becomes problematic. However, BODIPY dyes have no charge and less dye is required for quenching [255]. This should minimise the effects of dye conjugation on the protein substrate conformation and enzyme-active site interactions.

In addition, the pH dependence of fluorescein has prompted the use of the pH-insensitive BODIPY dyes in place of fluorescein in this type of assay.

5.2 PREPARATION OF BODIPY CONJUGATES

5.2.1 Procedures

BODIPY[®] 630 / 650 – X, SE: α -casein conjugates (from this point referred to as BODIPY for ease) were prepared using the method described in section 2.2.10. A concentration of 2.5 mg / ml α -casein was used and reaction mixture labelling ratios of 5:1, 15:1, and 45:1 BODIPY: α -casein were prepared. To determine the final dye protein (D:P) conjugate ratios, the Beer-Lambert Law was used to calculate dye and protein concentrations, as shown in section 2.2.11.

All fluorescence spectra were obtained using an Hitachi F-4500 fluorescence spectrophotometer with a standard 3 ml quartz fluorescence cuvette.

All absorbance measurements were taken using an Unicam 8700 UV / Visible Spectrometer. A standard 3 ml quartz absorbance cuvette was used and baselines were taken with buffer prior to measurements.

An absorbance spectrum of a solution of BODIPY was taken and used to calculate the BODIPY absorbance at 280 nm as a percentage of that at 630 nm. This was found to be 9.5 % (calculation in appendix H), which was taken into account in final D.P ratio calculations. The molar extinction coefficient of α -casein was also determined. A 0.1 % solution of α -casein was made up, the absorbance measured, and these absorbance values applied to the Beer-Lambert Law to calculate the molar extinction coefficient. This was found to be $21\,151\text{ cm}^{-1}\text{ M}^{-1}$ as shown in the calculations in appendix I. A molar extinction coefficient of $100\,300\text{ cm}^{-1}\text{ M}^{-1}$ was used for BODIPY. Absorbance values were corrected for dye absorbance at the protein wavelength of 280 nm (9.5 %) and for protein absorbance at the dye wavelength of 630 nm (0 %).

5.2.2 Results

Calculated final D:P ratios along with initial reaction mixture ratios for three BODIPY: α -casein conjugates are given in table 5.1.

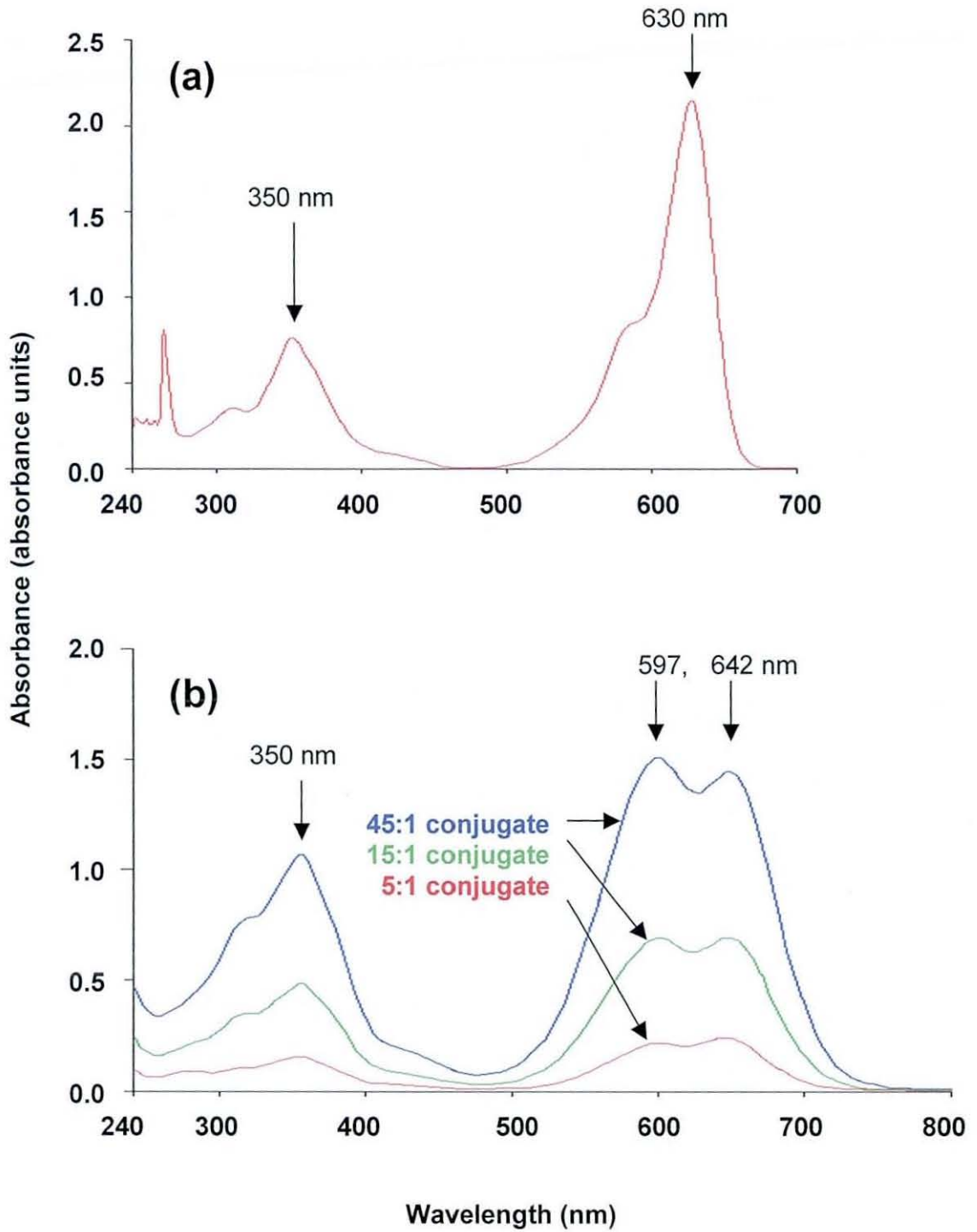
REACTION MIXTURE RATIO	CALCULATED DYE:PROTEIN RATIO
5:1	0.55:1
15:1	0.46:1
45:1	0.52:1

Table 5.1 – Initial reaction mixture and calculated final dye:protein ratios for BODIPY: α -casein conjugates

The results in table 5.1 show that as the amount of BODIPY in the reaction mixture was increased relative to the amount of α -casein, the actual dye:protein ratio did not change. However, this seems illogical and suggests that for some reason the rules of D:P ratio calculations have broken down.

The absorbance spectra that were taken of the free BODIPY dye (figure 5.1 (a)) and of the three BODIPY: α -casein conjugates (figure 5.1 (b)) for the D:P calculations are shown below. As shown in figure 5.1, upon conjugation, the absorption peak at 630 nm of the BODIPY solution (a) unexpectedly split into two peaks at 597 nm and 642 nm in the conjugates (b).

Figure 5.1 – Absorbance spectra of BODIPY solution (a) and BODIPY conjugates (b)



This phenomenon may be explained by H-dimer formation. This is a non-fluorescent complex of two neighbouring and aligned dye moieties that has an absorption maximum that is typically 20 – 40 nm shorter than the usual absorption maximum for a given chromophore. Similar behaviour has been demonstrated with tetramethylrhodamine and Cy dyes [287 - 289]. If this interpretation is correct then the following should be observed:

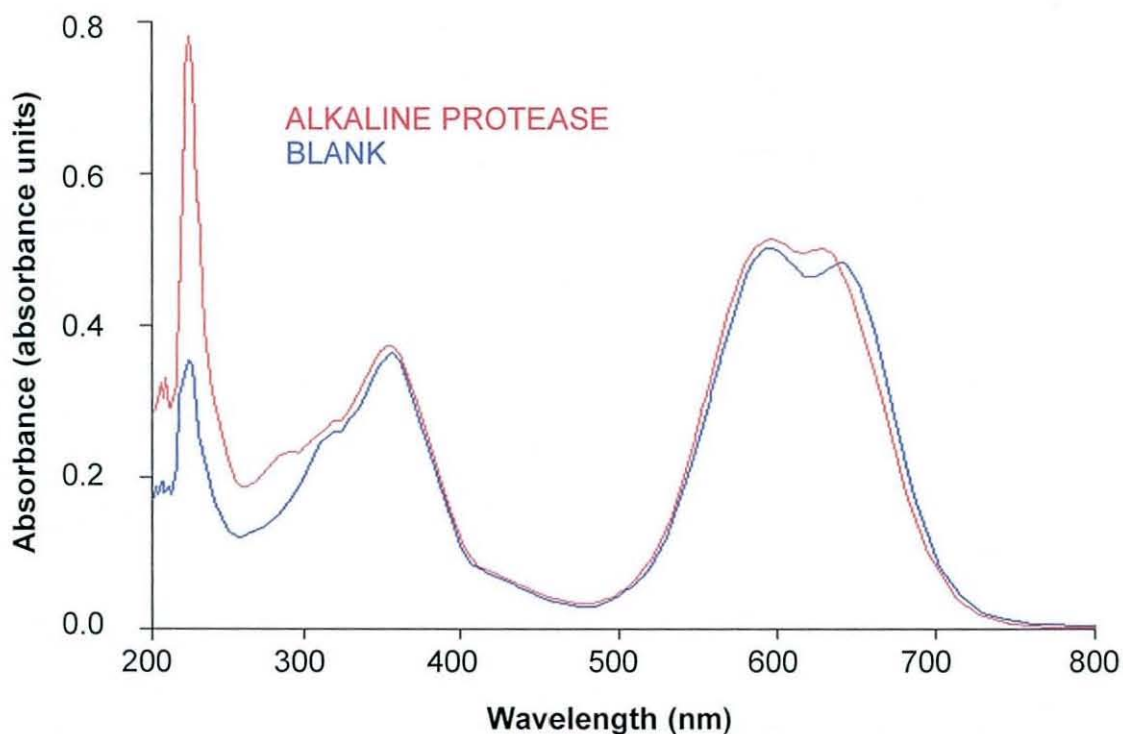
- Upon protease digestion of the labelled protein the 597 nm band should decrease relative to the 642 nm band
- Upon denaturation of the labelled protein the 597 nm band should decrease relative to the 642 nm band
- The 597 nm band should increase relative to the 642 nm band as the number of dyes attached per protein is increased
- The 597 nm peak should be absent on the fluorescence excitation spectrum of the labelled protein because it is due to absorption by the non-fluorescent dimer

Experiments have therefore been carried out to establish whether the above criteria are observed.

5.2.2.1 Effect of protease digestion on the absorption characteristics of BODIPY: α -casein

As shown in figure 5.2, upon incubation with the protease alkaline protease, the absorbance spectrum of BODIPY: α -casein changed such that the 597 nm band decreased relative to the 642 nm band.

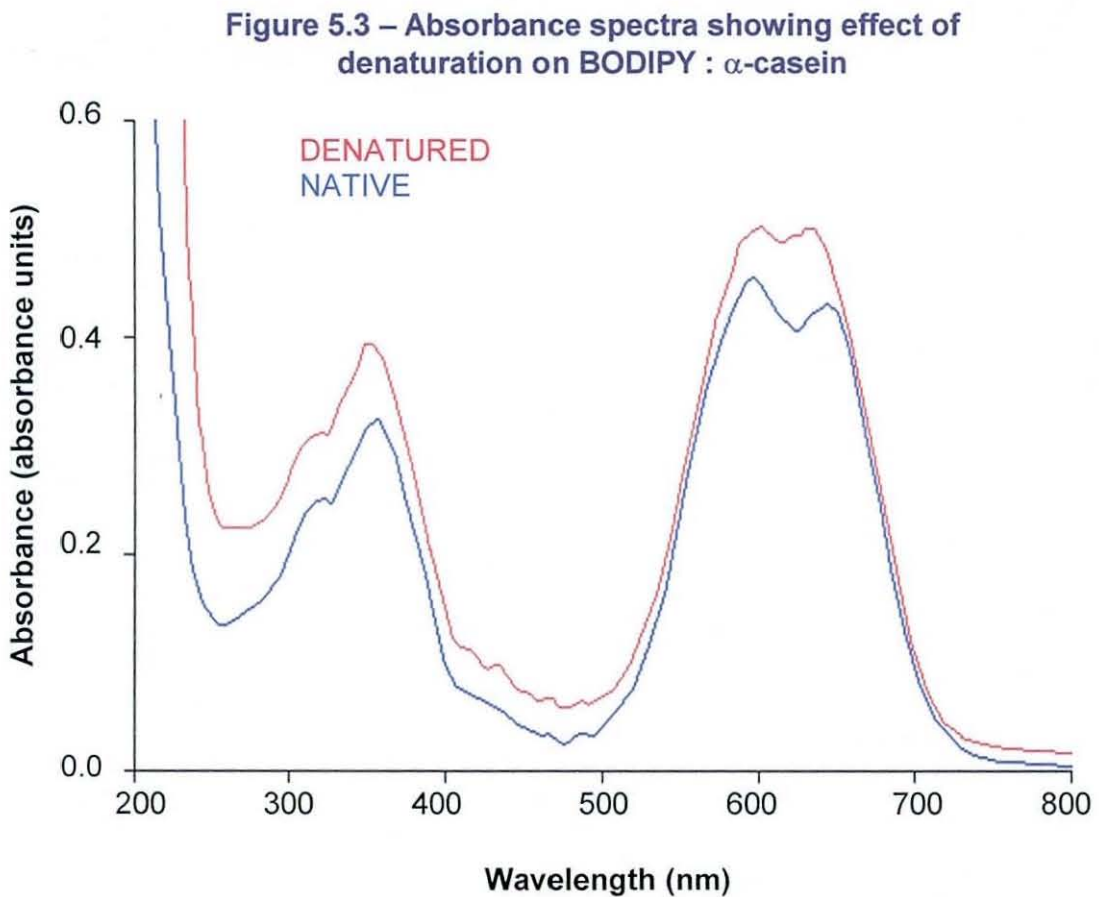
Figure 5.2 – Absorbance spectra showing effect of protease digestion on BODIPY : α -casein



The fact that protease digestion of BODIPY: α -casein had the above effect suggests H-dimer formation.

5.2.2.2 Effect of denaturation on the absorption characteristics of BODIPY: α -casein

Figure 5.3 shows the effect of denaturation through heating and urea upon the absorbance spectrum of BODIPY: α -casein conjugate.



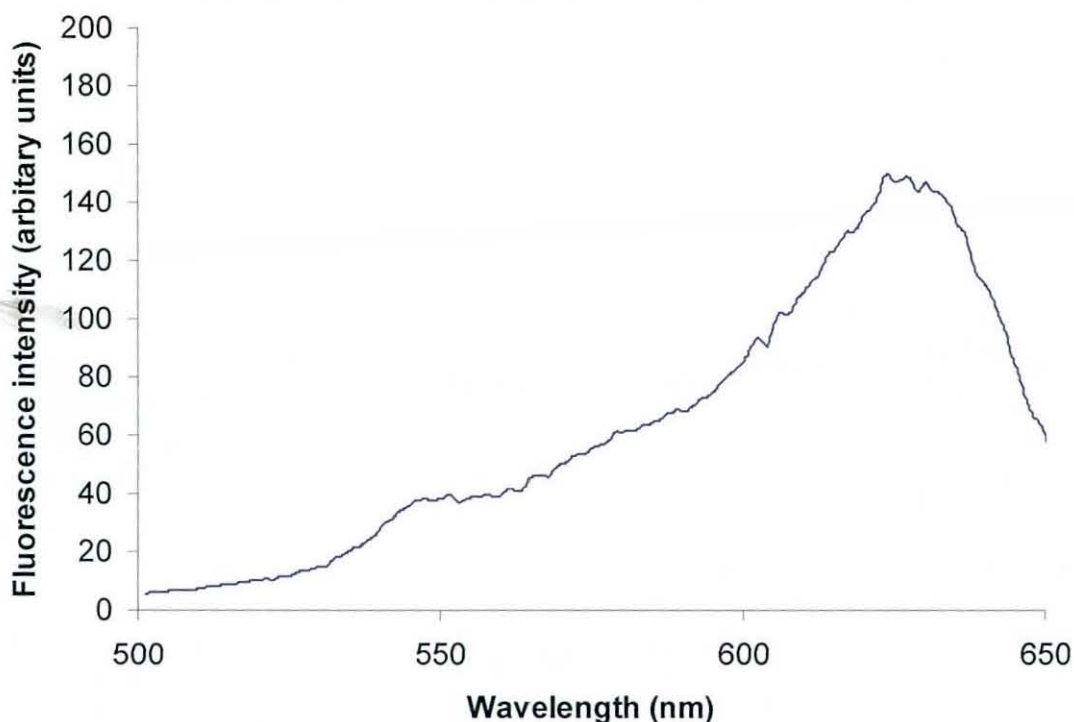
It can be seen that the 597 nm band decreased relative to the 642 nm band upon denaturation, again suggesting the formation of H-dimers between the BODIPY molecules.

5.2.2.3 Effect of the number of BODIPY dyes attached to α -casein on the absorbance spectra of the conjugates

The absorbance spectra of the three BODIPY conjugates – 5:1, 15:1, and 45:1, were shown above in figure 5.1 (b). From these spectra it can be seen that as the number of BODIPY molecules attached to α -casein was increased, the 597 nm band increased relative to the 642 nm band. This evidence further supports the H-dimer theory and also indicates that the D:P calculations shown in table 5.1 are incorrect. If the H-dimer theory is indeed correct, it follows that as the 597 nm band increased relative to the 642 nm band with increasing amounts of BODIPY in the reaction mixture, the number of BODIPY molecules attached to the α -casein molecule also increased. The D:P calculations did not suggest this, hence adding more weight to the fact that the calculations are wrong.

5.2.2.4 Comparison of the fluorescence excitation spectrum with the absorbance spectrum of BODIPY: α -casein

The fluorescence excitation spectrum of BODIPY: α -casein was obtained using excitation and emission bandwidths of 10 nm, a scan speed of 240 nm / min, and an emission wavelength of 655 nm. The excitation spectrum (figure 5.4) showed a lack of the 597 nm peak, which was apparent on the absorbance spectrum (figure 5.1(b)). This evidence also supports the theory that BODIPY H-dimers have been formed in the conjugate, as the dimer is non-fluorescent. However, the dimer does absorb light. As a change in structure has occurred, the dimer could really be thought of as another chromophore and so a double peak appears on the absorbance spectrum. The first peak is due to absorption by the dimer, the second by the single fluorophore molecule.

Figure 5.4 – Excitation spectrum of BODIPY: α -casein

5.2.2.5 Re-evaluation of D:P calculations

Calculation of D:P ratios makes one big assumption: that the spectral characteristics of the free dye and that of the conjugated dye are identical. Clearly this is not the case with BODIPY and BODIPY: α -casein, as shown by figure 5.1. The shape of the absorbance spectrum changes upon conjugation. This would greatly affect the D:P calculations if not taken into consideration and would help to explain why the D:P ratios given in table 5.1 seem so illogical.

With this in mind, the D:P calculations were altered such that a wavelength of 350 nm was chosen to calculate the dye concentration using the Beer-Lambert Law. As highlighted by figures 5.1 (a) and (b), the shape of the BODIPY

spectrum doesn't significantly change around 350 nm upon conjugation and so this wavelength was chosen for further calculations.

D:P calculations were carried out by measuring absorbances at 280 nm for α -casein and 350 nm for BODIPY, and were corrected for any dye contribution at the protein wavelength and *vice-versa*. α -casein absorbance at 350 nm was found to be 11 % of that at 280 nm (appendix J), and BODIPY absorbance at 280 nm was found to be 35 % of that at 350 nm (appendix K). Molar extinction coefficients of 21, 151 $\text{cm}^{-1} \text{M}^{-1}$ (appendix I) and 41, 627 $\text{cm}^{-1} \text{M}^{-1}$ (appendix L) were used for α -casein and BODIPY respectively.

Four BODIPY: α -casein conjugates were prepared as described in section 2.2.10 using reaction mixture ratios of 2:1, 5:1, 10:1, and 15:1. Final D:P ratios were calculated using the re-evaluated D:P calculation method with a dye wavelength of 350 nm. Initial reaction mixture and calculated final D:P ratios are given in table 5.2.

REACTION MIXTURE RATIO OF DYE:PROTEIN	FINAL DYE:PROTEIN RATIO
2:1	0.71:1
5:1	2.35:1
10:1	3.93:1
15:1	5.10:1

Table 5.2 – Initial reaction mixture and calculated final dye:protein ratios for various BODIPY: α -casein conjugates

As would be expected, as the amount of dye in the reaction mixture was increased relative to the protein concentration, a concomitant increase was seen in the final D:P ratio. As this ratio was increased, it might be expected that the suitability of the substrate as a quenched protease substrate would also have increased. In practice, however, the conjugate with a final D:P ratio of 2.35 gave the best fluorescence enhancement after treatment with enzymes, and so this conjugate was used in all subsequent work.

5.2.2.6 Calculation of percentage quenching in the 2.35 BODIPY: α -casein conjugate

In order to determine the amount of quenching that there was in the BODIPY: α -casein conjugate chosen for all further work, solutions of free BODIPY dye and conjugate were taken and absorbance readings taken at 350 nm. These solutions were adjusted such that the absorbance value of both solutions was identical i.e. the concentration of dye was the same in each.

Fluorescence emission spectra were then taken of each solution to compare the fluorescence of free and conjugated dye to calculate the amount of fluorescence quenching in the substrate. An excitation wavelength of 605 nm was used with excitation and emission bandwidths of 5 nm. Fluorescence intensities were corrected for any buffer fluorescence. Intensities of 389.14 and 1444.24 were obtained for conjugate and free dye, respectively. This indicated that the 2.35 BODIPY: α -casein conjugate was 73 % quenched.

73 % quenching in the conjugate indicated that BODIPY: α -casein should indeed act as an efficient intramolecularly quenched protease substrate. However, if more time were available this percentage quenching could be improved which would increase assay sensitivity.

5.2.2.7 Effect of altering excitation wavelength on the emission spectrum of BODIPY: α -casein

Figure 5.4 showed that the optimum excitation wavelength of BODIPY: α -casein was 625 nm. However, the optimum emission wavelength of BODIPY: α -casein is 644 nm, which gives a Stokes shift of just 19 nm. Using excitation and emission bandwidths of 10 nm and a solution of BODIPY: α -casein in a standard 3 ml quartz cuvette, emission spectra were taken using a variety of excitation wavelengths.

Figure 5.5 – Effect of altering excitation wavelength on the emission spectrum of BODIPY: α -casein

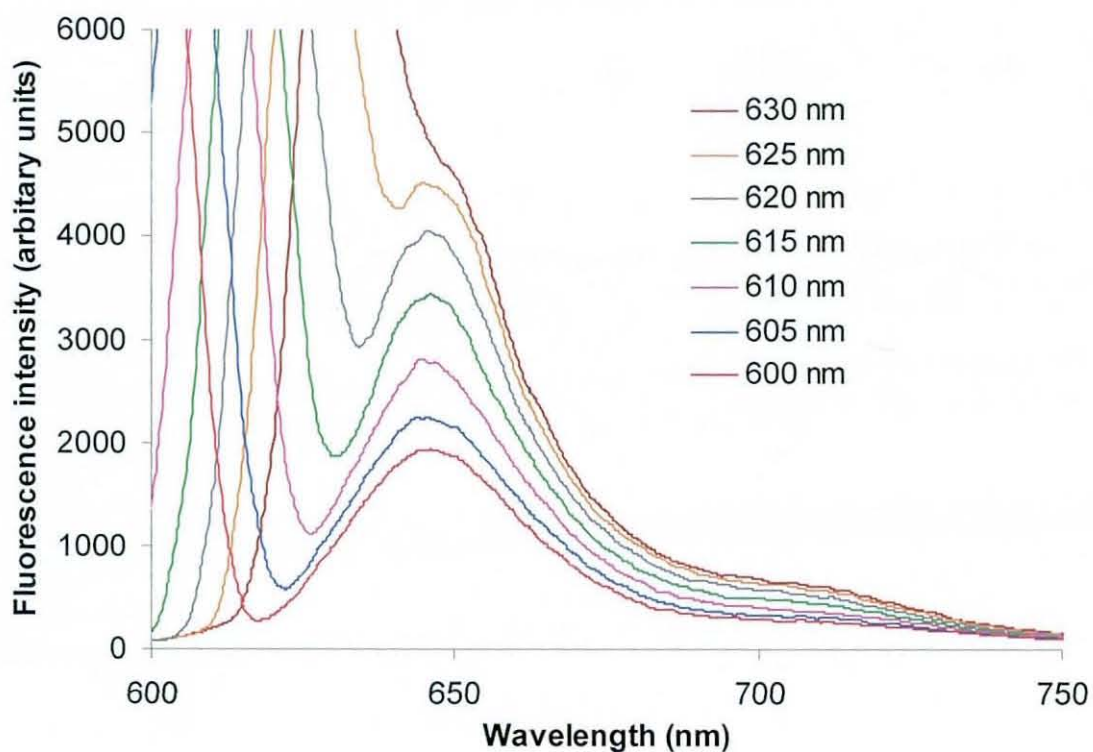


Figure 5.5 illustrates the increasing interference from the scatter peak on the emission peak as the excitation wavelength was increased to the optimum excitation wavelength. The use of the optimal excitation wavelength of 625 nm was therefore not practical because the Stokes shift was so small. Although a loss in fluorescence intensity was seen with lower excitation wavelengths, which will therefore decrease the sensitivity of the protease assays, a balance between sensitivity and interference was required and a sub-optimal wavelength of 605 nm was chosen for use in all subsequent experiments.

5.2.2.8 Investigation into the effect of additives on the absorbance and emission spectra of BODIPY: α -casein

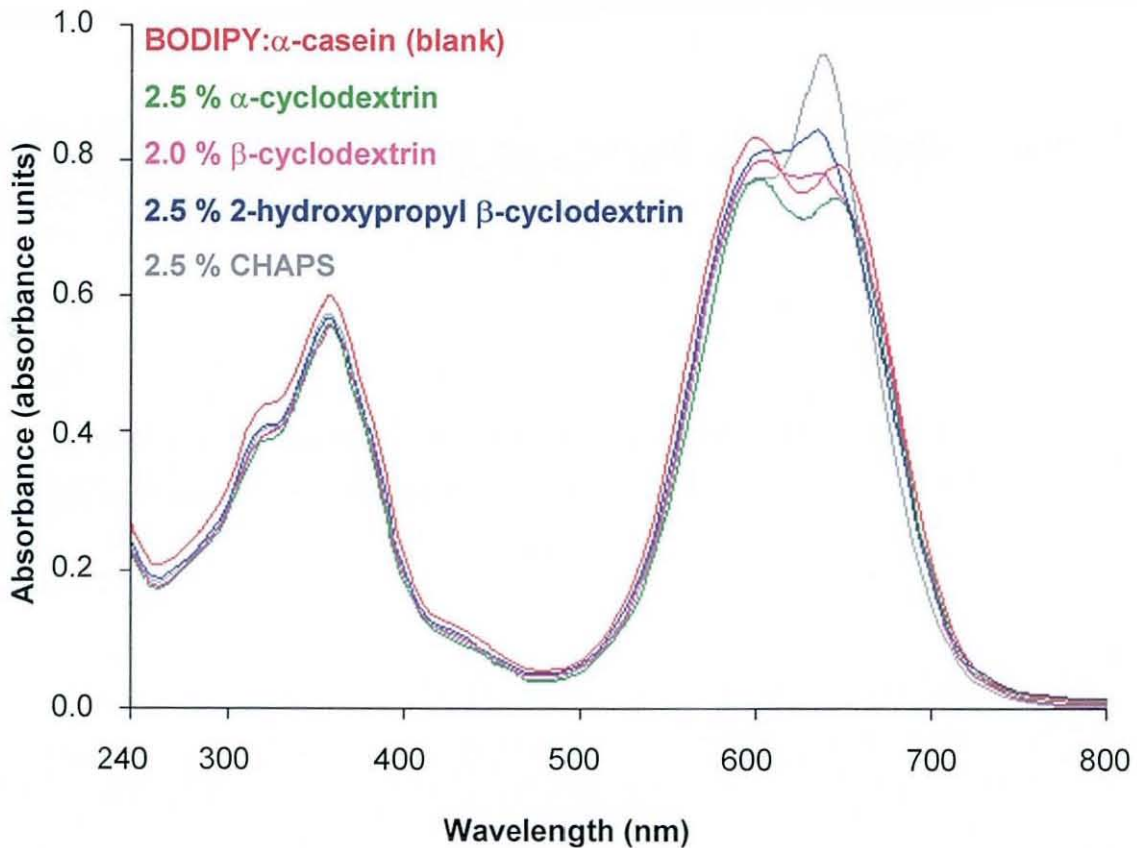
It has previously been shown in our lab that the fluorescence properties of a fluorophore can be changed by the appropriate choice of solvent environment [290]. This paper described the dependence of the optimum excitation wavelength of naphthofluorescein on the presence of additives such as cyclodextrins and (3-[3-cholamidopropyl]-dimethylamino)-1-propane sulfonate (CHAPS). Such effects were used to red-shift the excitation wavelength to match the output of a 635 nm diode laser for use in a simple and sensitive fluorescence detector.

The effect of cyclodextrins and CHAPS on the absorbance and emission wavelengths of BODIPY was therefore investigated to see if this phenomenon could be applied to assays involving BODIPY: α -casein.

It can be seen from figure 5.6 that the inclusion of different cyclodextrins and CHAPS into the solvent environment of BODIPY: α -casein had no great effect upon the peak absorbance wavelengths. The use of these additives to shift the

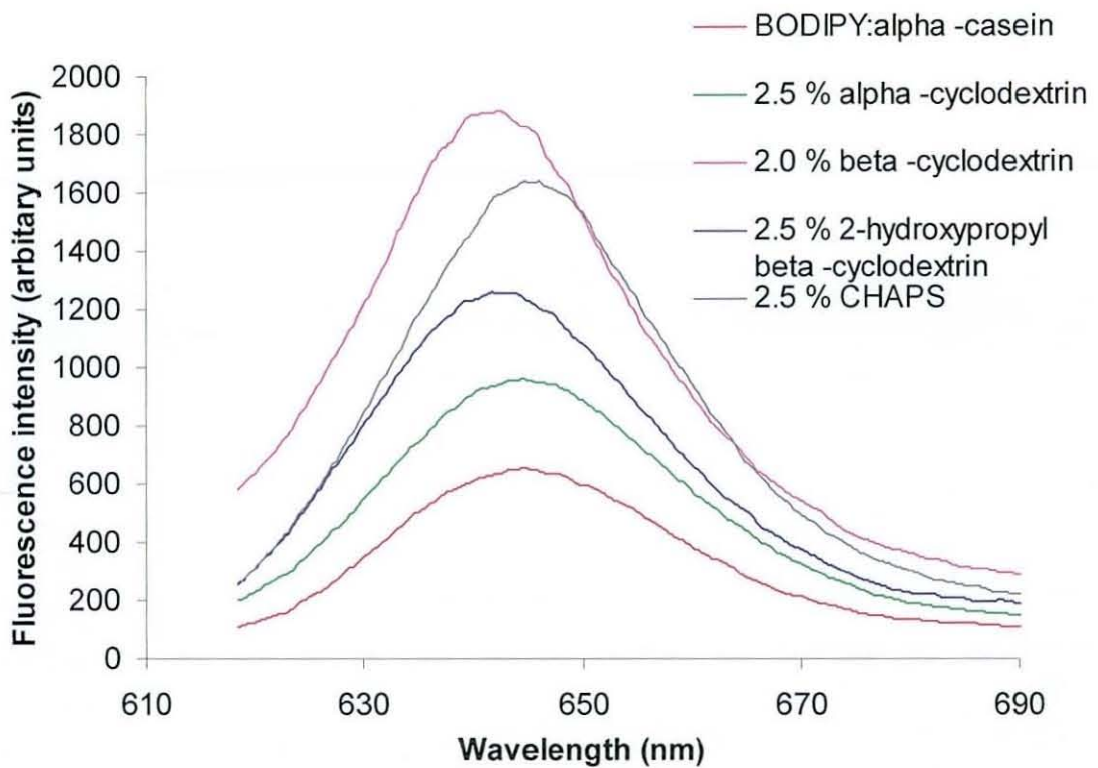
optimum excitation wavelength of BODIPY: α -casein towards that of the output of a 635 nm diode laser is therefore not applicable.

Figure 5.6 – Effect of cyclodextrins and CHAPS on the absorbance spectrum of BODIPY: α -casein



From figure 5.7 it is apparent that the inclusion of neither CHAPS nor the different cyclodextrins tested had much effect upon the emission wavelength of BODIPY: α -casein. The greatest effect (if any) was seen with 2.0 % β -cyclodextrin, and this was to shift the optimum to a lower wavelength. This would have no benefit at all, as it would only serve to lower the Stokes shift and introduce more error through scatter.

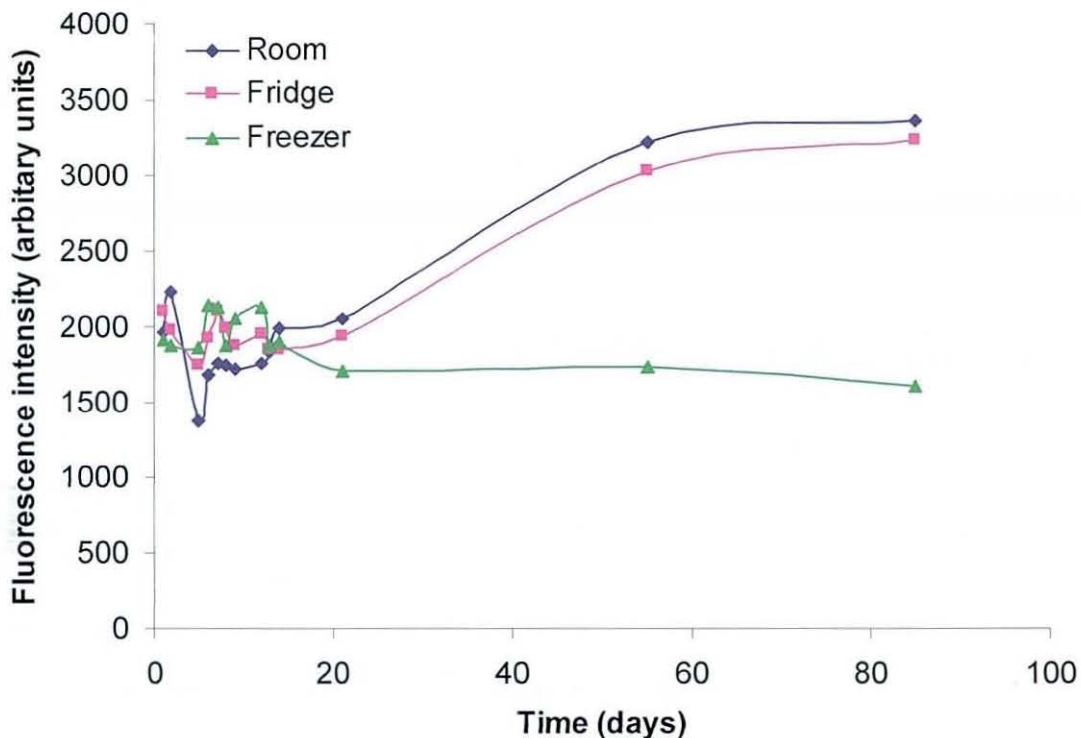
Figure 5.7 – Effect of cyclodextrins and CHAPS on the fluorescence emission spectrum of BODIPY: α -casein



5.2.2.9 Stability of BODIPY: α -casein

In order to determine the best condition for BODIPY: α -casein storage, BODIPY: α -casein was kept in brown bottles in a cupboard at room temperature and in the fridge, and also in aliquots in microcentrifuge tubes wrapped with black tape in the freezer. Over a period of 3 months samples were taken and the fluorescence monitored. All samples were first left to equilibrate to room temperature and then incubated in a water bath at 18 °C for 5 minutes. Emission spectra were then taken using an excitation of 605 nm and excitation and emission bandwidths of 10 nm. All readings were taken first thing in the morning. The effects of these different storage conditions are shown in figure 5.8.

Figure 5.8 – Effect of storage conditions on the fluorescence characteristics of BODIPY: α -casein

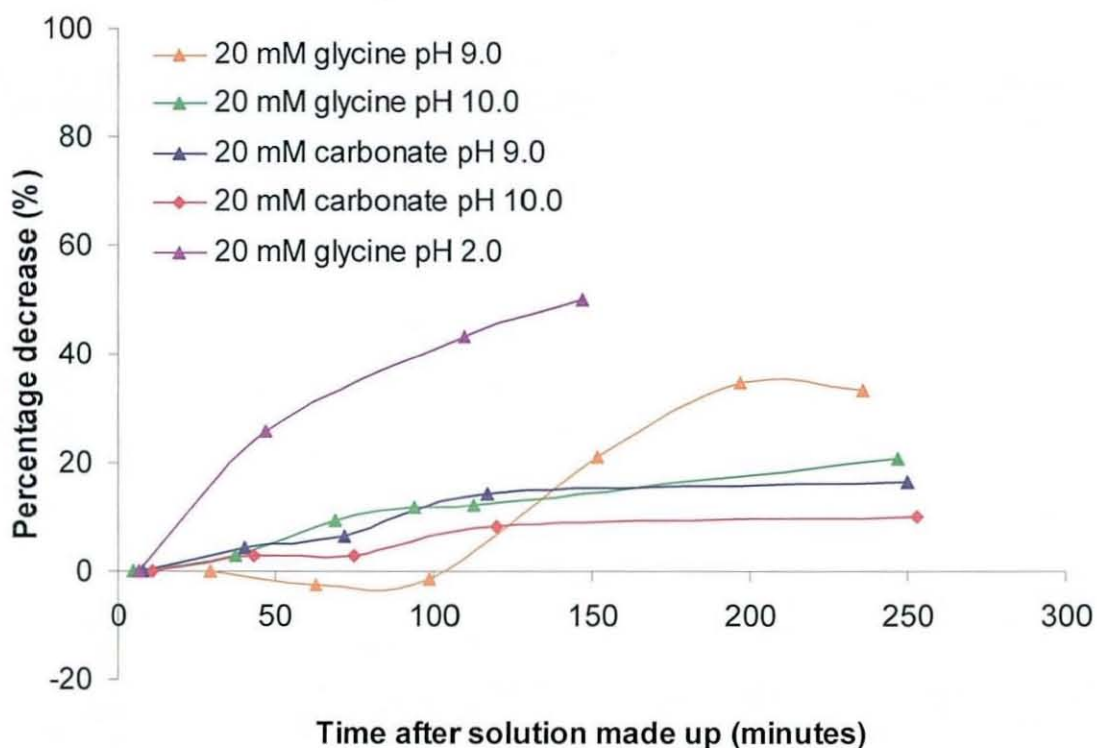


From figure 5.8 it is evident that the ideal storage condition for BODIPY: α -casein was in the freezer. For the first 20 days the stability of BODIPY: α -casein with respect to its fluorescence properties remained relatively constant with all three storage conditions. After 20 days however, both the fridge and room temperature samples began to increase in fluorescence intensity. This was most likely due to degradation of the quenched conjugate causing an increase in fluorescence due to abolished internal energy transfer. The freezer samples however remained relatively constant over a period of 3 months. All BODIPY: α -casein conjugate was thus stored in the freezer in 30 μ l aliquots in microcentrifuge tubes surrounded by black tape, and thawed as required.

As BODIPY: α -casein was made up in buffer for use in protease and protease inhibition experiments, the stability of BODIPY: α -casein in solution was tested.

Conjugate was prepared in glycine and carbonate buffers at different pH values. The fluorescence of the conjugate was measured and plotted as a percentage decrease in fluorescence over time after the solution had been made whilst sitting at room temperature in the lab (figure 5.9).

Figure 5.9 – Stability of BODIPY: α -casein in aqueous solution represented by the percentage decrease in fluorescence intensity over time



It was noticeable from figure 5.9 that in lower pH solution the BODIPY: α -casein conjugate was unstable. At higher pH values the substrate was more stable and it was seen that BODIPY: α -casein was most stable in carbonate buffer at a pH of 10.0. BODIPY: α -casein diluted in this buffer gave a percentage decrease in fluorescence intensity of 10 % over 253 minutes, which was adequate for carrying out experiments. The drop in fluorescence over time was most likely due to photodecomposition.

5.3 PROTEASE KINETICS

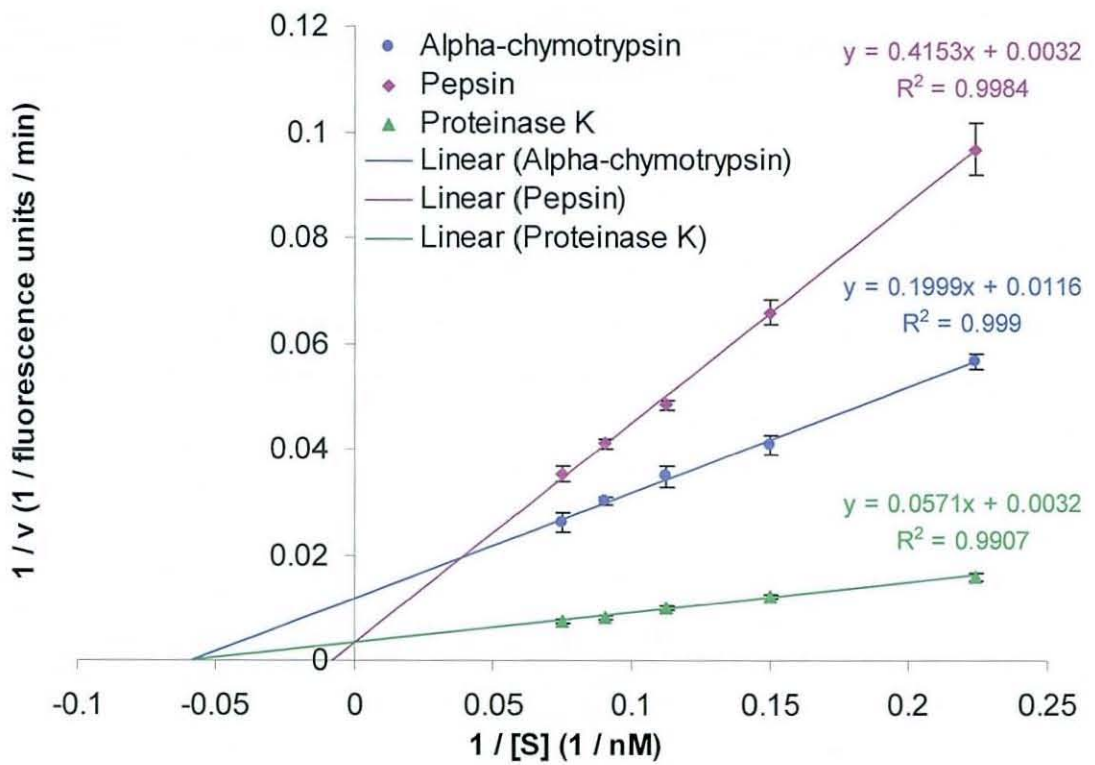
Simple experiments were carried out to illustrate the use of the fluorogenic BODIPY: α -casein substrate in enzyme kinetics. This was not part of the objective of this work and was purely to demonstrate that enzyme kinetics can be done. The results of these experiments are therefore not strictly important, the aim was to show the principle. A discussion of enzyme kinetics was given in section 1.6.3.

5.3.1 Procedures

Fluorescence measurements were carried out statically in a 3 ml standard quartz cuvette using an Hitachi F-4500 fluorescence spectrophotometer with an excitation wavelength of 605 nm and excitation and emission bandwidths of 10 nm. A range of increasing substrate concentrations was used and the fluorescence intensity measured and converted to fluorescence intensity units per minute. Lineweaver-Burk graphs were then plotted for the proteolytic cleavage of BODIPY: α -casein by proteinase K, α -chymotrypsin, and pepsin, as shown in figure 5.10. Measurements were carried out in triplicate and error bars represent plus and minus one standard deviation.

5.3.2 Results

Figure 5.10 – Lineweaver-Burk plots for the proteolytic digestion of BODIPY: α -casein by various proteases



From figure 5.10 values of K_M and V_{max} were determined and these are given in table 5.3.

PROTEOLYTIC ENZYME	K_M (nM)	V_{max} (fluorescence units / min)
PROTEINASE K	17.86	312.5
α -CHYMOTRYPSIN	17.24	86.21
PEPSIN	129.72	312.5

Table 5.3 – K_M and V_{max} values obtained experimentally for various proteolytic enzymes on the substrate BODIPY: α -casein

Figure 5.10 demonstrates that the proteolytic digestion of BODIPY: α -casein by proteinase K, α -chymotrypsin, and pepsin obeyed classical Michaelis-Menten kinetics. Values of K_M and V_{max} have also been determined (table 5.3). Comparing K_M values indicates that BODIPY: α -casein is a more efficient substrate for proteinase K and α -chymotrypsin than for pepsin.

5.4 PROTEASE ASSAYS IN A STATIC SYSEM

5.4.1 Procedures

Fluorescence measurements were made for the protease assays using an Hitachi F-4500 fluorescence spectrophotometer with excitation and emission bandwidths of 10 nm and an excitation wavelength of 605 nm. Static measurements were carried out in a standard quartz fluorescence cuvette.

Five protease enzymes, each with varying pH optima, ranging from pH 2.5 – 9.0, were investigated in these experiments – alkaline protease, proteinase K, α -chymotrypsin, trypsin, and pepsin. A description of each is given in section 1.6.4.

In order to test the stability of the F-4500 fluorescence spectrophotometer, the fluorescence of a rhodamine B reference block was monitored over time. An excitation wavelength of 558 nm with excitation and emission bandwidths of 2.5 nm was used. An emission scan was performed immediately after the spectrophotometer had been switched on and then every 30 minutes thereafter. The shutter between the excitation beam and reference block was shut between readings so as not to heat or photobleach the block. A measurement was also taken of the Xe lamp output along side the fluorescence readings. These results are shown below in figure 5.11.

Figure 5.11 – Fluorescence intensity of a reference block and output of the Xe lamp light source over time for the F-4500 spectrophotometer

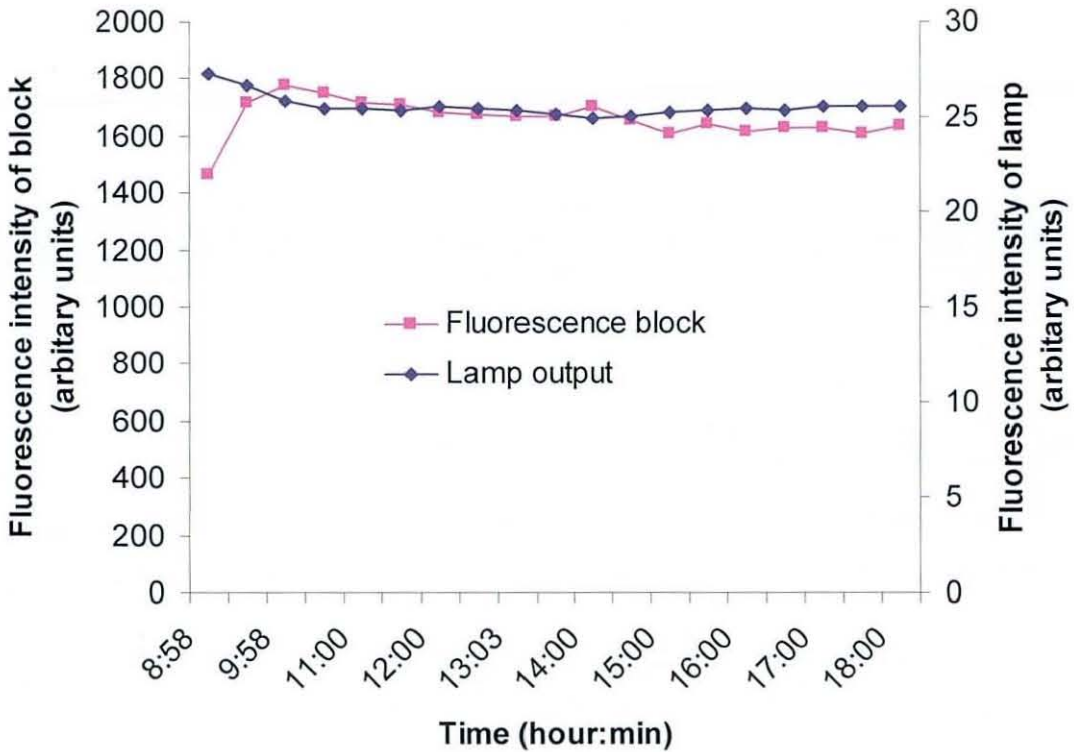


Figure 5.11 indicates that although the Xe lamp output remains relatively constant over 9 hours, the fluorescence reading of a reference block increases rapidly over the first hour after the F-4500 had been switched on. After this initial instability the readings were sufficiently stable. For all experiments that were carried out therefore, the F-4500 was switched on and allowed to stabilise for 1 hour before fluorescence readings were taken.

5.4.2 Results

5.4.2.1 Optimisation of protease assay conditions

Assay conditions for each enzyme were optimised univariately. The effect of the following parameters on the enzyme assay were studied:

- [enzyme]
- incubation time
- temperature
- pH
- buffer system
- [buffer]

The assay parameters studied and optimum conditions found for each enzyme are shown below in table 5.4.

KEY: CA = Carbonate, CI = Citrate, GL = Glycine, PH = Phosphate

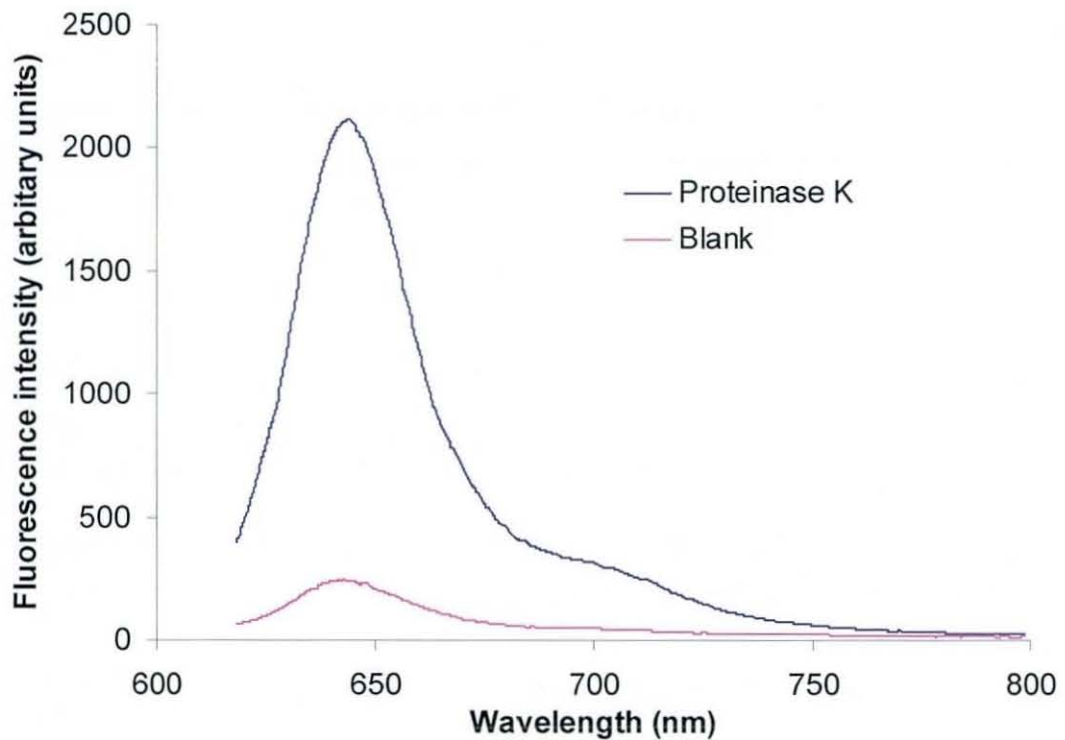
	[ENZYME] ($\mu\text{g/ml}$)	TIME (minutes)	TEMPERATURE ($^{\circ}\text{C}$)	pH	BUFFER	[BUFFER] (mM)
ALKALINE PROTEASE						
RANGE STUDIED	0 – 7	0 – 30	20 – 55	7 – 11	PH / CA	5 – 75
OPTIMUM	2.65	10	40	9.0	CA	10
PROTEINASE K						
RANGE STUDIED	0 – 17	1 – 20	20 – 45	7 – 11	PH / CA	6 – 90
OPTIMUM	9.9	5	40	7.0	PH	8
α- CHYMOTRYPSIN						
RANGE STUDIED	0 – 17	1 – 20	20 – 45	7 – 9	PH	5 – 100
OPTIMUM	3.3	10	40	7.5	PH	10
PEPSIN						
RANGE STUDIED	0 – 17	1 – 20	20 – 55	1 – 3	GL / CI	10 – 100
OPTIMUM	13.2	10	50	2.5	GL	50
TRYPSIN						
RANGE STUDIED	0 – 22	1 – 20	20 – 50	5 – 9	CI / PH	8 – 90
OPTIMUM	4.29	15	45	7.0	PH	10

Table 5.4 - Assay parameters studied and optimum conditions for various proteolytic enzymes using the fluorogenic substrate BODIPY : α -casein in a static system

As optimisation experiments will be described in detail for the protease assays carried out in a flowing system, individual optimisation experiments for the assay conditions for the 5 proteolytic enzymes investigated here will not be described.

Under the optimised conditions given in table 5.4, BODIPY: α -casein was incubated with the protease proteinase K. As shown in figure 5.12, incubation of conjugate with proteinase K gave a fluorescence increase of approximately 9-fold, indicating that BODIPY: α -casein is promising as a fluorogenic protease substrate.

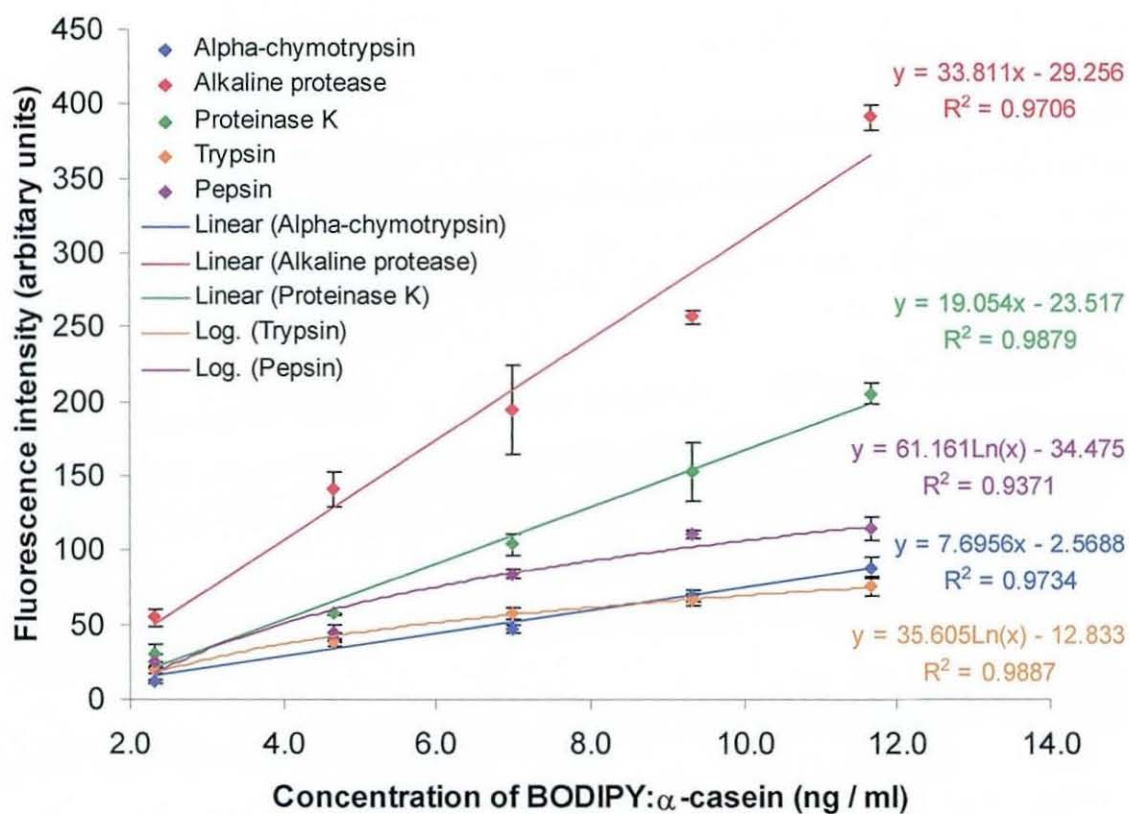
Figure 5.12 – Fluorescence emission spectra showing enhancement seen upon incubation of BODIPY: α -casein with a protease enzyme (proteinase K)



5.4.2.2 Calibration curves for BODIPY: α -casein

The effect of substrate concentration was examined for each of the proteolytic enzymes under optimised conditions. Readings were taken in triplicate and error bars show plus and minus one standard deviation.

Figure 5.13 – Influence of BODIPY: α -casein concentration on the production of BODIPY when incubated with a variety of proteases in a static system under optimised conditions



The activities of a variety of proteolytic enzymes on the substrate BODIPY: α -casein were compared, as shown in figure 5.13. These enzymes represent proteases with activities over a wide pH range – 2.5 to 9.0. It was therefore possible to compare the activities of acid, neutral, and alkaline proteases of different classes (pepsin being an aspartic protease and alkaline protease, proteinase K, trypsin, and α -chymotrypsin being serine proteases) on the same graph using the same substrate merely by changing the buffer system. It can be seen that alkaline protease is by far the most efficient protease, followed by proteinase K. Trypsin and α -chymotrypsin have extremely similar efficiencies, as would be expected from these closely related enzymes. Pepsin also has a lower activity towards BODIPY: α -casein than both alkaline protease and proteinase K.

5.4.2.3 Determination of limits of detection

Limits of detection, defined as the analyte concentration giving a signal equal to the blank signal plus 3 standard deviations of the blank [291], were determined for each of the five proteolytic enzymes in the static system. Fluorescence intensity against enzyme concentration was plotted for the linear portion of the graphs for each of the enzymes, which are shown in figures 5.14 to 5.18. Measurements were taken in triplicate and error bars are given which represent plus and minus one standard deviation.

Figure 5.14 – Fluorescence intensity against enzyme concentration to calculate the limit of detection of alkaline protease

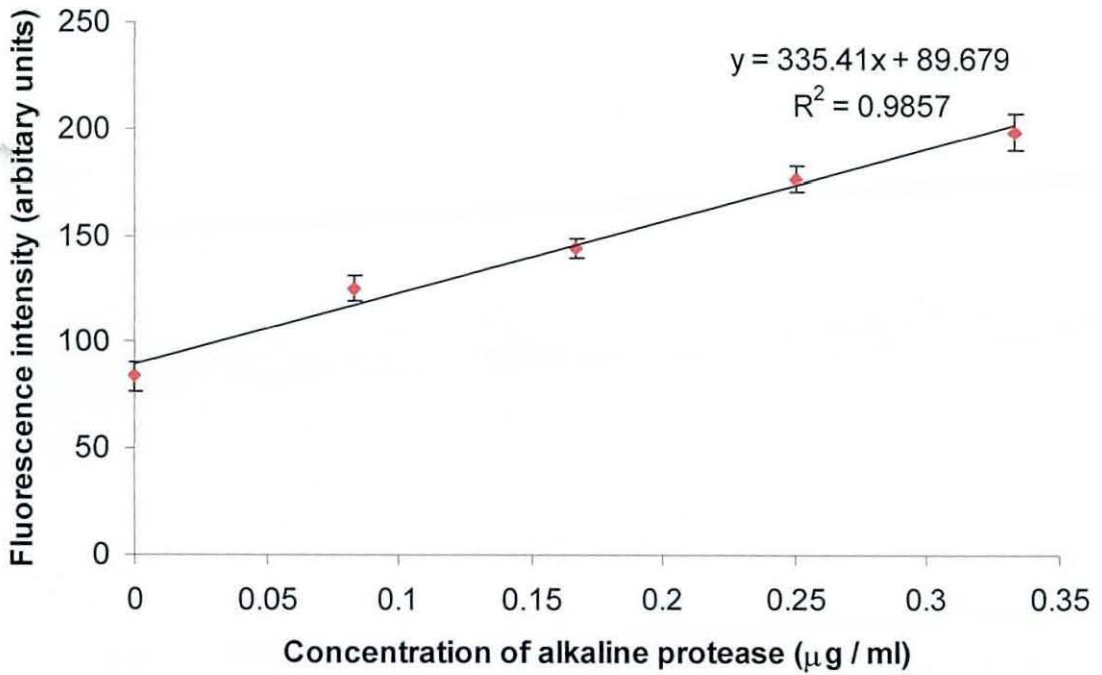


Figure 5.15 – Fluorescence intensity against enzyme concentration to calculate the limit of detection of proteinase K

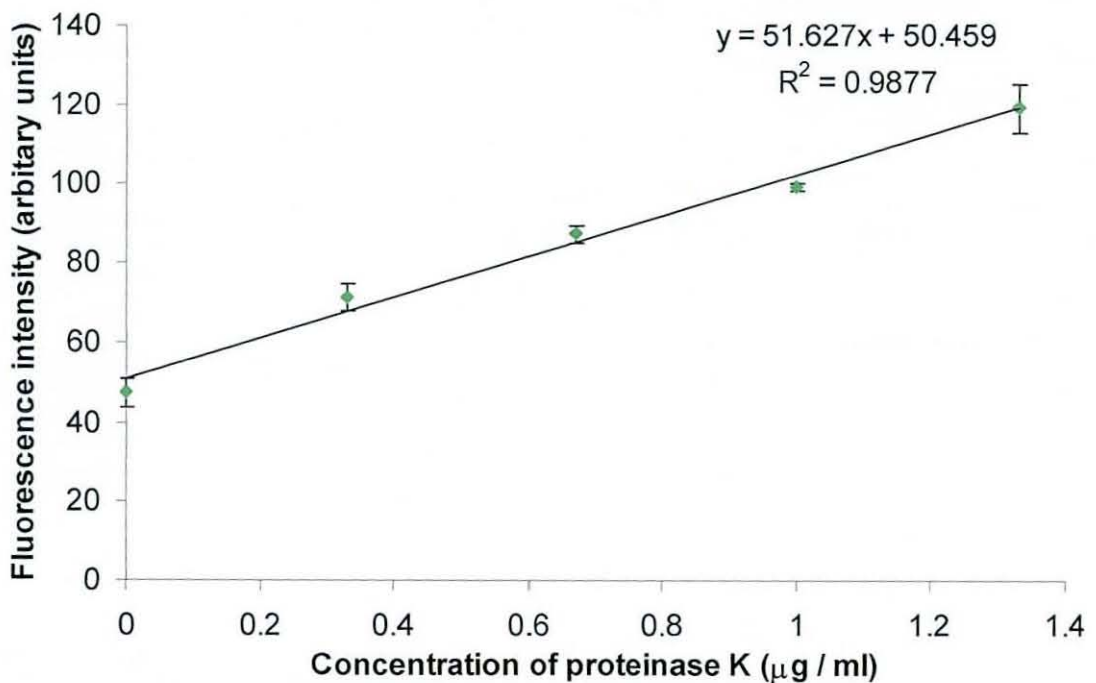


Figure 5.16 – Fluorescence intensity against enzyme concentration to calculate the limit of detection of α -chymotrypsin

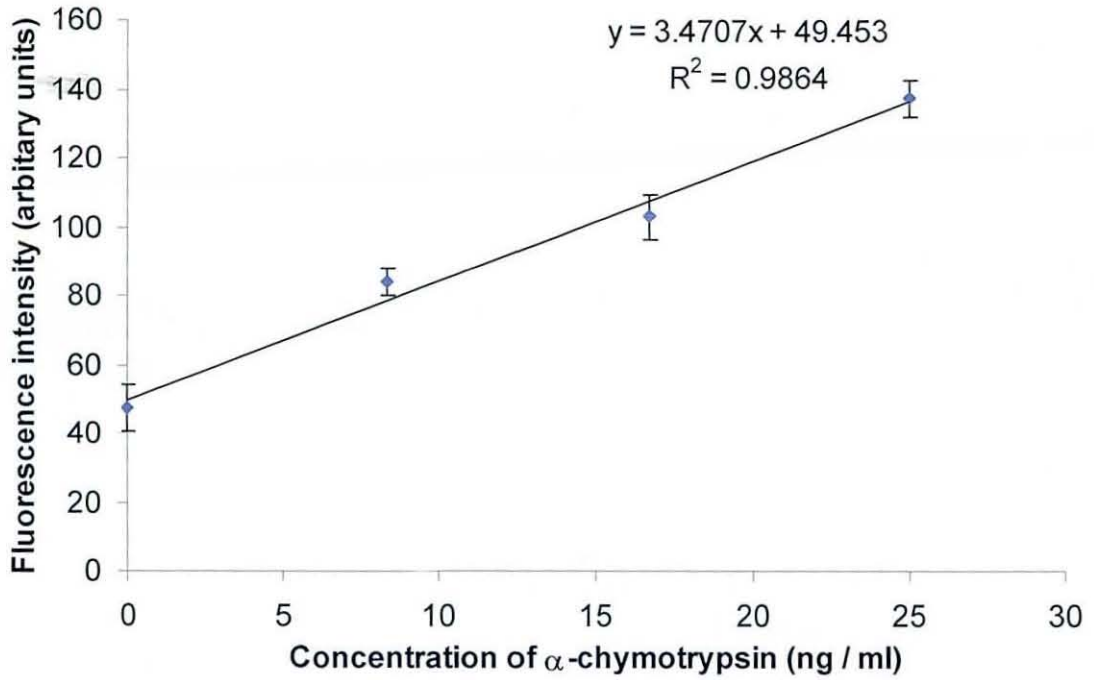


Figure 5.17 – Fluorescence intensity against enzyme concentration to calculate the limit of detection of trypsin

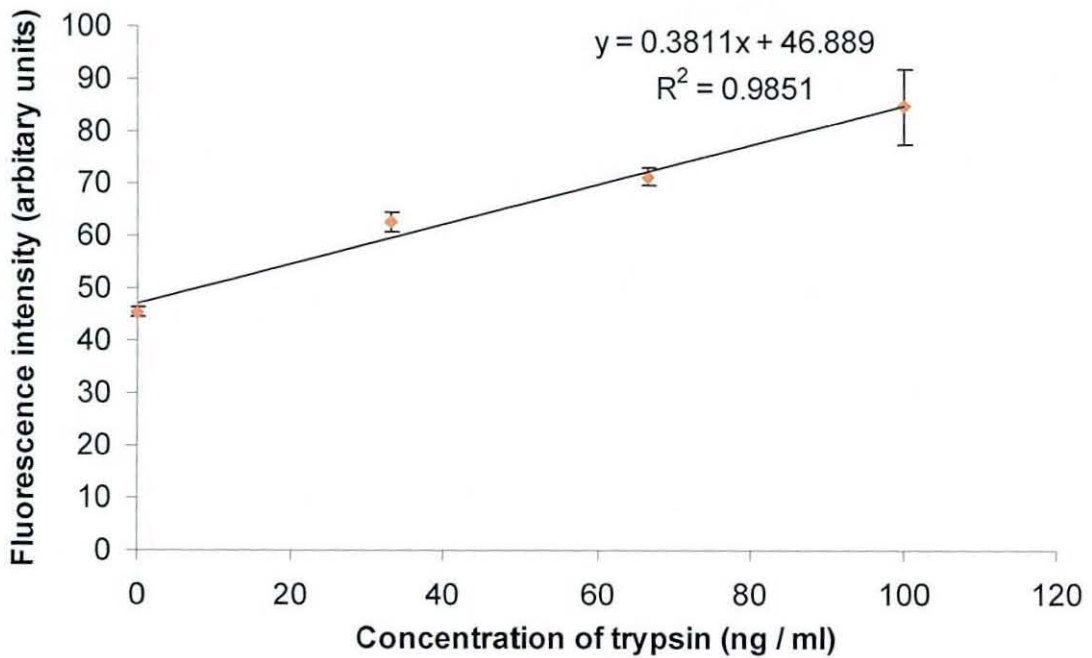
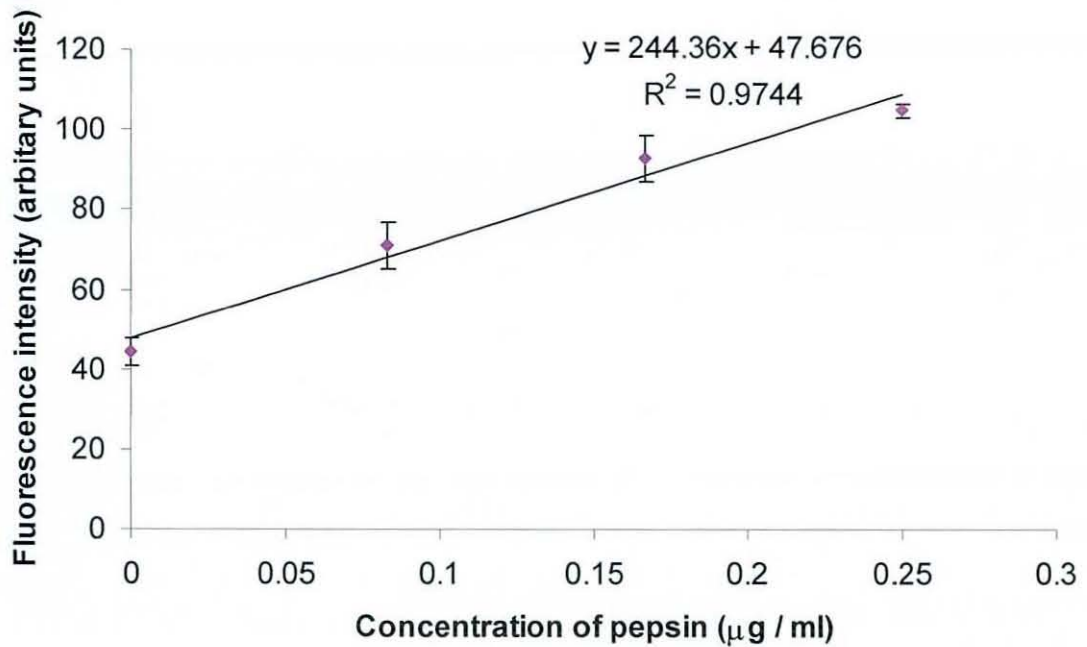


Figure 5.18 – Fluorescence intensity against enzyme concentration to calculate the limit of detection of pepsin



Limits of detection for each proteolytic enzyme using the substrate BODIPY: α -casein are given in table 5.5.

PROTEOLYTIC ENZYME	LIMIT OF DETECTION (nM)
ALKALINE PROTEASE	0.880
PROTEINASE K	4.630
α -CHYMOTRYPSIN	0.208
TRYPSIN	0.148
PEPSIN	0.771

Table 5.5 – Limits of detection determined experimentally for various proteolytic enzymes using the substrate BODIPY: α -casein in a static system

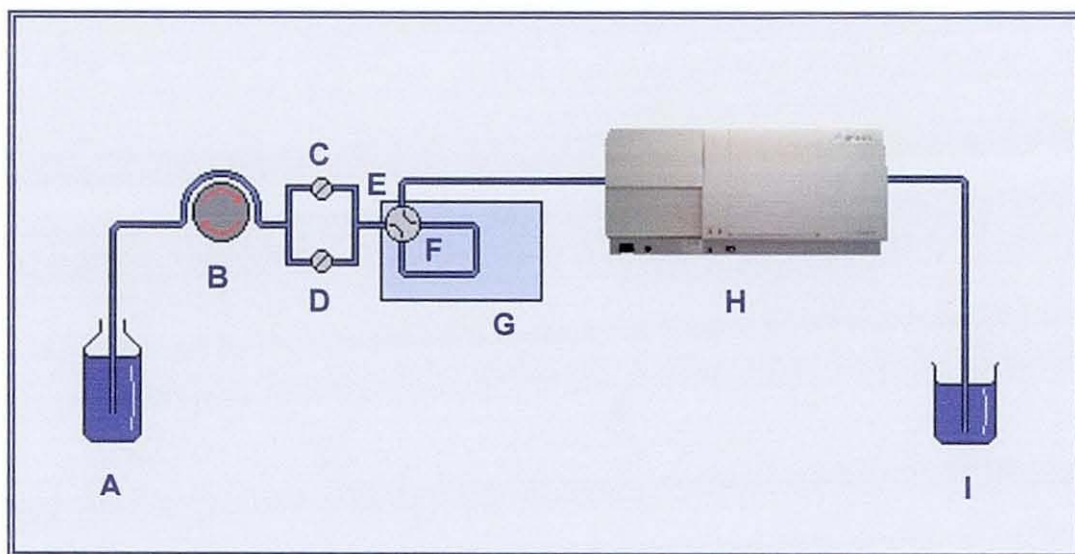
Limits of detection in the nM region have been achieved for all five proteolytic enzymes using this system. These experiments indicate that BODIPY: α -casein is promising as a sensitive fluorogenic substrate for the assay of proteolytic enzymes. As these preliminary experiments have proven successful, BODIPY: α -casein was studied in a flow analysis manifold with four enzymes at different pH values. Such a flow set-up would be well suited for use in the high throughput screening of candidate drug molecules.

5.5 PROTEASE ASSAYS IN A FLOWING SYSTEM

5.5.1 Procedures

Fluorescence measurements were made using an F-4500 fluorescence spectrophotometer with excitation and emission bandwidths of 10nm and time scans were performed with excitation and emission wavelengths of 605 nm and 644 nm respectively. Flow system measurements were carried out using the set-up shown in figure 5.19.

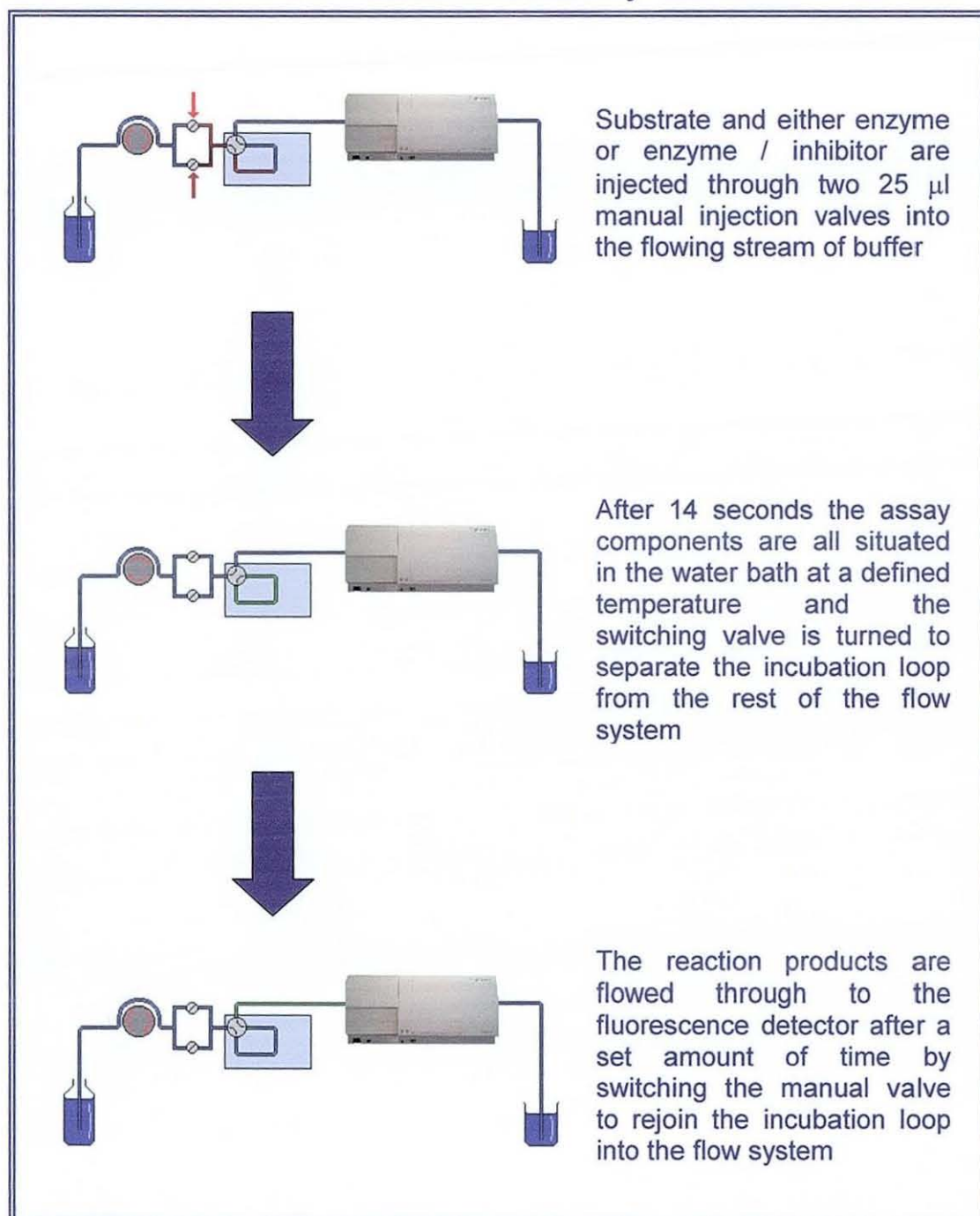
Figure 5.19 – Manifold for flow injection enzyme and enzyme inhibition assays



(A = buffer, B = peristaltic pump, C = conjugate injection valve, D = enzyme or enzyme / inhibitor injection valve, E = switching valve, F = incubation loop, G = thermostatted water bath, H = fluorescence detector, I = waste)

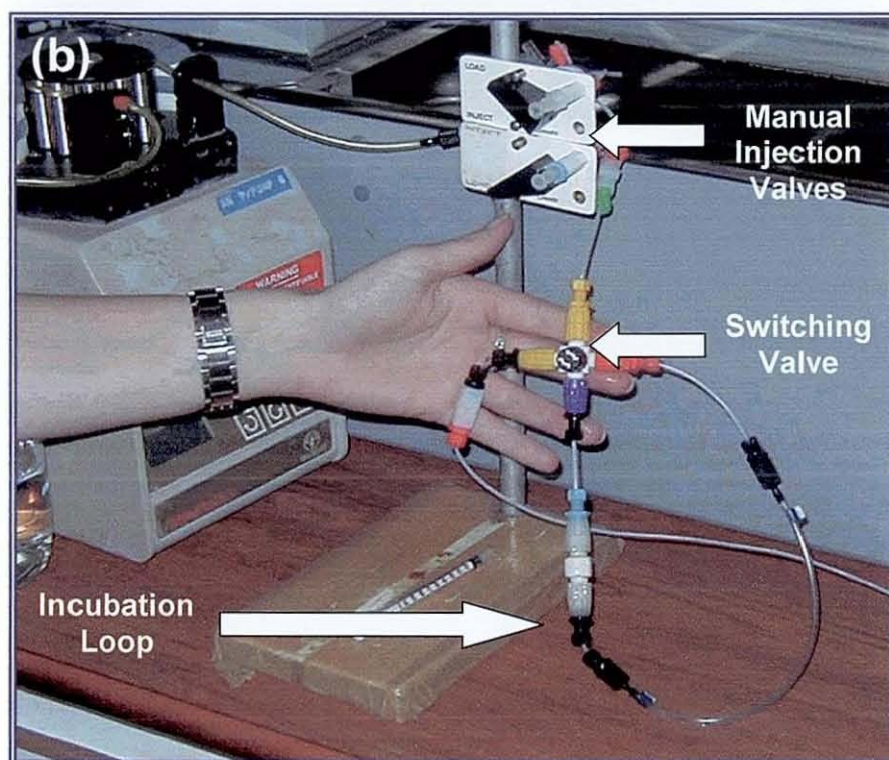
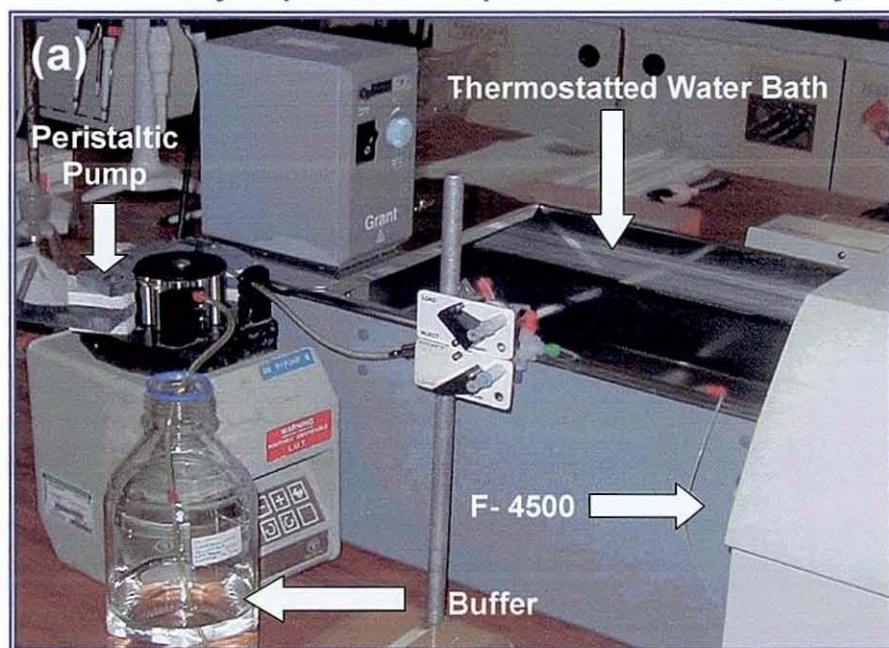
The sequences of events that occur in these flow system experiments are depicted in figure 5.20.

Figure 5.20 – Sequence of events for the determination of proteases and their inhibitors in a flow system



Photos are shown below depicting the actual set-up of this flow system in our laboratory (figures 5.21 (a) and (b)).

Figures 5.21 (a) and (b) – Actual layout and equipment used for flow analysis protease and protease inhibition assays



A flow rate of 1.85 ml / min (calculations in appendix M) was used for all flow system experiments. The volume of the incubation loop was calculated to be 670 μ l (calculations in appendix N).

Four protease enzymes, each with varying pH optima, ranging from pH 2.5 – 9.5, were investigated in these experiments – alkaline protease, proteinase K, α -chymotrypsin, and pepsin. A description of each is given in section 1.6.4.

5.5.2 Results

Assay conditions for each enzyme were optimised. The effect of the following parameters on the enzyme assay were studied:

- [enzyme]
- incubation time
- temperature
- pH
- buffer system
- [buffer]

The assay parameters studied and optimum conditions found for each enzyme are shown below in table 5.6.

KEY: CA = Carbonate, CI = Citrate, GL = Glycine, PH = Phosphate

	[ENZYME] ($\mu\text{g/ml}$)	TIME (minutes)	TEMPERATURE ($^{\circ}\text{C}$)	pH	BUFFER	[BUFFER] (mM)
ALKALINE PROTEASE						
RANGE STUDIED	0 – 1.87	1.0 – 3.0	25 – 50	7 – 11	PH / CA	10 – 100
OPTIMUM	0.75	2.0	40	9.5	CA	10
PROTEINASE K						
RANGE STUDIED	0 – 1.87	1.0 – 3.0	25 – 50	7 – 11	PH / CA	10 – 100
OPTIMUM	1.49	1.5	35	9.5	CA	20
α- CHYMOTRYPSIN						
RANGE STUDIED	0 – 1.49	1.0 – 3.0	25 – 50	7 – 9	PH	10 – 100
OPTIMUM	0.75	1.5	35	7.5	PH	10
PEPSIN						
RANGE STUDIED	0 – 44.78	1.0 – 3.0	25 – 50	1 – 3	GL / CI	10 – 100
OPTIMUM	37.31	1.5	35	2.0	GL	20

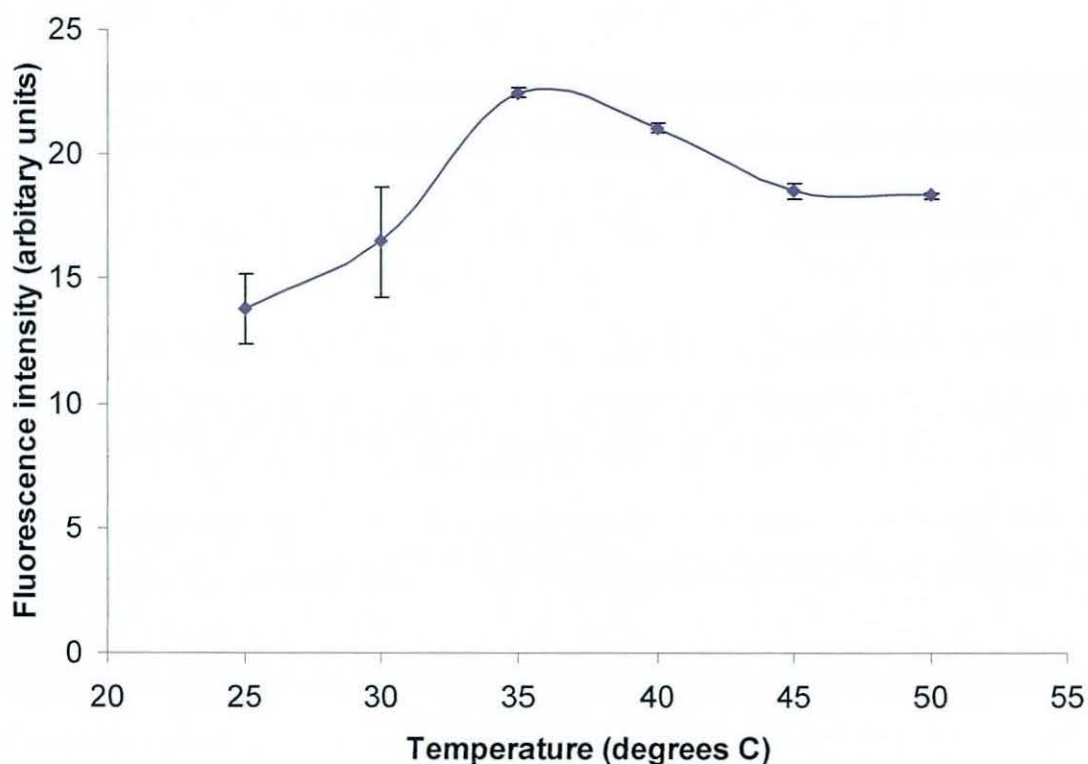
Table 5.6 - Assay parameters studied and optimum conditions for various proteolytic enzymes using the fluorogenic substrate BODIPY : α -casein in a flow system

The effect of assay parameters only on the proteolytic enzyme pepsin will be discussed in detail as it was found that similar trends were observed with all four proteolytic enzymes. Optimisation experiments were carried out in duplicate and error bars represent plus and minus one standard deviation. All readings were corrected for any effects of the assay parameters upon the conjugate.

5.5.2.1 Effect of temperature

Effect of temperature on the activity of pepsin was examined over the range 25 – 50 °C using a thermostatted water bath. As shown in figure 5.22, there was an increase in the reaction rate as the temperature was increased from 25 °C to 35 °C. A maximum level was reached at 35 °C after which the reaction rate declined with further increase of temperature. At high temperature, rapid denaturation of the quaternary protein structure occurred leading to a disruption of the enzymatic activity.

Figure 5.22 – Effect of temperature on the activity of pepsin

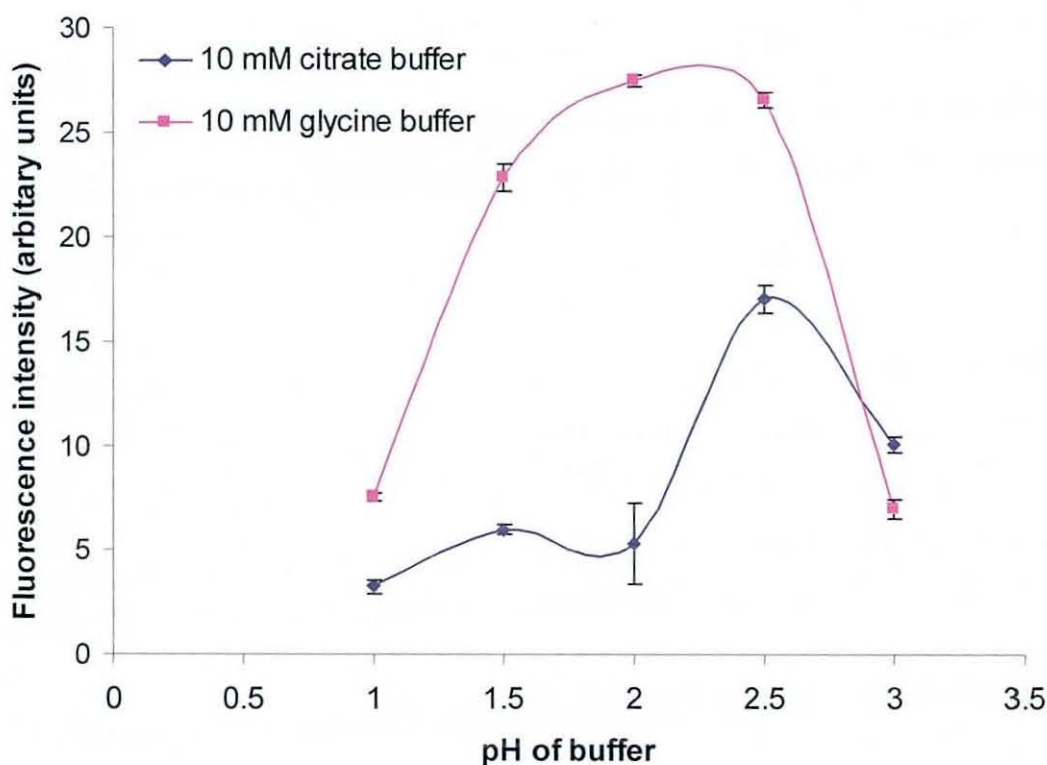


A temperature of 35 °C was selected for subsequent experiments since a maximum reaction rate was seen at this temperature. This was expected as most biological enzymes operate at 37 °C.

5.5.2.2 Effect of pH and buffer system

Effect of buffer system and pH on the activity of pepsin was also investigated. A pH range from 1.0 – 3.0 in 0.5 increments was investigated using citrate and glycine buffers. As shown in figure 5.23, pH and buffer system both dramatically affected the reaction rate. Higher fluorescence intensities were observed with glycine buffer than citrate buffer and a pH optimum of 2.0 was seen with glycine buffer. On both sides of this pH value, the fluorescence dropped drastically.

Figure 5.23 – Effect of pH and buffer system on the activity of pepsin

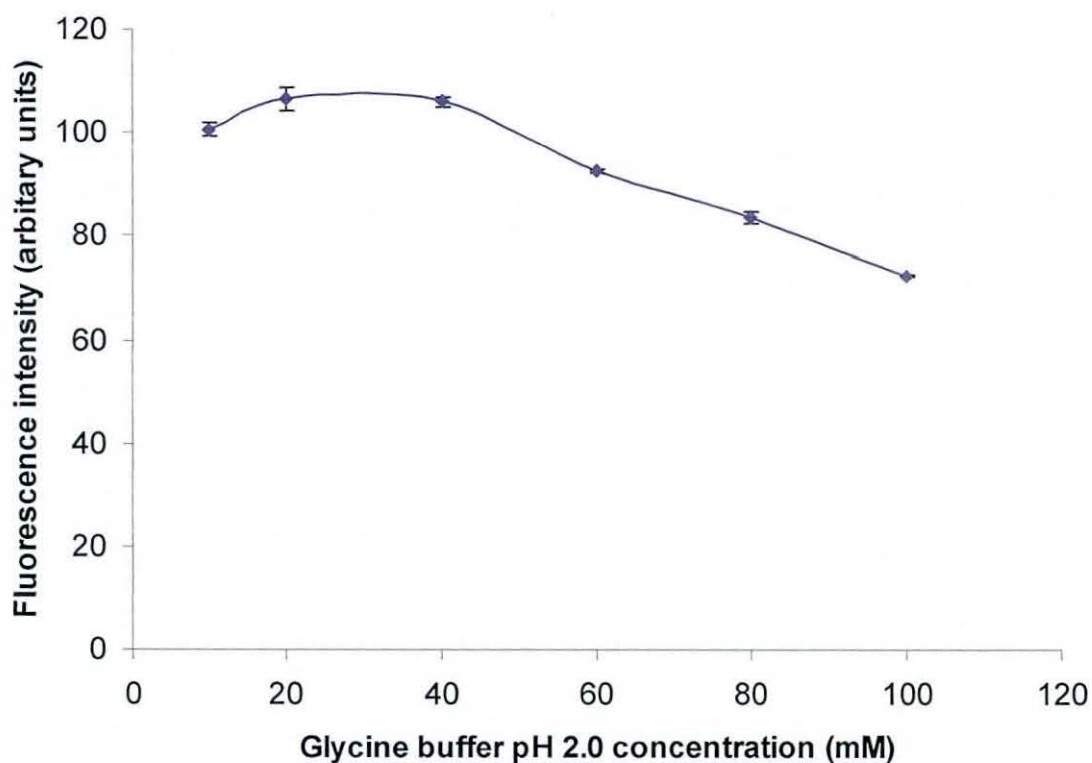


As a maximum reaction rate was achieved with glycine buffer at a pH of 2.0, this buffer system and pH was used for all subsequent experiments.

5.5.2.3 Effect of buffer concentration

The effect of glycine buffer pH 2.0 concentration was optimised. A concentration range of 10 to 100 mM was studied and as shown in figure 5.24, a peak in pepsin activity was seen at a concentration of 20 mM. At higher buffer concentrations a drop in the reaction rate occurred.

Figure 5.24 – Effect of buffer concentration on the activity of pepsin

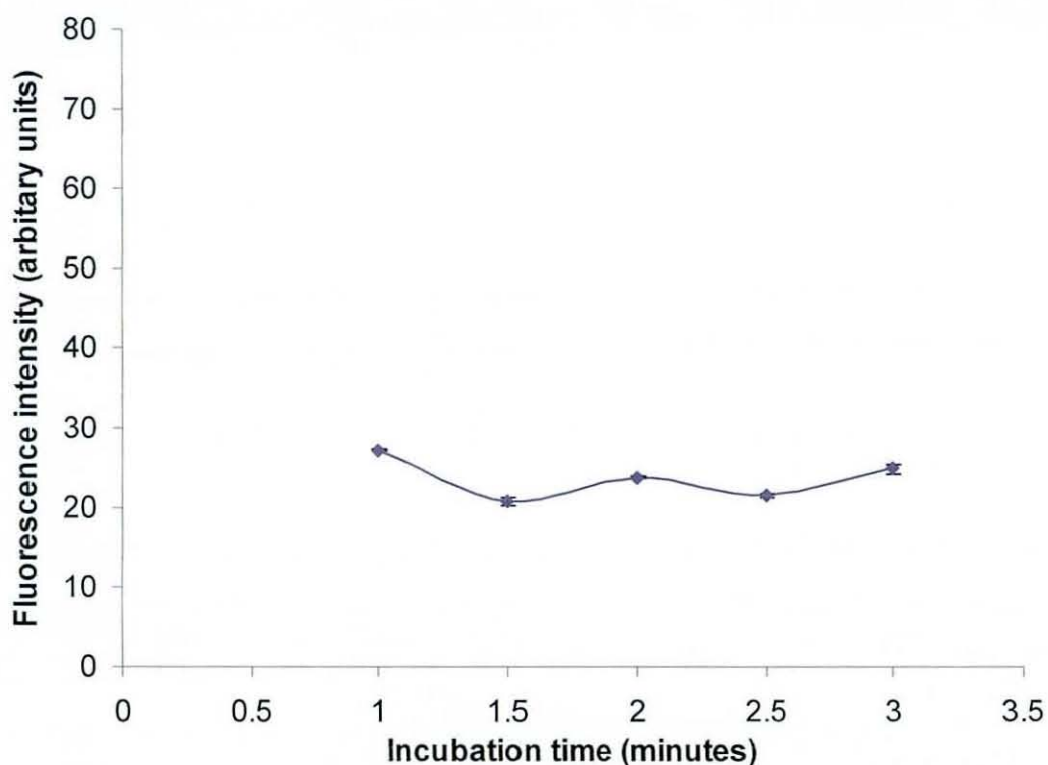


Buffer concentration affected the reaction rate of pepsin on the substrate BODIPY: α -casein. A concentration of 20 mM was chosen for further work as this concentration gave an optimal reaction rate and was sufficient to maintain buffering capacity.

5.5.2.4 Effect of incubation time

The time of incubation of pepsin and BODIPY: α -casein together was investigated over the range 1.0 to 3.0 minutes in 0.5 minute increments. Figure 5.25 demonstrates that incubation time did not greatly affect the reaction rate.

Figure 5.25 – Effect of incubation time on the activity of pepsin

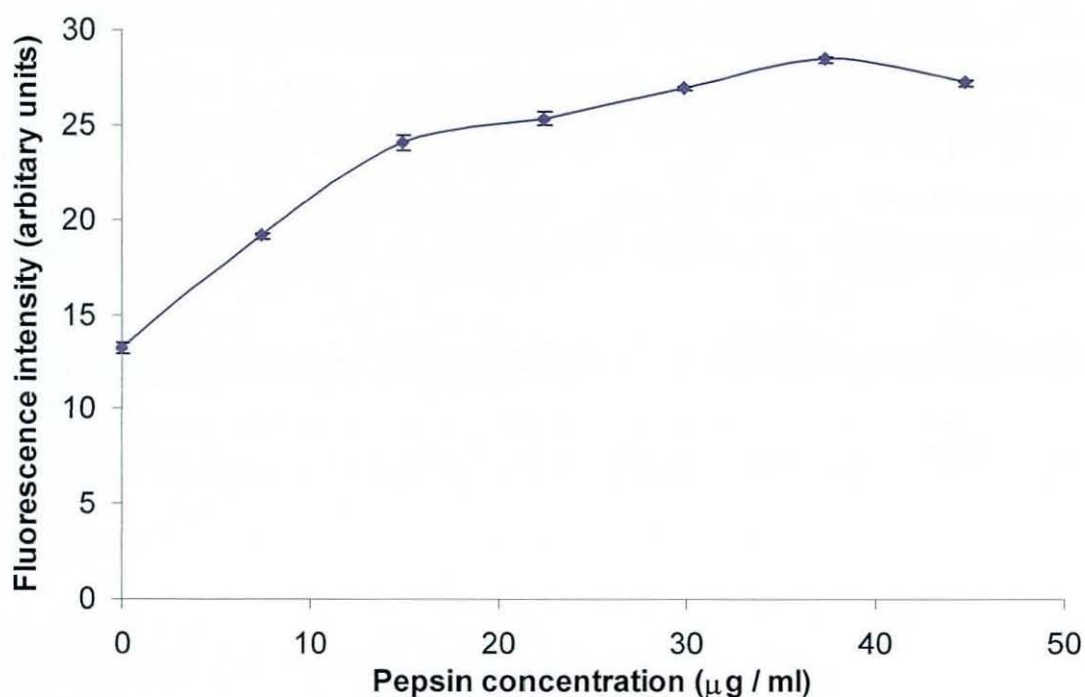


As incubation time had no great effect upon the enzymatic activity of pepsin, an incubation time of 1.5 minutes was selected for further experiments. This was chosen as it is beneficial to carry out experiments for high throughput screening rapidly and difficulties were found using an incubation time of 1.0 minute when carrying out injections and switching manually.

5.5.2.5 Effect of pepsin concentration on the reaction rate

Pepsin concentration was varied between 0 and 44.78 $\mu\text{g} / \text{ml}$. It can be seen from figure 5.26 that between 0 and 15 $\mu\text{g} / \text{ml}$ the formation of product proceeds at a rate which is linear with increasing enzyme concentration. In this region therefore, the reaction is independent of substrate concentration i.e. substrate is present in excess and the reaction is a 'zero order' reaction. However, as substrate is used up, the enzyme's active sites are no longer saturated, and substrate concentration becomes rate limiting and the reaction becomes first order. This is signified by the graph reaching a plateau. Ideally to measure enzyme activity, measurements must be made in that portion of the graph where the reaction is zero order because the reaction is then independent of substrate concentration and dependent only on enzyme concentration. Calculation of the limit of detection of pepsin was therefore carried out in the region 0 to 4 $\mu\text{g} / \text{ml}$, as described in section 5.5.2.7.

Figure 5.26 – Influence of pepsin concentration on the formation of product when incubated with BODIPY: α -casein



5.5.2.6 Calibration graphs for BODIPY: α -casein in the flowing system

Using the optimum assay parameters for each enzyme given in table 5.6, a calibration graph for BODIPY: α -casein was obtained by injecting a series of standard solutions of BODIPY: α -casein into the flow system with each of the enzymes. Measurements were made in triplicate and error bars show plus and minus one standard deviation. Each measurement was carried out separately. The BODIPY: α -casein conjugate that was used with alkaline protease was a different batch from that used with proteinase K, α -chymotrypsin, and pepsin. For this reason alkaline protease has been plotted on a separate graph.

Figure 5.27 – Influence of substrate concentration on the production of BODIPY fluorescence when incubated with alkaline protease in a flowing system

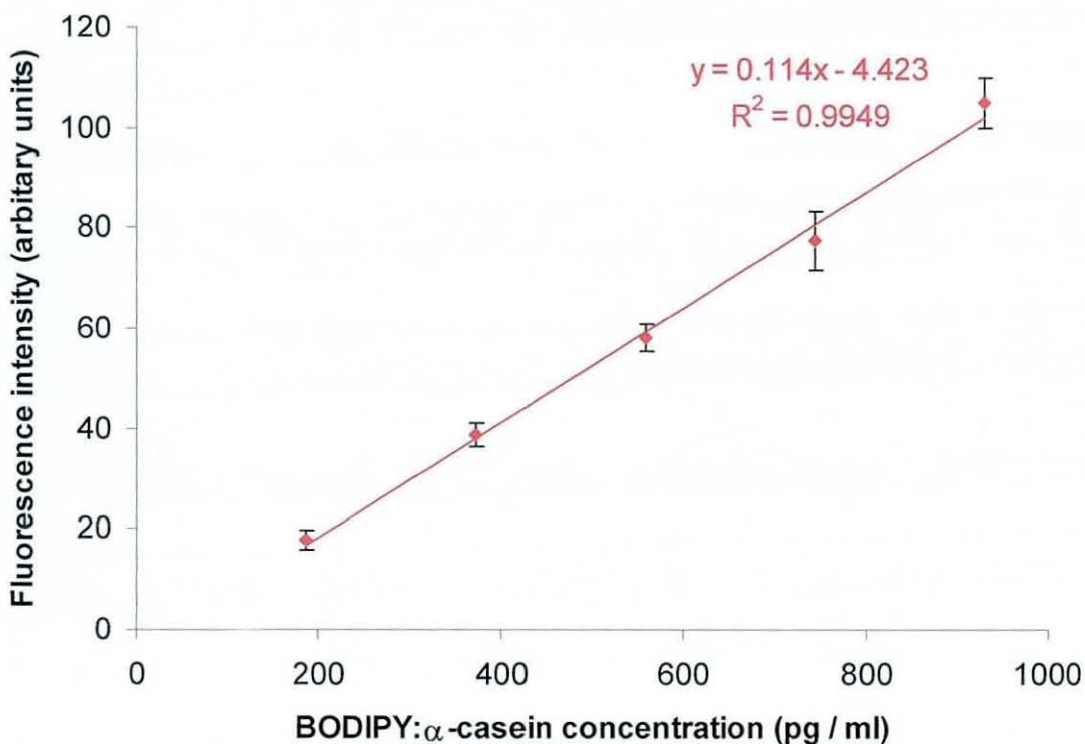
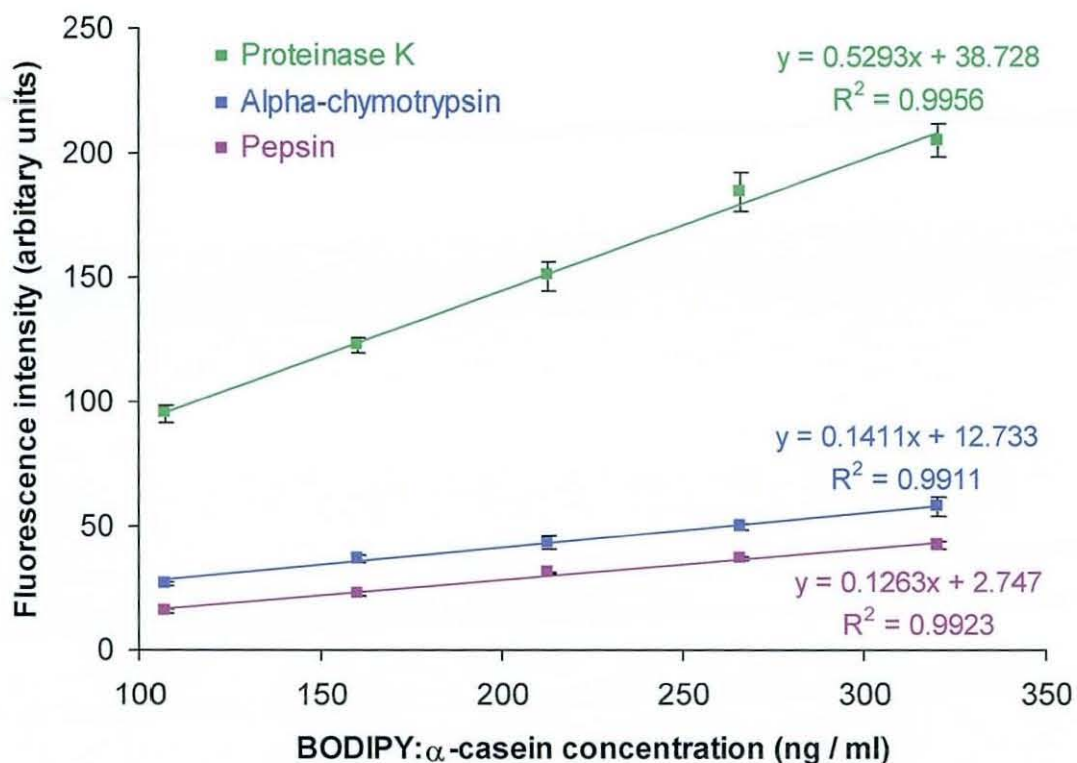


Figure 5.28 – Influence of substrate concentration on the production of BODIPY fluorescence when incubated with a range of proteolytic enzymes in a flowing system



The calibration graphs obtained for BODIPY:α-casein show good linearity in the ranges investigated. In this region enzyme is not limiting and so increases in substrate concentration are accompanied by an increase in reaction rate, as more substrate is available to be converted to product by the enzyme.

Figure 5.28 shows that different proteases act upon the substrate with different efficiencies. Proteinase K cleaves the substrate with a greater activity than either α-chymotrypsin or pepsin. These results do show that the activities of a variety of enzymes with different pH optima can be compared using the same substrate merely by altering the pH and buffer system.

5.5.2.7 Determination of limits of detection

Limits of detection, defined as the analyte concentration giving a signal equal to the blank signal plus 3 standard deviations of the blank [291], were determined for each of the four proteolytic enzymes in the flow analysis system. Fluorescence intensity against enzyme concentration was plotted for the linear portion of the graphs for each of the enzymes, which is the analytical useful region for the determination of enzymes due to the fact that the reaction is independent of substrate concentration i.e. zero order reaction. These results are shown in figures 5.29 to 5.32. Measurements were taken in triplicate and error bars are given which represent plus and minus one standard deviation.

Figure 5.29 – Fluorescence intensity against enzyme concentration to calculate the limit of detection of alkaline protease

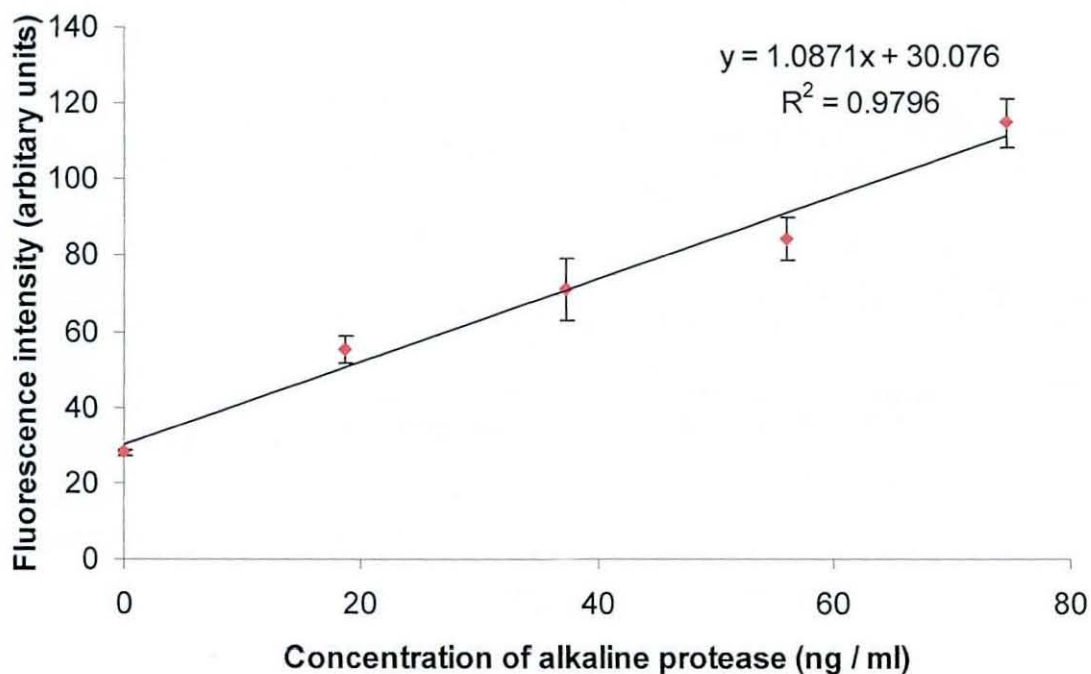


Figure 5.30 – Fluorescence intensity against enzyme concentration to calculate the limit of detection of proteinase K

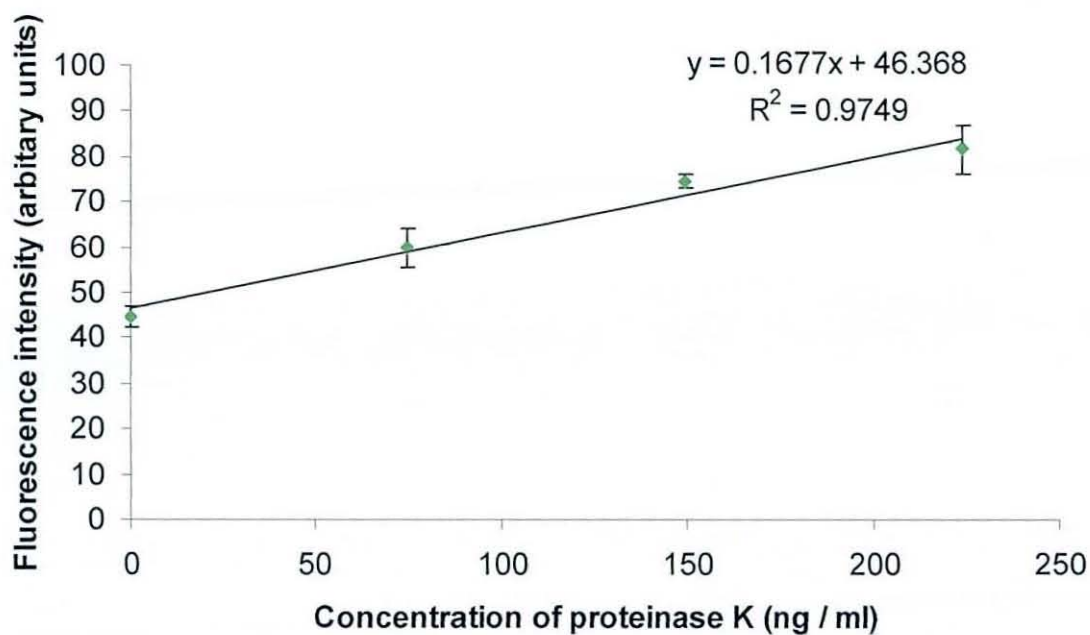


Figure 5.31 – Fluorescence intensity against enzyme concentration to calculate the limit of detection of α -chymotrypsin

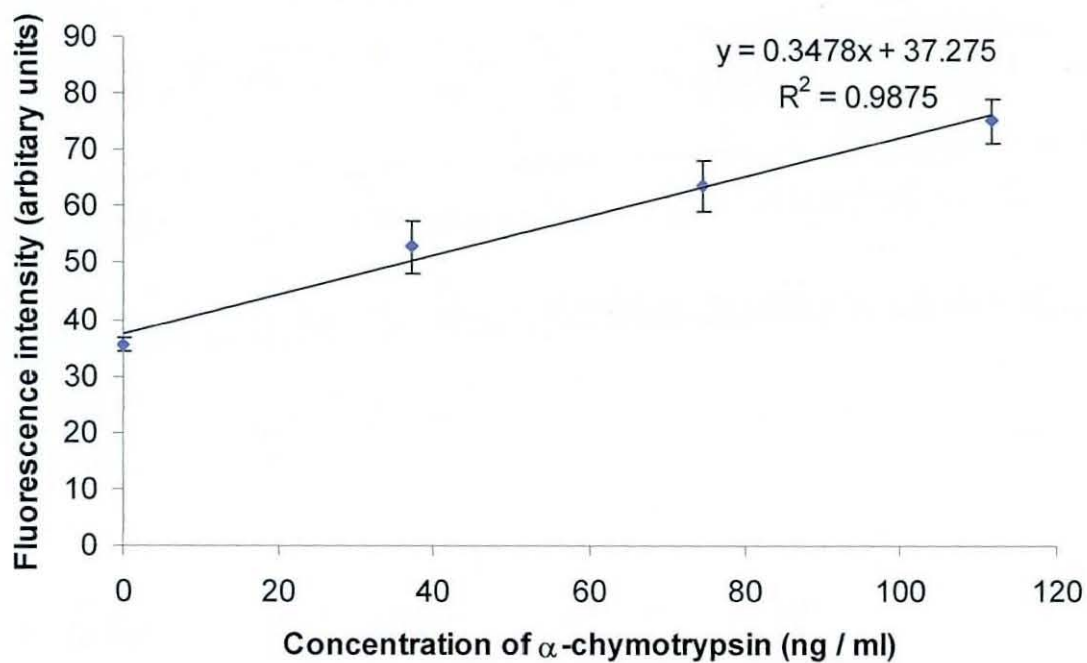
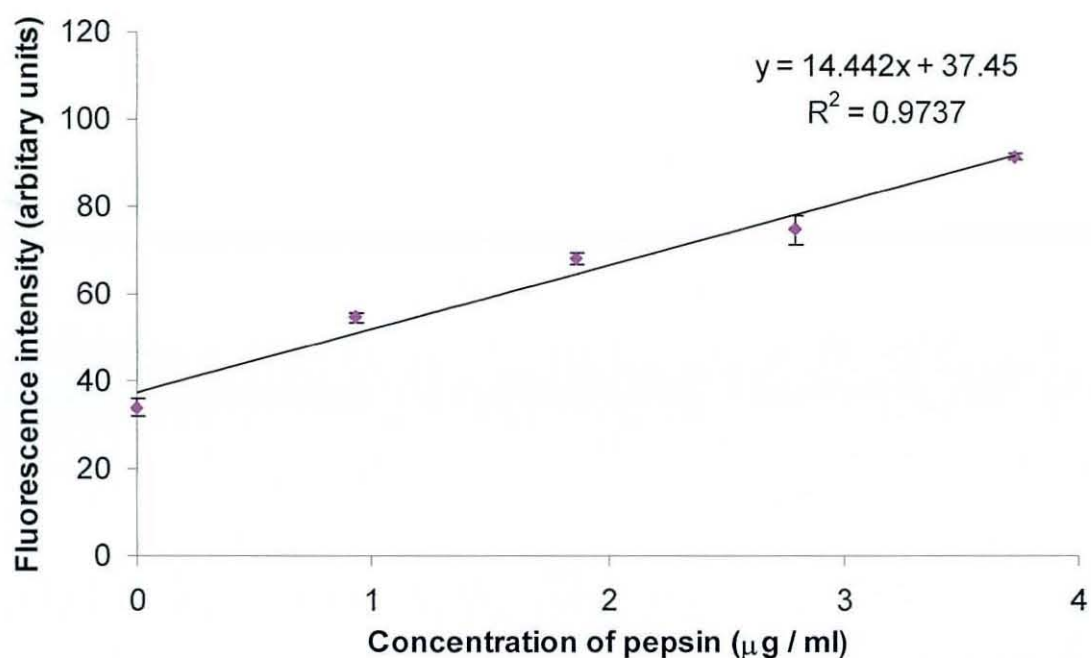


Figure 5.32 – Fluorescence intensity against enzyme concentration to calculate the limit of detection of pepsin



Calculated limits of detection for each proteolytic enzyme using the substrate BODIPY: α -casein in a flow analysis system are given in table 5.7.

PROTEOLYTIC ENZYME	LIMIT OF DETECTION
ALKALINE PROTEASE	10 pM
PROTEINASE K	1.07 nM
α -CHYMOTRYPSIN	0.21 nM
PEPSIN	4.49 nM

Table 5.7 – Limits of detection determined experimentally for various proteolytic enzymes using the substrate BODIPY: α -casein in a flowing system

Sensitive detection limits have been determined for these proteolytic enzymes in the nM (proteinase K, α -chymotrypsin, and pepsin) and pM (alkaline protease) range. If these are compared with those obtained in the static system (table 5.5) it can be seen that the detection limits of both alkaline protease and proteinase K have been significantly improved. Those of α -chymotrypsin are identical, whereas with pepsin it was found that the static system gave a lower limit of detection than the flowing system. However, in all cases the time taken to carry out the assays was much improved in the flowing system compared with the static system as depicted in table 5.8. This has a great advantage when applying these protease assays to a high throughput screening format.

PROTEOLYTIC ENZYME	INCUBATION TIME IN STATIC SYSTEM (mins)	INCUBATION TIME IN FLOWING SYSTEM (mins)
ALKALINE PROTEASE	10	2.0
PROTEINASE K	5	1.5
α -CHYMOTRYPSIN	10	1.5
PEPSIN	10	1.5

Table 5.8 – Comparison of assay incubation times for various proteolytic enzymes in static and flow system formats

The experiments that have been carried out in the flow analysis system demonstrate that the substrate BODIPY: α -casein is a sensitive fluorogenic protease substrate and that the flow-based procedure that has been described here is well suited for use in high throughput screening.

5.5.2.8 Comparison with previously published work

A quenched BODIPY dye-labelled casein substrate for the assay of proteases has previously been described by Jones *et al.* [255] and was described in section 1.8.3.2.2. In this study a 96-well microtiter plate format was used with the BODIPY dyes BODIPY FL and BODIPY TR-X. These had excitation / emission wavelengths of 485 nm / 530 nm, and 590 nm / 645 nm, respectively. The limits of detection (LOD) and the times taken to carry out the assays are given in table 5.9 and compared with those that have been found in this study. Limits of detection have been given in terms of enzyme units as defined by the supplier. To convert the limits of detection given in table 5.7 from molar concentrations to enzyme units, enzyme activities defined by the supplier of 21 Units / mg protein, 10.2 Units / mg protein, 51 Units / mg protein, and 4500 Units / mg protein were used for alkaline protease, proteinase K, α -chymotrypsin, and pepsin respectively. Use of detection limits in enzyme units allows for a fair comparison.

	M.L.Beaumont		Jones <i>et al.</i>	
	LOD (Units)	TIME (mins)	LOD (Units)	TIME (mins)
ALKALINE PROTEASE	2.1×10^{-6}	2.0	1×10^{-4}	60
PROTEINASE K	6.3×10^{-5}	1.5	1×10^{-4}	60
α -CHYMOTRYPSIN	5.5×10^{-5}	1.5	2×10^{-4}	60
PEPSIN	1.4×10^{-1}	1.5	5×10^{-3}	60

Table 5.9 – Comparison of enzyme limits of detection between two different methods using similar protease substrates

Comparing the limits of detection for the work carried out in our lab with those of Jones *et al.*, it can be seen that in each of the cases apart from pepsin the limit of detection has been significantly improved. The time taken to carry out the assays has also been vastly improved. The limits of detection that have been achieved in our lab could also be improved further. A sub-optimal wavelength of 605 nm was used to excite the BODIPY: α -casein substrate. With the use of laser induced fluorescence and cut-off filters an optimal excitation wavelength of 625 nm could be used, which would improve the sensitivity of the system giving improved limits of detection.

5.6 PROTEASE INHIBITION ASSAYS

5.6.1 Procedures

The effect of a variety of reversible inhibitors on the proteolytic enzymes was investigated. Enzyme – inhibitor pairs were chosen from the enzymes already studied and a selection of inhibitors consisting of 4-hydroxycoumarin, warfarin, 3-nitrophenylboronic acid, aprotinin, leupeptin, and pepstatin A. A description of the inhibitors used during this work is given in section 1.9.2.

Protease inhibition assays were carried out using the set-up that was shown in figures 5.19 and 5.20. Enzyme and inhibitor were pre-mixed before injection and then mixed with substrate in the incubation loop.

5.6.2 Results

Preliminary experiments were carried out by incubating each proteolytic enzyme with increasing amounts of each inhibitor. It was found that neither 4-hydroxycoumarin nor warfarin had an inhibitory effect upon any of the proteolytic enzymes tested. However, it was found that the remaining inhibitors – 3-nitrophenylboronic acid (3-NPBA), aprotinin, leupeptin, and pepstatin A – each inhibited the four proteolytic enzymes to varying degrees. Enzyme – inhibitor pairs were selected depending on which inhibitor exerted the best inhibitory effect upon which enzyme.

The following enzyme – inhibitor pairs were used for all subsequent experiments

- 3-nitrophenylboronic acid and proteinase K
- aprotinin and α -chymotrypsin
- leupeptin and alkaline protease
- pepstatin A and pepsin

In addition to the enzyme assay parameters previously optimised (table 5.6), additional inhibition assay conditions were optimised using univariate methods. All other conditions such as pH, buffer system, and temperature, were as those conditions previously optimised.

The effect of the following parameters on the inhibition assays were optimised:

- pre-incubation time
- incubation time
- [substrate]
- [enzyme]

The assay parameters studied and the optimum values found for each enzyme – inhibitor pair are given below in table 5.10.

	PRE-INCUBATION TIME (mins)	INCUBATION TIME (minutes)	[SUBSTRATE] (ng ml ⁻¹)	[ENZYME] (μg ml ⁻¹)
3-NPBA + PROTEINASE K RANGE STUDIED OPTIMUM	0 – 5 2	1 – 5 2	53.3 – 266.4 106.6	0 – 1.866 0.746
APROTININ + α-CHYMOTRYPsin RANGE STUDIED OPTIMUM	0 – 4 2	1 – 5 1	53.3 – 266.4 106.6	0 – 1.119 0.373
LEUPEPTIN + ALKALINE PROTEASE RANGE STUDIED OPTIMUM	0 – 5 4	1 – 5 1	53.3 – 266.4 213.1	0 – 0.233 0.093
PEPSTATIN A + PEPSIN RANGE STUDIED OPTIMUM	0 – 4 2	1 – 5 1	53.3 – 266.4 106.6	0 – 37.31 0.373

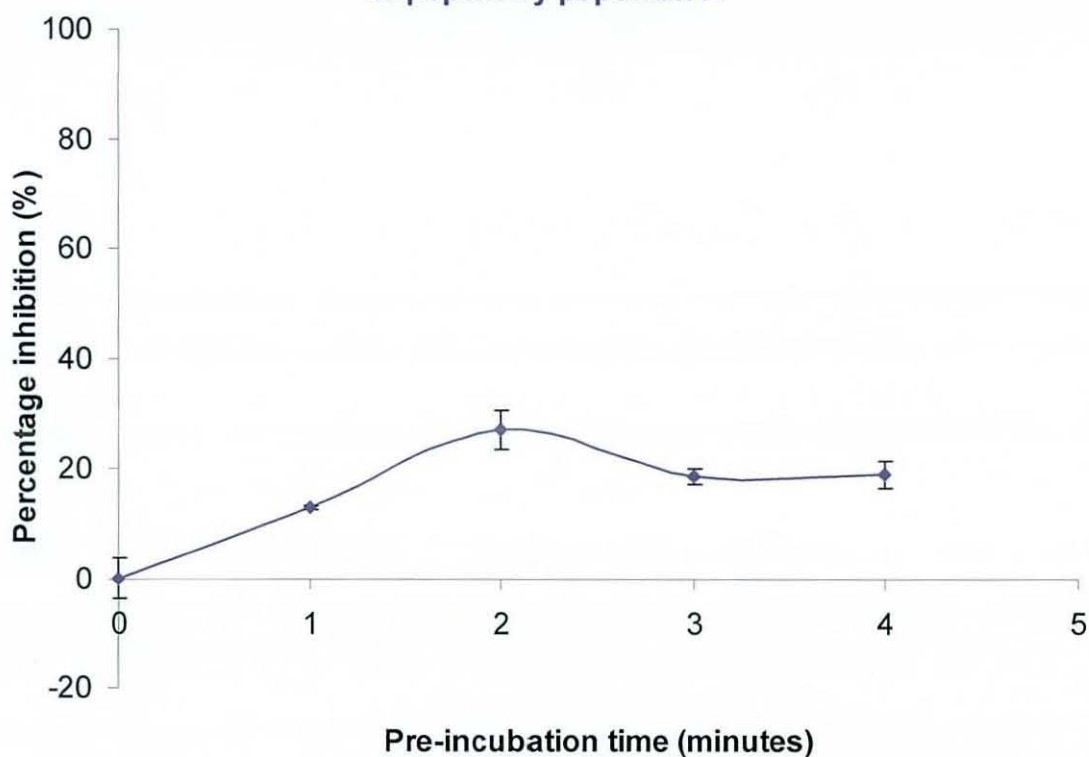
Table 5.10 - Assay parameters studied and optimum conditions for various enzyme – inhibitor pairs using the fluorogenic substrate BODIPY:α-casein in a flow system

The optimisation of inhibition parameters will be discussed only for pepsin and pepstatin A. Optimisation experiments were carried out in duplicate and error bars represent plus and minus one standard deviation.

5.6.2.1 Effect of pre-incubation time

Enzyme and inhibitor were pre-mixed in a glass vial and incubated for a set amount of time before injection into the flowing system and incubation with substrate. The effect of varying this pre-incubation time was investigated over 0 to 4 minutes in 1 minute increments. The results of this are shown in figure 5.33.

Figure 5.33 – Influence of pre-incubation time on the inhibition of pepsin by pepstatin A

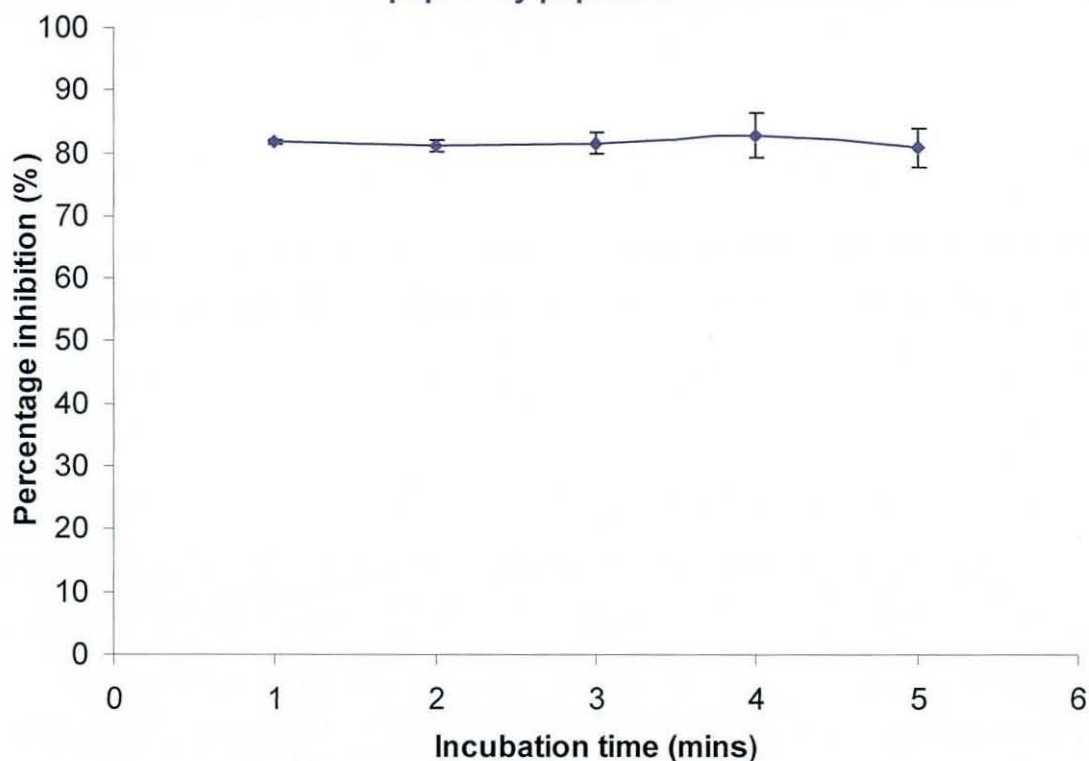


The effect of pre-incubation time on the inhibition of pepsin by pepstatin A wasn't significant. This was expected as pepstatin A has been reported as an uncompetitive inhibitor of pepsin [281] and as described in section 1.9.1.1, uncompetitive inhibitors bind only with the ES complex and not with the free enzyme. Increasing the time spent for the enzyme and inhibitor together will therefore not increase inhibition. A pre-incubation time of 2 minutes was therefore chosen for further studies.

5.6.2.2 Effect of incubation time

Incubation time is defined as the time spent for the substrate, enzyme, and inhibitor together in the incubation loop in the thermostatted water bath after injection of each into the flowing system. This assay parameter was investigated over the range 1 to 5 minutes in 1 minute increments. Figure 5.34 shows the effect of increasing incubation time on the amount of enzyme inhibition seen.

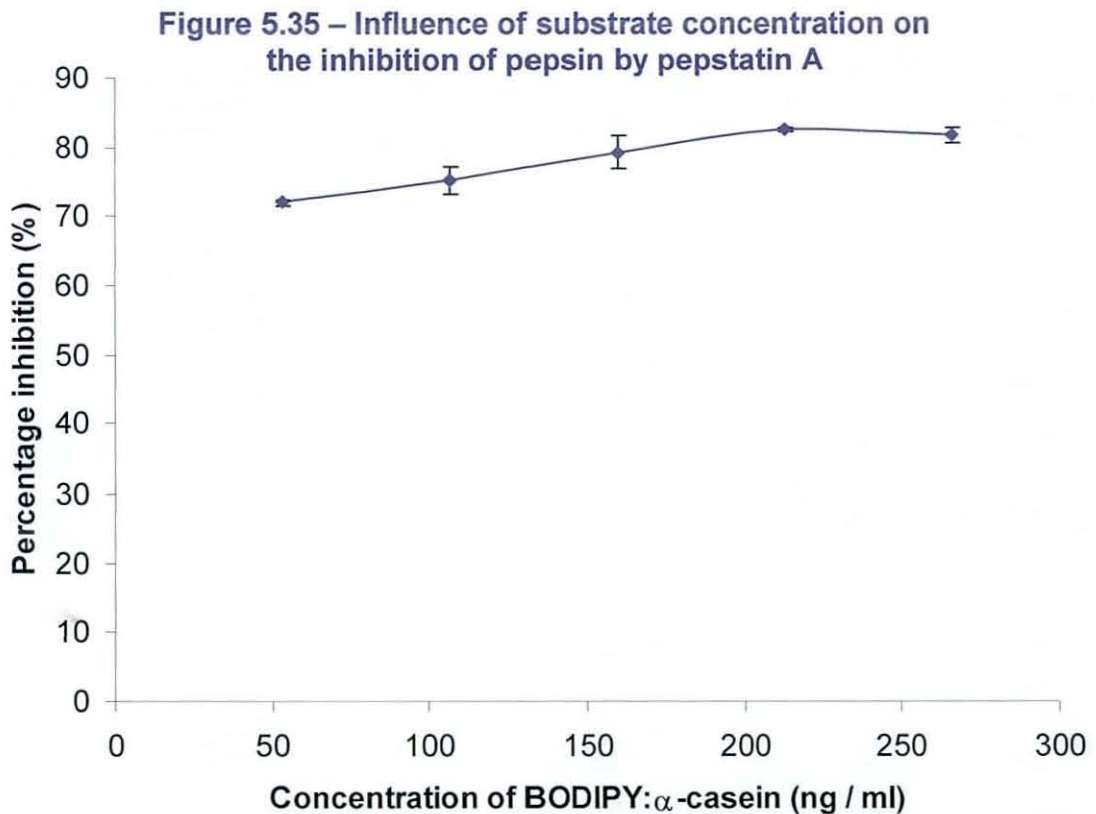
Figure 5.34 – Influence of incubation time on the inhibition of pepsin by pepstatin A



From figure 5.34 it is evident that the inhibition of pepsin by pepstatin A was not affected by incubation time. As increases in the incubation time of the substrate and inhibitor together did not cause an increase in the percentage inhibition, this suggests that the binding of inhibitor to the enzyme – substrate complex proceeds instantaneously. An incubation time of 1 minute was thus selected for further work so as to keep the assay time to a minimum.

5.6.2.3 Effect of substrate concentration

Increasing concentrations of substrate were injected into the flowing system with enzyme and inhibitor to determine the effect of increasing the substrate concentration on the inhibition of pepsin by pepstatin A. Figure 5.35 shows the results of this experiment.

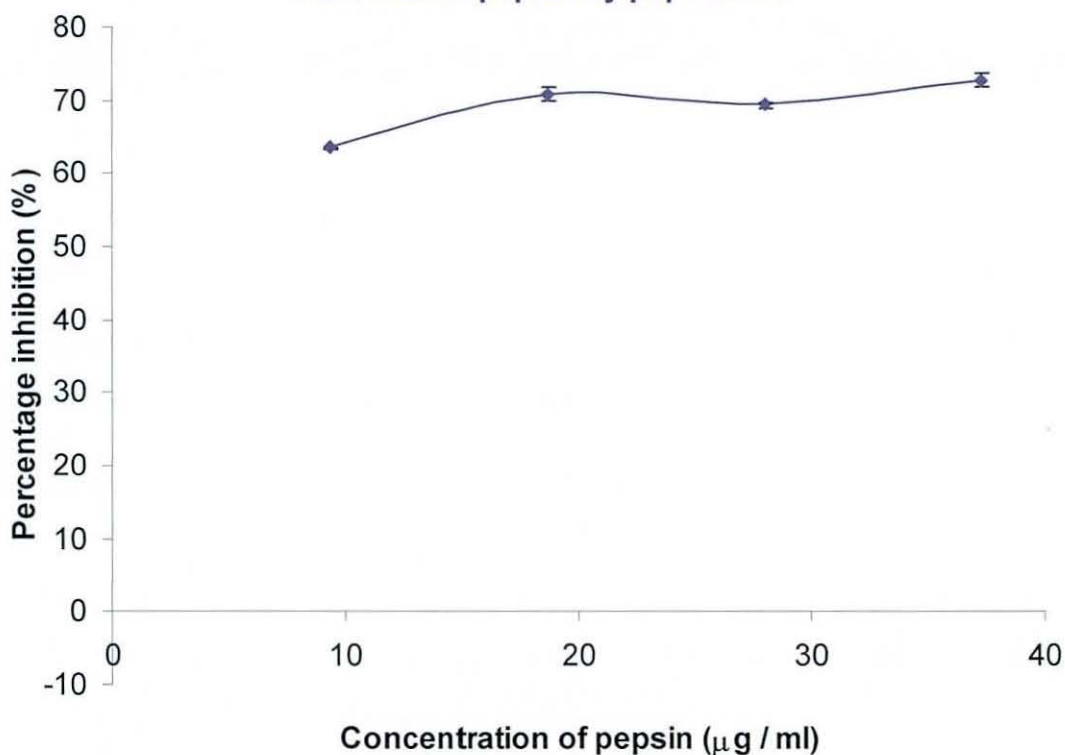


The effect of increasing the substrate concentration was to increase the inhibition of pepsin by pepstatin A (figure 5.35). As pepstatin A is an uncompetitive inhibitor of pepsin, the inhibitor binds only with the enzyme – substrate complex, not with free enzyme. In this case therefore, as described in section 1.9.1.1 increased substrate concentration increases the inhibition. A high concentration of substrate will not reverse the inhibition, as is the case with reversible competitive inhibition. A substrate concentration of 106.6 ng ml^{-1} was used for further work.

5.6.2.4 Effect of enzyme concentration

The effect of increasing the concentration of pepsin was determined by injecting a series of increasing enzyme concentrations into the flowing system and detecting the amount of fluorescence (figure 5.36).

Figure 5.36 – Influence of pepsin concentration on the inhibition of pepsin by pepstatin A



As shown in figure 5.36, as the concentration of pepsin was increased the percentage inhibition also increased, although not to a great extent. Since the inhibitor only binds with the enzyme – substrate complex and not with free enzyme the effect will be minimal as increasing the enzyme concentration would only cause an increase in the inhibition through the formation of more enzyme – substrate, and not through the increased free enzyme. A concentration of 18.66 $\mu\text{g} / \text{ml}$ was used for further work as after this concentration increase in percentage inhibition is minimal.

5.6.2.5 Problems with inhibitors

A number of delays were introduced into this work due to problems encountered with the inhibitors.

Leupeptin hemisulfate salt was very problematic with respect to its stability. In powder form leupeptin is stable for 2 years if stored frozen and kept very dry. However, a 10 mM aqueous solution is stable for a week at 4 °C and at working concentrations (10 to 100 μ M) a solution is stable only for a few hours and the stock solution should be stored on ice for intermitted use over several hours.

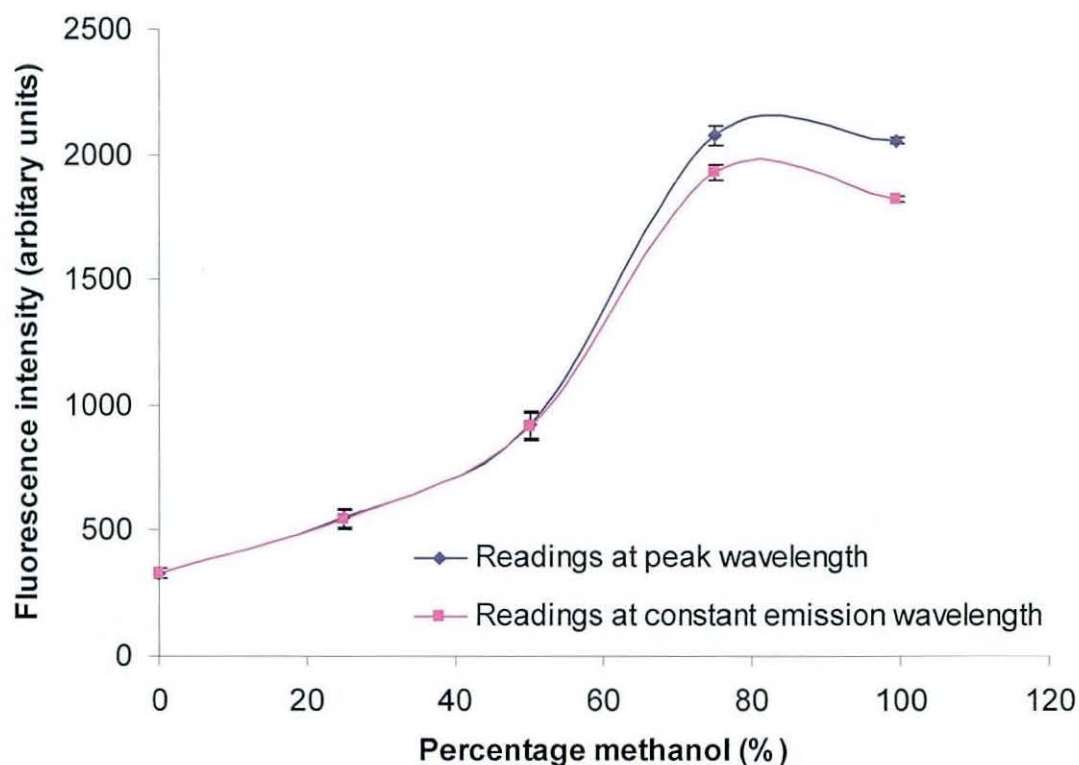
Leupeptin was purchased in 5 mg vials, which is equivalent to 2.1 mM. A stock solution was made up by adding 1 ml 10 mM carbonate buffer pH 9.5 to the vial to give a concentration of 5 mg / ml. This was to prevent loss of leupeptin that would occur if weighing out and to prevent inaccuracies due to weighing out of very small quantities. These solutions were therefore stable for just one week. All experiments involving leupeptin therefore needed to be carried out rapidly and this is reflected in the poorer accuracy found with the leupeptin data than with the other inhibitors. This accuracy was also affected by the fact that individual experiments needed to be carried out in a few hours, as the solutions were stable for just several hours when kept on ice.

Leupeptin has working concentrations of 10 to 100 μ M. As shown in figure 5.45, the leupeptin inhibition curve was carried out using concentrations ranging from 0 to 3.73 μ g / ml. This is equivalent to molar concentrations of 0 to 7.8 μ M. The inhibition curve therefore has been carried out at low inhibitor concentrations, which is reflected in the low percentage inhibition. However due to the instability of leupeptin, which therefore makes it an extremely difficult inhibitor to work with, it was felt that spending more money on higher concentrations was not valid as it

was only really important to obtain the linear portion of the graph to determine the limit of detection.

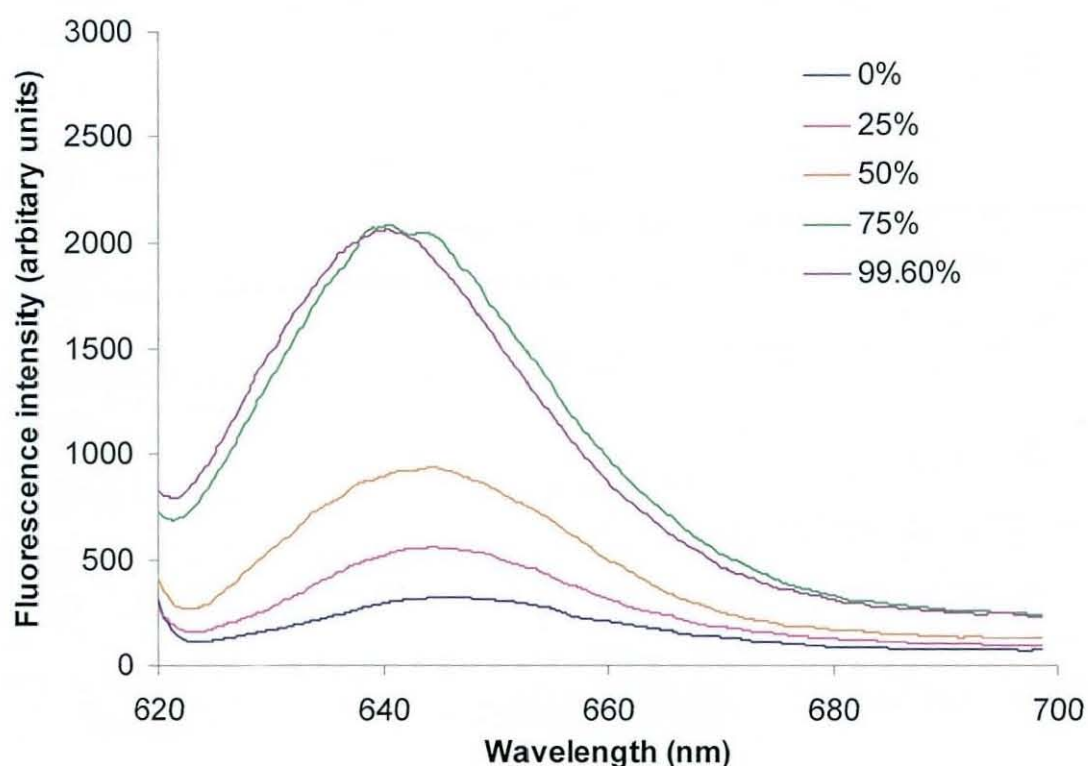
A second source of difficulty was found with pepstatin A. This inhibitor has poor solubility and does not dissolve in aqueous solutions. Pepstatin A is soluble in both dimethyl sulphoxide (DMSO) and methanol. Experiments were carried out and it was found that in the presence of inhibitor, the fluorescence intensity increased, the opposite of what was expected. The fluorescence of the substrate was therefore compared in buffer with that in methanol. As shown in figure 5.37, methanol dramatically affected the fluorescence of BODIPY. With increasing methanol concentration a concomitant increase in BODIPY fluorescence was noted. This response was unexpectedly non-linear.

Figure 5.37 – Influence of methanol concentration on the fluorescence of BODIPY: α -casein



The two plots in figure 5.37 represent the fluorescence intensity at the emission wavelength of BODIPY: α -casein and also at the peak emission wavelength found. This was because it was also found that the addition of increasing amounts of methanol caused a small wavelength shift of the emission maximum towards lower wavelengths, as shown in figure 5.38.

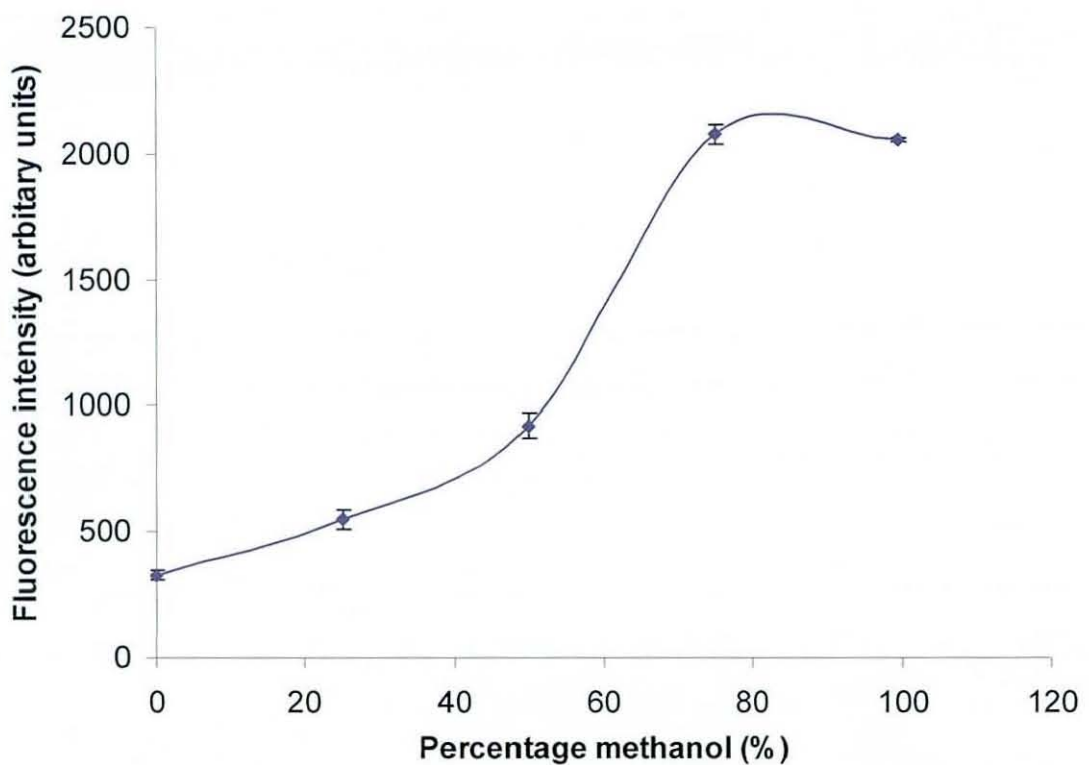
Figure 5.38 – Influence of percentage concentration of methanol on the emission wavelength of BODIPY: α -casein



It has been noted in the past that methanol dramatically enhances serine protease activity under anhydrous conditions [292]. Certain solvents or 'additives' including dimethylsulfoxide, formamide, and ethylene glycol have also been reported in the past to increase enzyme activity when added into the organic media along with small amounts of water [293, 294].

In order to determine whether methanol affects the fluorophore only or whether the enzyme is also affected by the presence of methanol, methanol concentration was increased in the presence of substrate and enzyme (figure 5.39).

Figure 5.39 – Influence of percentage methanol on the fluorescence of BODIPY: α -casein and pepsin

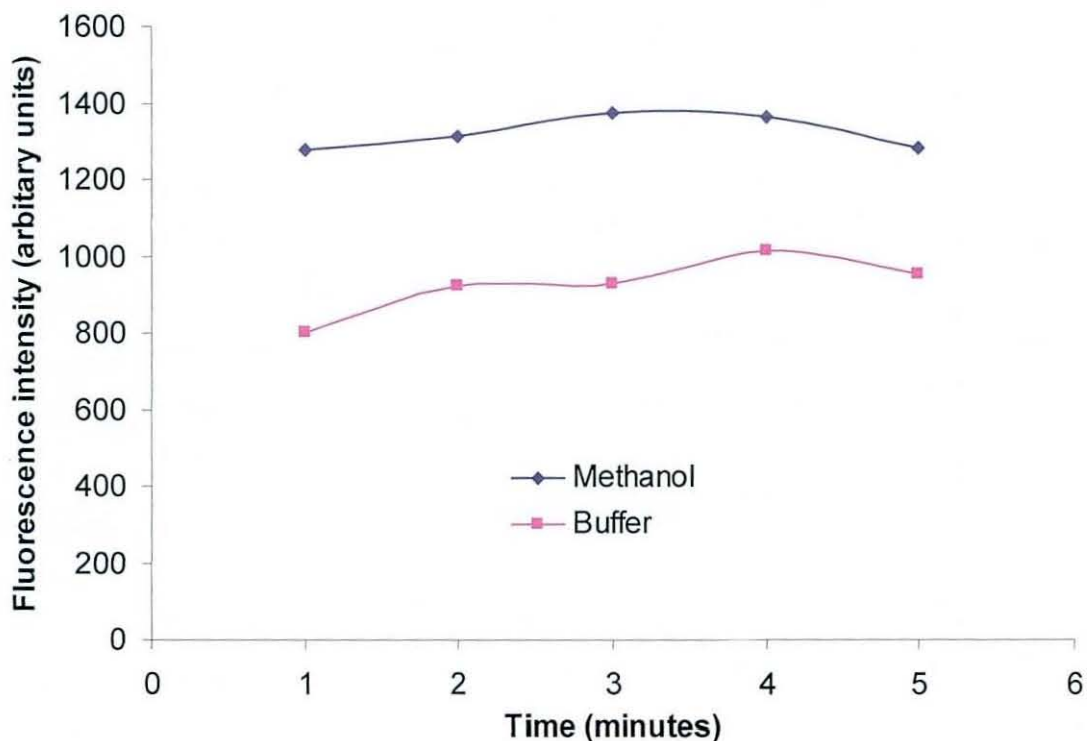


If the shapes of the two graphs shown in figures 5.37 and 5.39 are compared it can be seen that the shapes are extremely similar. This suggests that the effect of methanol is purely on the BODIPY: α -casein substrate and not on pepsin, as if pepsin were also affected, the shape of the graph would have altered when compared to that of figure 5.37.

The solvent dependent effect that has been observed with BODIPY has been documented in the past by a number of researchers for various fluorophores [295, 296]. In fact, the Molecular Probes Inc. website records many of the BODIPY spectra in methanol as it greatly enhances the fluorescence [297].

The effect of incubating BODIPY: α -casein and pepsin over time in the presence of a constant concentration of methanol was also investigated. As shown in figure 5.40, the presence of methanol has a constant effect over time demonstrated by the similar shapes of the curves representing the presence of methanol and the absence of methanol (buffer).

Figure 5.40 – Influence of incubation time on the fluorescence of BODIPY: α -casein incubated with pepsin in the presence and absence of methanol



As DMSO is also a solvent for pepstatin A, the effect of inclusion of DMSO in the medium on the fluorescence intensity and emission wavelength of BODIPY: α -casein was also studied.

From figure 5.41 it can be deduced that DMSO also causes an enhancement of the fluorescence of BODIPY, although at lower percentage concentrations the effect is not as dramatic as that seen with methanol. However, the effect of DMSO on the optimum emission wavelength was opposite to that caused by methanol as shown in figure 5.42. Whilst methanol caused a blue shift of around 5 nm at 99.6 %, DMSO caused a red shift of about 7 nm at 99.6 %.

Figure 5.41 – Influence of percentage methanol and DMSO on the fluorescence of BODIPY: α -casein

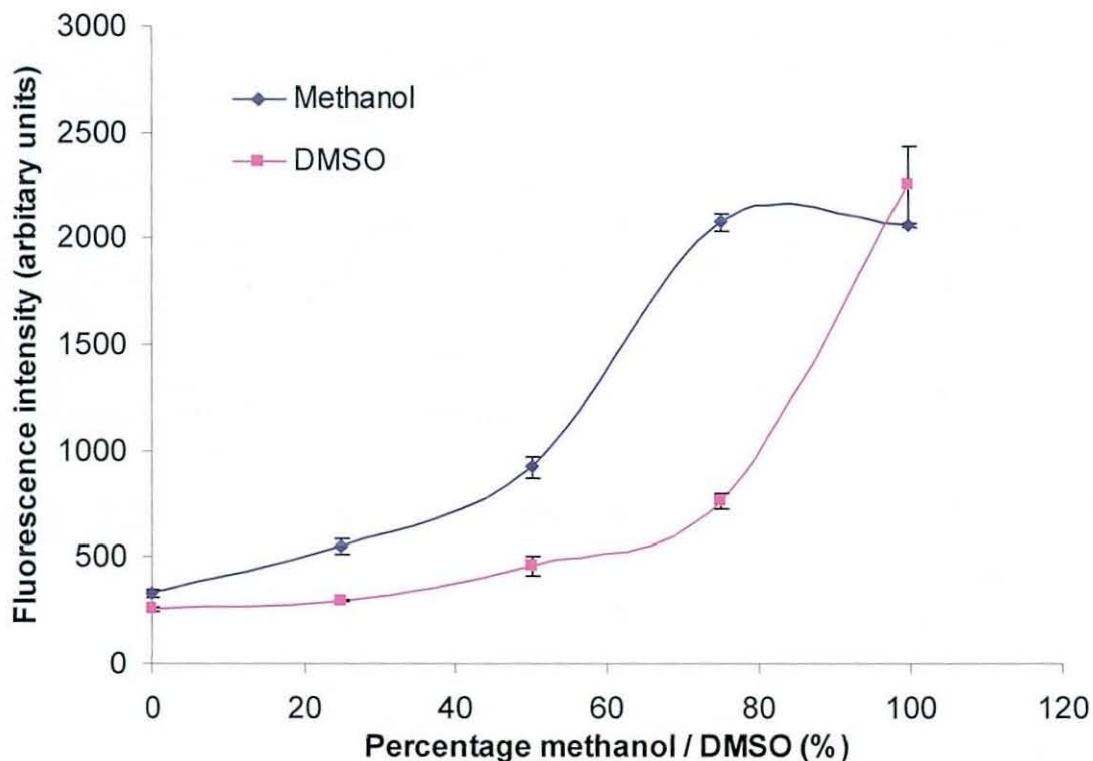
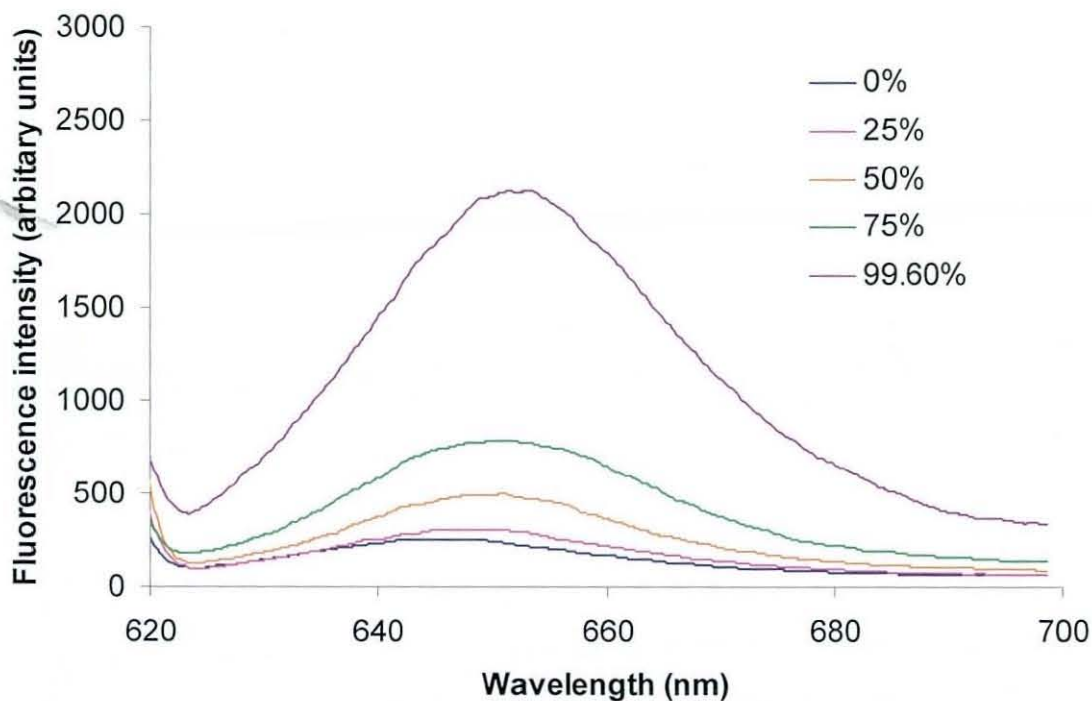


Figure 5.42 – Influence of DMSO percentage concentration on the optimum emission wavelength of BODIPY: α -casein



As DMSO is more toxic than methanol and as pepstatin A was found to dissolve more easily in methanol than DMSO, the decision was taken to use methanol as the solvent for DMSO even though the fluorescence enhancement effects were greater with methanol than with DMSO.

A 10 mg / ml stock solution of pepstatin A in methanol was stored in aliquots in the freezer where they were stable for months. An aliquot was used for each experiment and was made up in 20 mM glycine buffer pH 10.0, which was stable for at least one day at room temperature. Accounting for dilution in buffer and for additional dilution in the flow system, the actual percentage of methanol present during optimisation experiments amounted to only 0.09 %. To take the effect of this methanol upon BODIPY: α -casein into account, the same percentage of methanol was also included in the blank reading. For the generation of the pepstatin A inhibition curve, the amount of methanol in the series of standard solutions of pepstatin A injected into the flowing system was kept constant.

5.6.2.6 Generation of inhibition curves

Under the optimum conditions already described, a series of inhibitor standard solutions were mixed with enzyme and injected into the flow analysis system to be incubated with substrate. The reaction products were detected and the fluorescence measured. Measurements were taken in triplicate and error bars represent plus and minus one standard deviation. Inhibition curves were obtained for each of the four enzyme – inhibitor pairs.

Figure 5.43 – Inhibition of proteinase K by 3-nitrophenylboronic acid

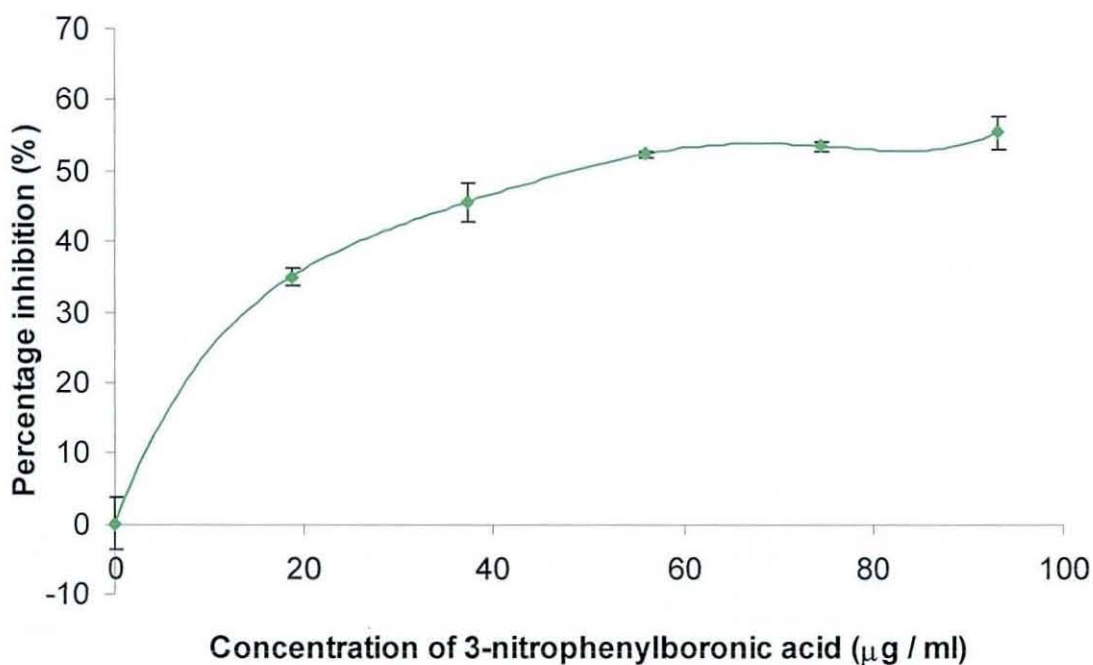


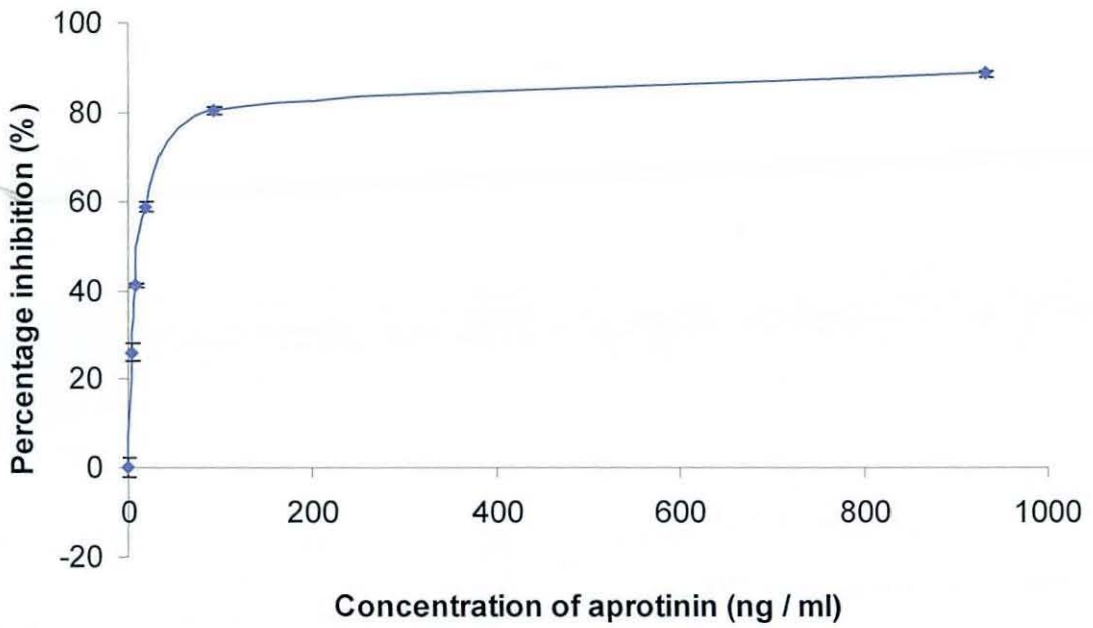
Figure 5.44 – Inhibition of α -chymotrypsin by aprotinin

Figure 5.45 – Inhibition of alkaline protease by leupeptin

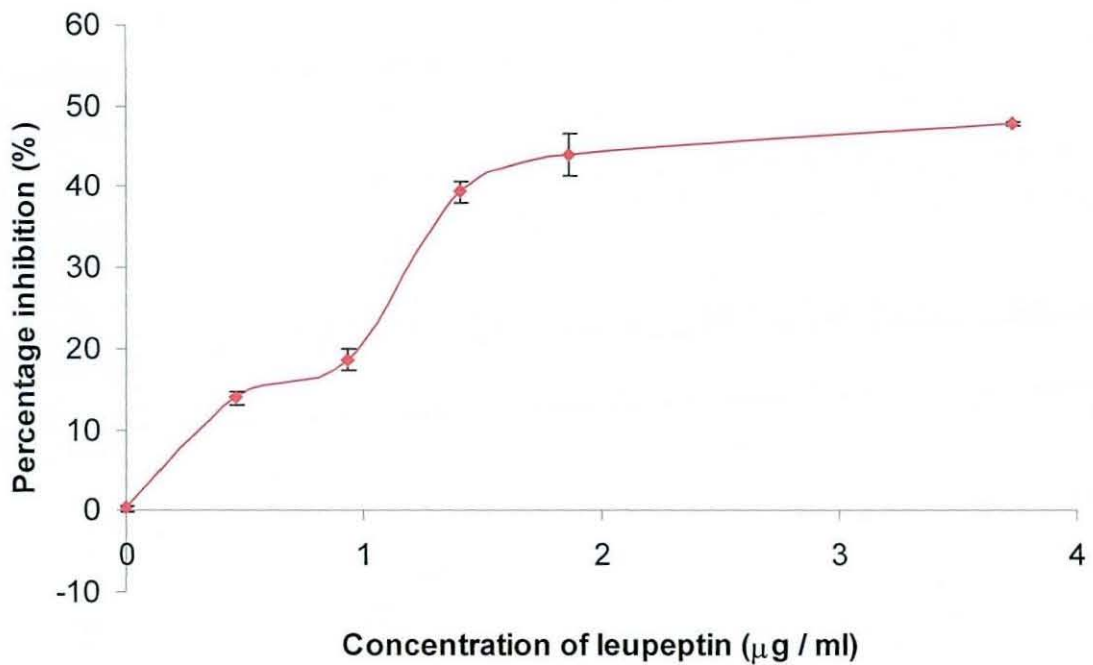
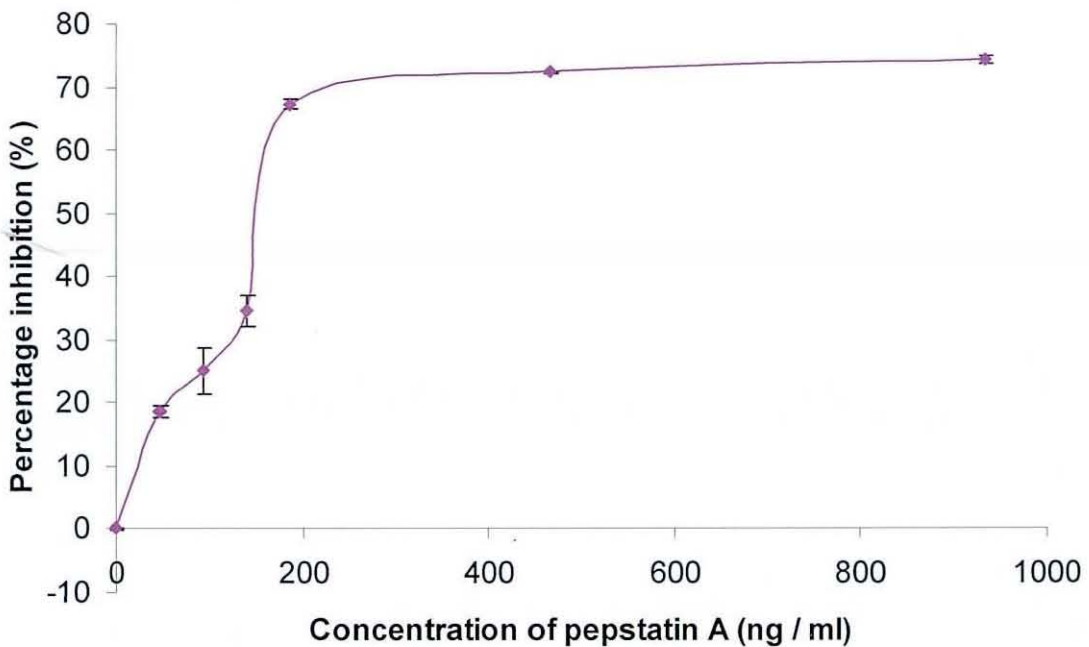


Figure 5.46 – Inhibition of pepsin by pepstatin A

Figures 5.43 to 5.46 show the inhibition of the enzymes proteinase K, α -chymotrypsin, alkaline protease, and pepsin by the inhibitors 3-nitrophenylboronic acid, aprotinin, leupeptin, and pepstatin A respectively. As inhibitor concentration was increased there was an increase in percentage inhibition until a plateau point was reached. For the competitive inhibitors (3-nitrophenylboronic acid, aprotinin, and leupeptin), as inhibitor concentration is increased there is more competition against the substrate for the active site of the enzyme and so percentage inhibition increases. At the plateau point of the inhibition curves, enzyme becomes limiting and further increases in inhibitor produce no increases in inhibition because there are no more enzyme sites to compete for. In the case of pepstatin A, which is an uncompetitive inhibitor, increases in inhibitor concentration cause more inhibitor to bind with the enzyme – substrate complex, preventing the formation of product. This is reflected by an increase in the percentage inhibition. However, at the plateau point the formation of the enzyme – substrate complex becomes limiting and further increases in

inhibitor concentration produce no further increases in inhibition because there is no more enzyme – substrate complex to inhibit.

For the competitive inhibitors at any given inhibitor concentration, if the amount of substrate is increased a drop in percentage inhibition would be seen as there would be more substrate to compete with the inhibitor for the enzyme active site.

5.6.2.7 Determination of limits of detection

Limits of detection, defined as the analyte concentration giving a signal equal to the blank signal plus 3 standard deviations of the blank [291], were determined for each of the four inhibitors in the flow analysis system. Percentage inhibition against inhibitor concentration was plotted for the linear portion of the graphs for each of the inhibitors. These results are shown in figures 5.47 to 5.50. Measurements were taken in triplicate and error bars are given which represent plus and minus one standard deviation.

Figure 5.47 – Percentage inhibition against inhibitor concentration in linear portion to calculate limit of detection

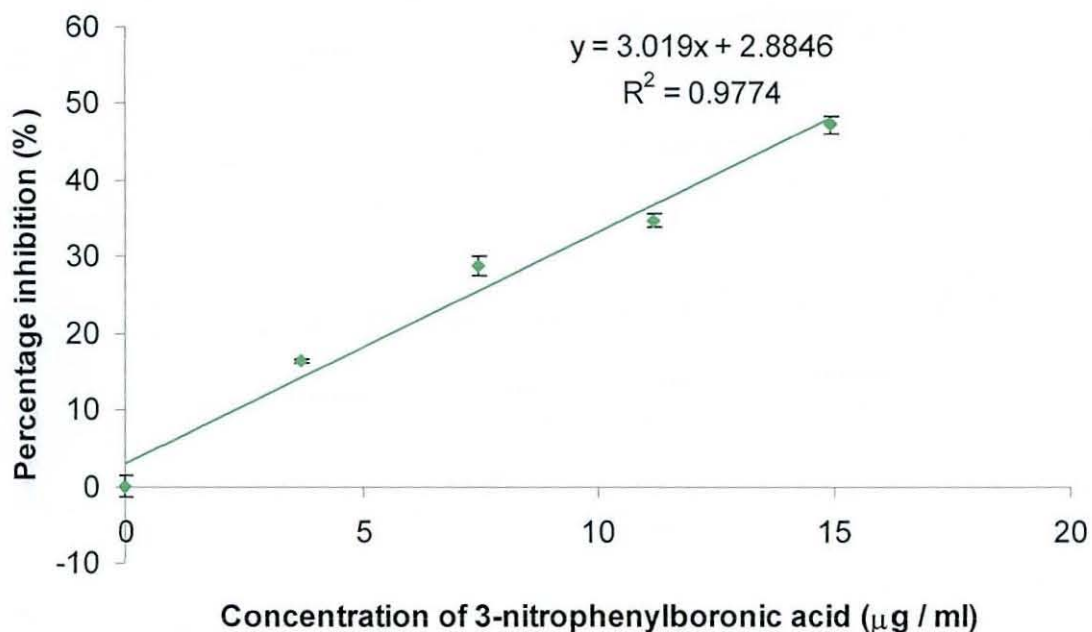


Figure 5.48 – Percentage inhibition against inhibitor concentration in linear portion to calculate limit of detection

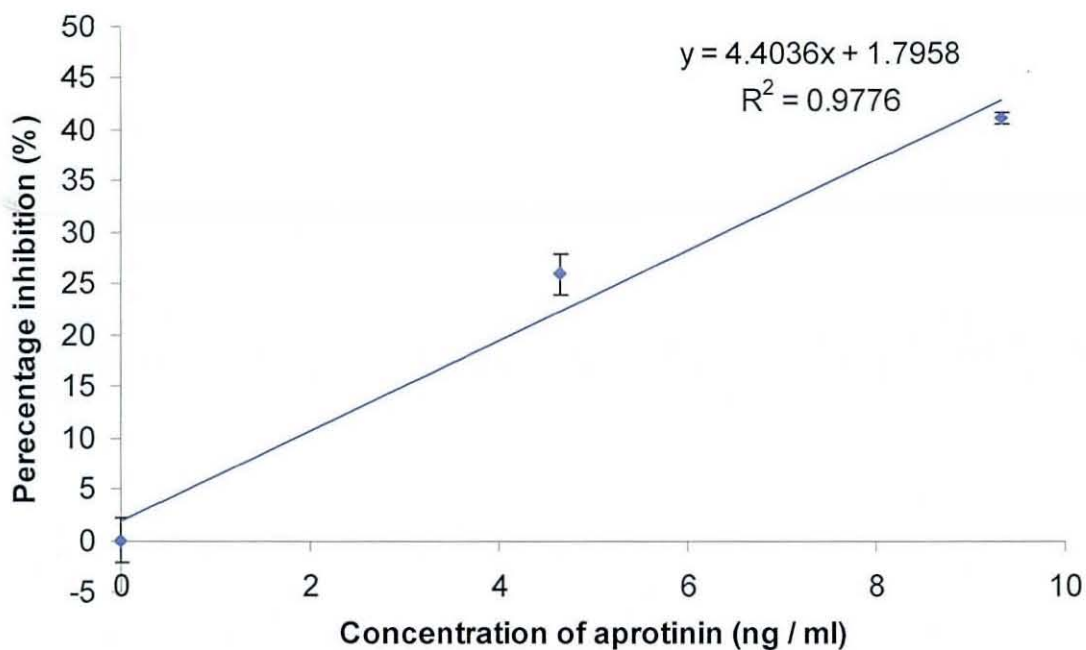


Figure 5.49 – Percentage inhibition against inhibitor concentration in linear portion to calculate limit of detection

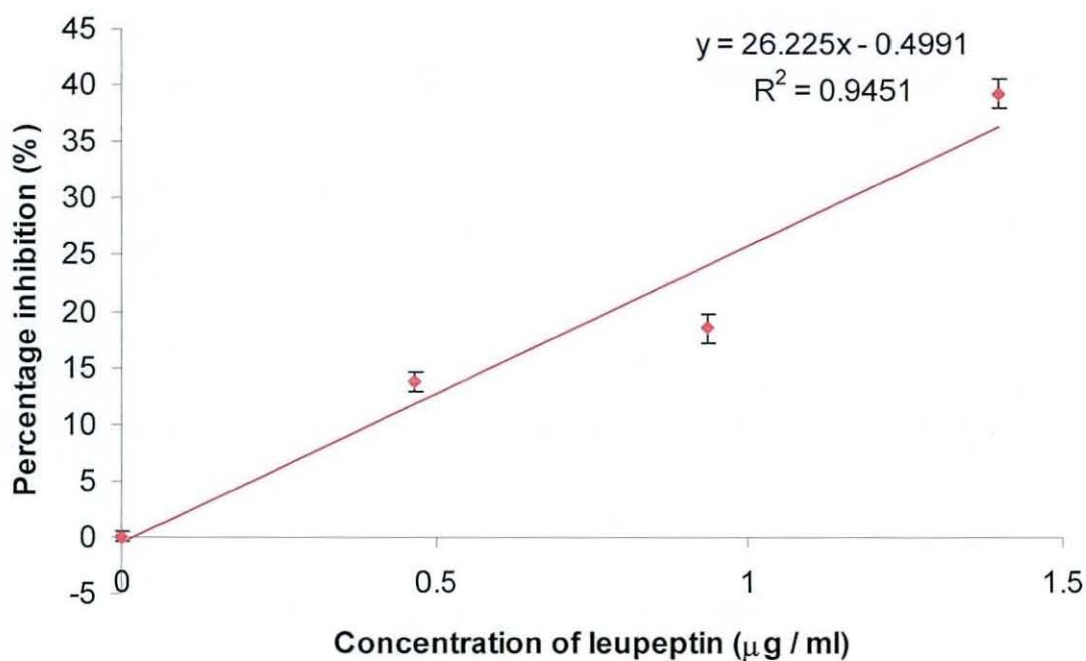
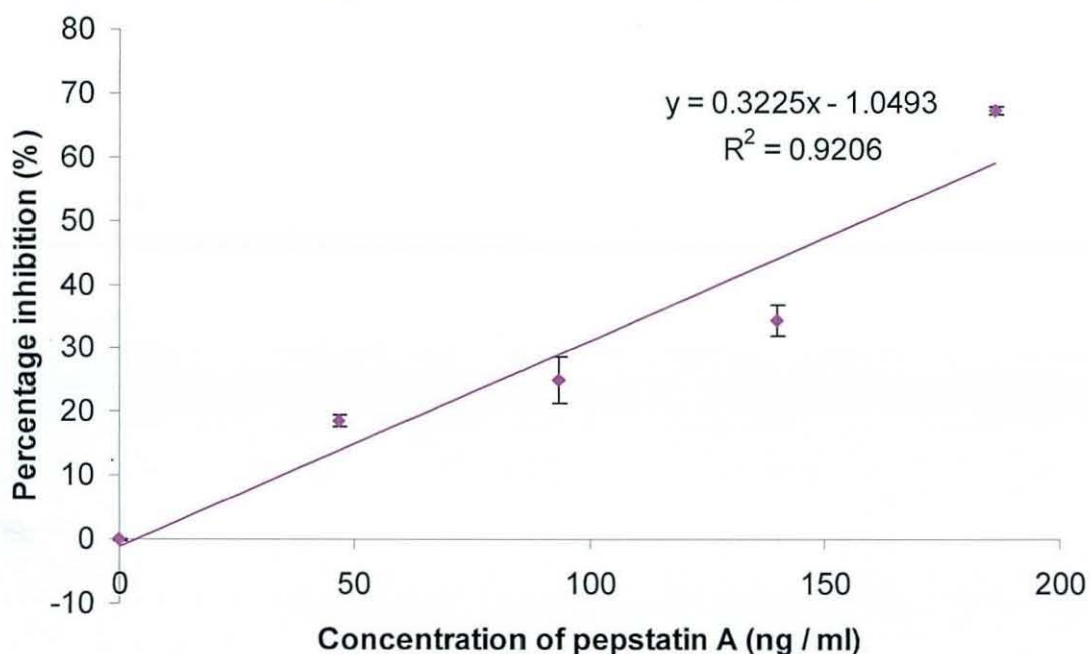


Figure 5.50 – Percentage inhibition against inhibitor concentration in linear portion to calculate limit of detection



Calculated limits of detection for each inhibitor using the substrate BODIPY: α -casein are given in table 5.11.

INHIBITOR	LIMIT OF DETECTION
3-NITROPHENYLBORONIC ACID	2.504 μ M
APROTININ	0.161 nM
LEUPEPTIN	0.140 μ M
PEPSTATIN A	7.20 nM

Table 5.11 - Limits of detection determined experimentally for various protease inhibitors using the substrate BODIPY: α -casein in a flowing system

Limits of detection in the μM (3-nitrophenylboronic acid and leupeptin) and nM (aprotinin and pepstatin A) regions were achieved using the substrate BODIPY: α -casein, showing that this fluorogenic substrate can successfully be applied to the sensitive determination of protease inhibitors.

From graphs 5.47 to 5.50 it is obvious that both aprotinin and pepstatin A are both potent serine and aspartate protease inhibitors respectively. This was seen by the high percentage inhibition achieved at low inhibitor concentrations. This was reflected in the nM limits of detection. Conversely, 3-nitrophenylboronic acid and leupeptin were less efficient serine protease inhibitors giving much lower percentage inhibitions at higher inhibitor concentrations. This was again reflected in the limits of detection, which were obtained in the μM region.

The time taken to carry out these inhibition assays was rapid taking at most 5 minutes for leupeptin and again the flow-based procedure that has been described would be easily automatable for use in high throughput screening assays.

The method that has previously been described in section 1.8 3 2 2 by Jones *et al.* [255] was also applied to the determination of aprotinin. This work was able to achieve limits of detection in the $\mu\text{g} / \text{ml}$ range using an assay that took 80 minutes. The method that has been described in this thesis has improved on this significantly. Aprotinin has been detected at a concentration of 1.1 ng / ml in just 3 minutes.

5.7 DISCUSSION

Calculations of dye protein ratios in fluorescent or fluorogenic conjugates usually assume that the absorbance characteristics of the conjugated dye are the same as those of the free dye. This was clearly not the case at wavelengths at ca. 600 nm in the present work. Experiments were carried out to establish whether the shape change that was observed between the absorbance spectra of the free and conjugated dye was due to the formation of covalent H-dimers between BODIPY molecules. The results of these experiments strongly suggested that this theory was indeed plausible. D:P calculations were therefore carried out using absorbance values at 280 nm and 350 nm to estimate protein and dye concentrations respectively. The latter wavelength was chosen because the absorbance spectra of the free dye and conjugated dye in this region are very similar. Using this method of calculation, it was found that a D:P ratio of 2.35 BODIPY molecules to 1 α -casein was optimal for use in these types of assays. This was in general agreement with the findings of Jones *et al.*, [255] who showed that conjugation of an average of 4.5 to 6 BODIPY FL or even fewer BODIPY TR-X dye groups to each casein molecule was optimal. Presumably, the formation of non-fluorescent dimers within the substrate would further help to produce a quenching effect due to the fact that the dimers can absorb the exciting light but will not fluoresce. Larger D:P ratios could be achieved by increasing the levels of dye in the reaction mixtures, but this resulted in an increase in background fluorescence, i.e. in the intensity of the unhydrolysed substrate, limiting the sensitivity of the assays.

The relatively low molar ratio of dye to protein used in the present work provides an ideal substrate for proteolytic enzymes. Worries that the use of highly derivatised molecules might sterically hinder the activity of some proteases are minimised. In addition, BODIPY dyes have no overall charge, so dye conjugation

should not greatly affect the protein substrate conformation and enzyme-active site interactions.

The application of BODIPY: α -casein to the determination of different proteolytic enzymes with varying pH optima has been demonstrated. Enzyme assays were carried out in just 1.5 minutes with the exception of alkaline protease (2 minutes). The pH insensitivity of BODIPY means that assays can be carried out in a single step without the need to carry out the proteolytic reaction at one pH and the measurement at another pH, as would be necessary sometimes in the case of fluorescein. The procedure does not require any process such as separation, precipitation or centrifugation.

The suitability of BODIPY: α -casein as a substrate in enzyme inhibition assays has also been investigated. Limits of detection of 2.50 μ M, 0.14 μ M, and 7.20 nM were obtained for 3-nitrophenylboronic acid, leupeptin, and pepstatin A respectively. The inhibitor aprotinin has been found to be of benefit during paediatric cardiac surgery to reduce blood loss with cardiopulmonary bypass [274]. The limit of detection for this inhibitor was 0.161 nM (1.1 ng ml⁻¹) using the current setup. This is extremely sensitive compared to the assay using a similar substrate described by Jones *et al* [255] who observed inhibition in the μ g ml⁻¹ range. In addition, the assay described by Jones *et al.* took 1 hour 20 minutes to carry out, whereas the assay described here was carried out in just 3 minutes.

The fluorogenic assays that have been described here are rapid, taking at most 2 minutes for the enzyme assays and 5 minutes for the inhibition assays. They combine the sensitivity of long-wavelength fluorescence with the ease of automation that can be applied to flow injection analysis and could be incorporated into a simple portable fluorescence instrument. They have many clinical applications, since proteases are involved in virtually all biological functions and dysfunctions and therefore are promising as drug targets.

The application of BODIPY: α -casein in a flow injection analysis system is a rapid, simple and sensitive method for the assay of proteases, essential requirements for a routine assay. The whole assay is easily automated and would be well suited for use in high throughput screening.

CHAPTER 6

Experimental: multi-determination protease inhibition assays

6.1 INTRODUCTION

The aim of these experiments was to apply those protease inhibition assays that had previously been optimised in chapter 5 to a flow injection analysis system such that the system would allow the determination of more than one inhibitor in the same assay i.e. multi-determination.

The need to improve productivity is imperative for drug discovery companies. One strategy is: test more discover more. The availability therefore of a simple, sensitive, and efficient method for the multi-determination of proteolytic enzymes and their inhibitors would be of much use in the high throughput screening of candidate drug molecules. This set-up would allow a much higher throughput accompanied by cost advantages.

An excellent review discusses the determination of two or more species in the same sample with a single flow injection analysis system [71]

6.2 MULTI-DETERMINATION PROTEASE INHIBITION ASSAYS USING CONTROLLED-PORE GLASS AS A SUPPORT

6.2.1 Procedures

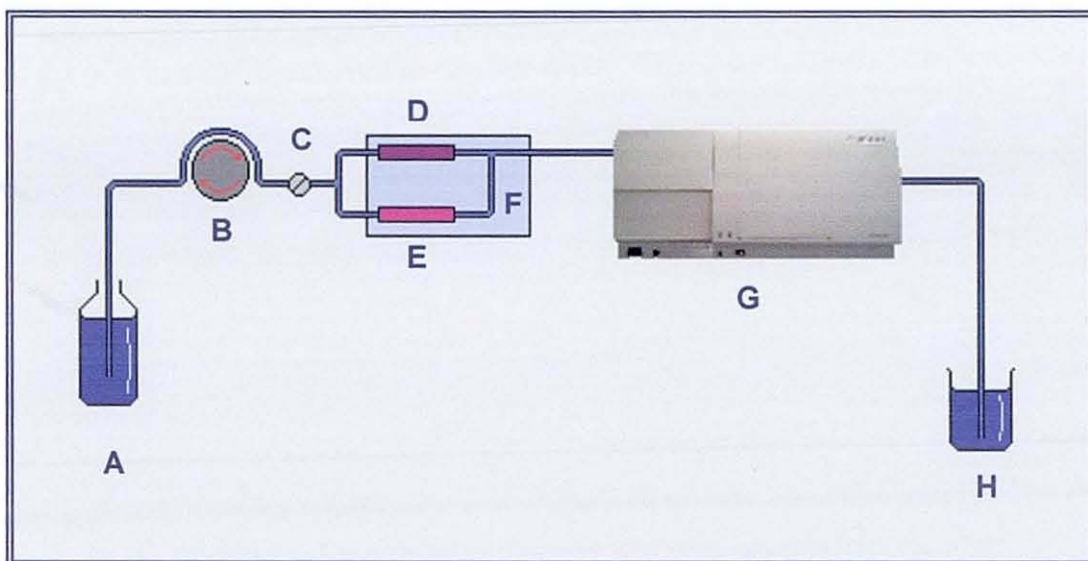
Fluorescence measurements were made using an F-4500 fluorescence spectrophotometer with excitation and emission bandwidths of 10nm. Time scans were performed with excitation and emission wavelengths of 605 nm and 644 nm respectively for BODIPY and BODIPY: α -casein, 280 nm and 350 nm for α -casein, 597 nm and 660 nm for naphthofluorescein, and 640 nm and 664 nm for Cy5.

As a starting point for these assays, it was decided that the simplest approach would be a dual protease inhibition assay using one buffer system. As it was shown that both alkaline protease and proteinase K operated at pH 9.5 (section 5.5.2, table 5.6), these two enzymes seemed like the obvious choice. The serine protease inhibitor 3-nitrophenylboronic acid was shown to inhibit proteinase K (section 5.6.2.6, graph 5.43) and so this inhibitor was selected for use in this dual system. The ideal set-up for a simple assay was to flow buffer slowly through two enzyme reactors, each containing either immobilised alkaline protease or immobilised proteinase K, as shown in figure 6.1. A substrate / inhibitor solution would be injected and the fluorescence detected through the time-dependent separation of the peaks emerging from each of the reactors by having different lengths of flow tubing between the enzyme reactors and the detector.

The other alternative to this set-up was to capture the assay components in each column using an incubation loop and 4-port manual switching valve. However, this approach seemed more problematic due to having two switching valves to

operate at exact times, and so the above approach depicted in figure 6.1 was chosen as the proposed system.

Figure 6.1 – Schematic of flow injection analysis system for multi-determination protease inhibition assays

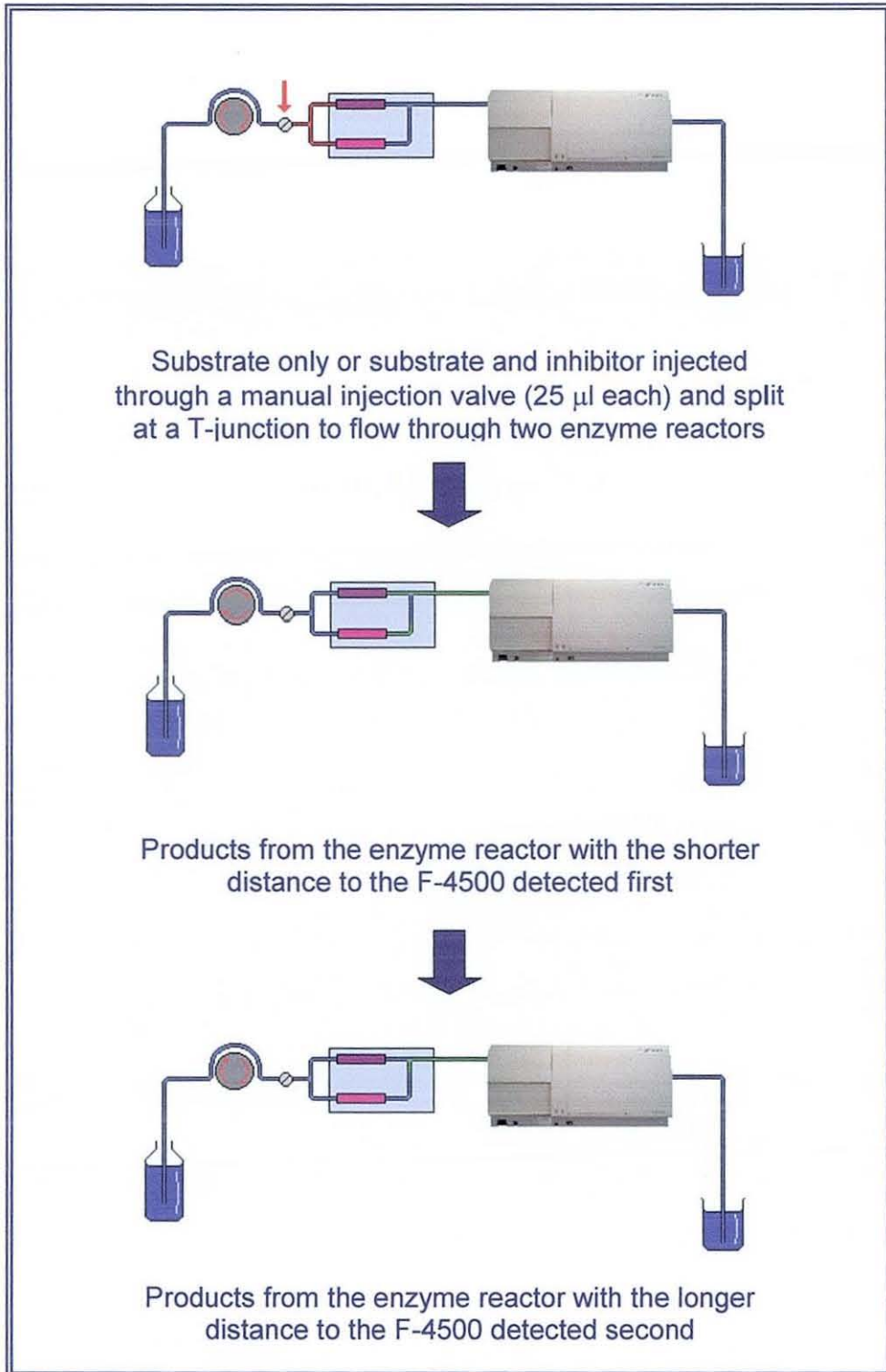


(A = buffer, B = peristaltic pump, C = substrate or substrate / inhibitor injection valve, D = enzyme reactor 1, E = enzyme reactor 2, F = thermostatted water bath, G = fluorescence detector, H = waste)

Temperature, pH and buffer system conditions for alkaline protease had previously been optimised (section 5.5.2, table 5.6) to be 10 mM carbonate buffer pH 9.5 and 40 °C. Those for proteinase K were 20 mM carbonate buffer pH 9.5, 35 °C. By comparing results for buffer concentration and temperature for each enzyme, it was decided that the best compromise for multi-determination assay conditions was 20 mM carbonate pH 9.5, 35 °C. These conditions were used in all experiments.

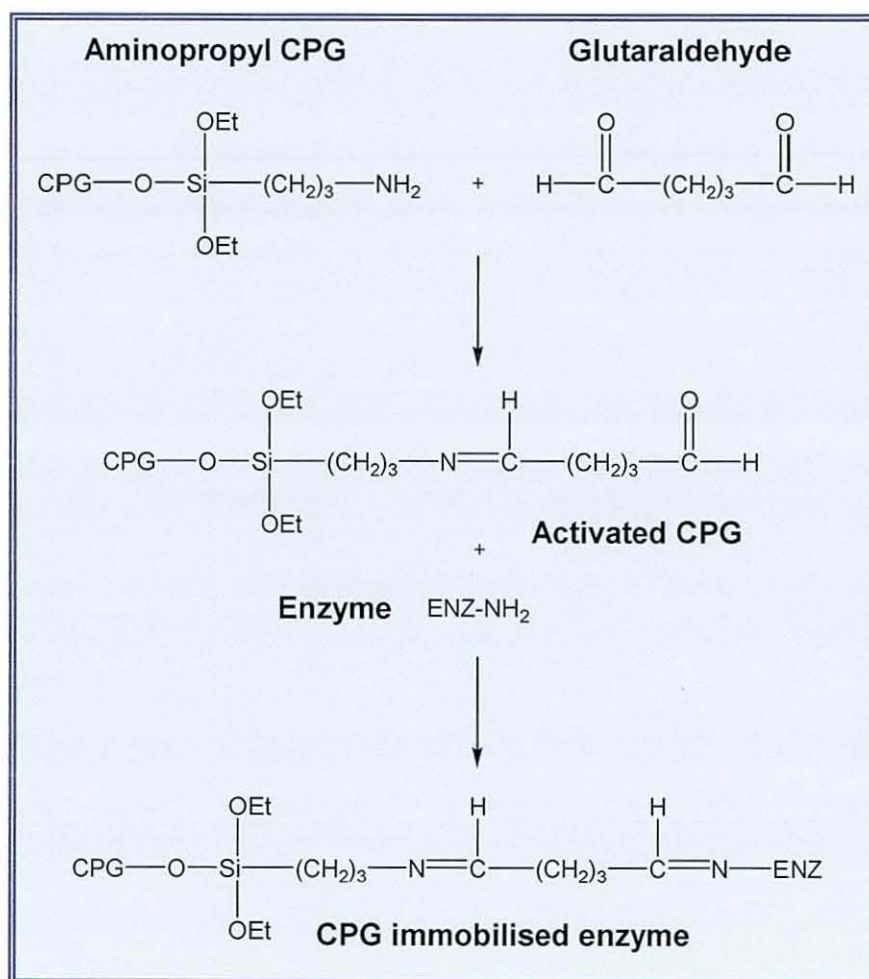
The sequence of events using this flow analysis set-up is shown in figure 6.2.

Figure 6.2 – Sequence of events for multi-determination protease inhibition assays using immobilised enzyme reactors



Controlled-pore glass (CPG) was purchased from Cambio Ltd. and evaluated as a support for use in these assays. Immobilisation of alkaline protease and proteinase K onto controlled pore glass was carried out as previously described by Masoom and Townshend [120], a description of which is given in section 2.2.12. 2.5, 5.0, and 7.5 mg alkaline protease and 4.0, 8.0, and 12.0 mg proteinase K were used for immobilisation onto 0.5 g CPG. Immobilised enzyme was stored in buffer at 4 °C when not in use. The reaction scheme for enzyme immobilisation is given in figure 6.3.

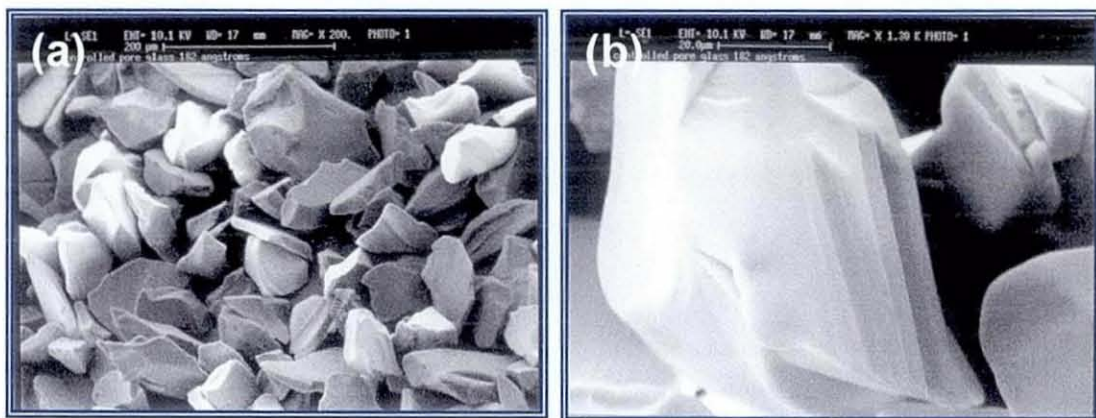
Figure 6.3 – Reaction scheme for the immobilisation of enzyme onto controlled pore glass



CPG was chosen as a support due to its high surface area, high mechanical strength, high flow rate, and rigid glass structure. Initial studies were carried out on aminopropyl CPG with a mean pore diameter of 182 Å, a particle size of 37 – 74 μm, and a capacity of 701.9 μmoles amine per g glass. CPG with a mean pore diameter of 972 Å, a particle size of 37 – 74 μm, and a capacity of 67 μmoles amine per g glass was also used.

A picture of the 182 Å aminopropyl CPG was taken using a Scanning Electron Microscope (SEM) as shown in figure 6.4.

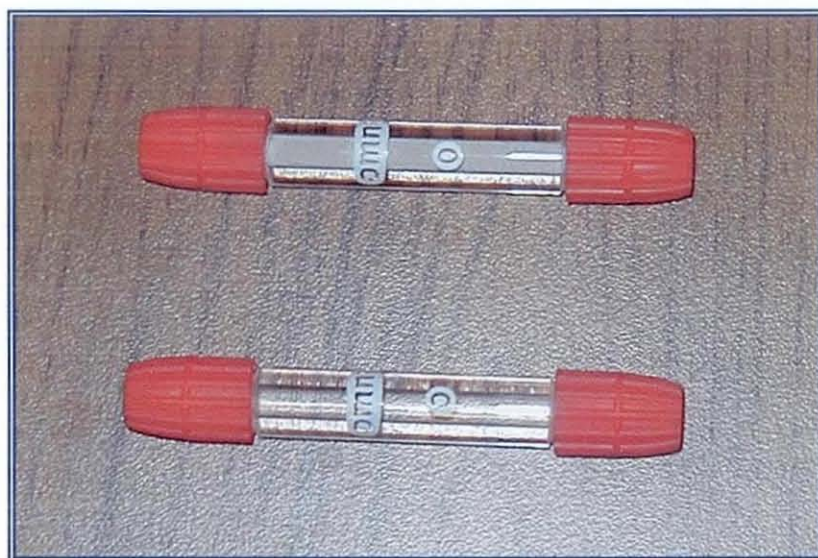
Figure 6.4 - SEM pictures showing (a) 182 Å aminopropyl glass and (b) an individual glass chip



It can be seen that CPG has the appearance of fine glass chips of irregular shape. The best resolution for the SEM is at distances of 100 – 200 μm and so the pores of the CPG were beyond the capability of the SEM.

Immobilised enzyme was packed into Omnifit glass columns with a bore of 3 mm, and a length of either 2.5 cm or 5.0 cm corresponding to reactor volumes of 175 μl and 350 μl respectively. Aluminium frits with 2 μm pores were used at both ends to prevent leakage of the CPG. The appearance of the enzyme reactor 5 cm in length is shown in figure 6.5. Columns were packed by mounting the columns vertically, and connecting the bottom of the column to a peristaltic pump that allowed suction of loaded CPG through the top end of the column downwards. The glass column was then incorporated into the flowing system and clamped vertically in the water bath. Buffer was flowed upwards through the column, which was opposite to the direction of packing. This ensured maximum contact of the buffer with the CPG and tighter packing.

Figure 6.5 – Appearance of the enzyme reactors: top – packed glass column, bottom – empty glass column



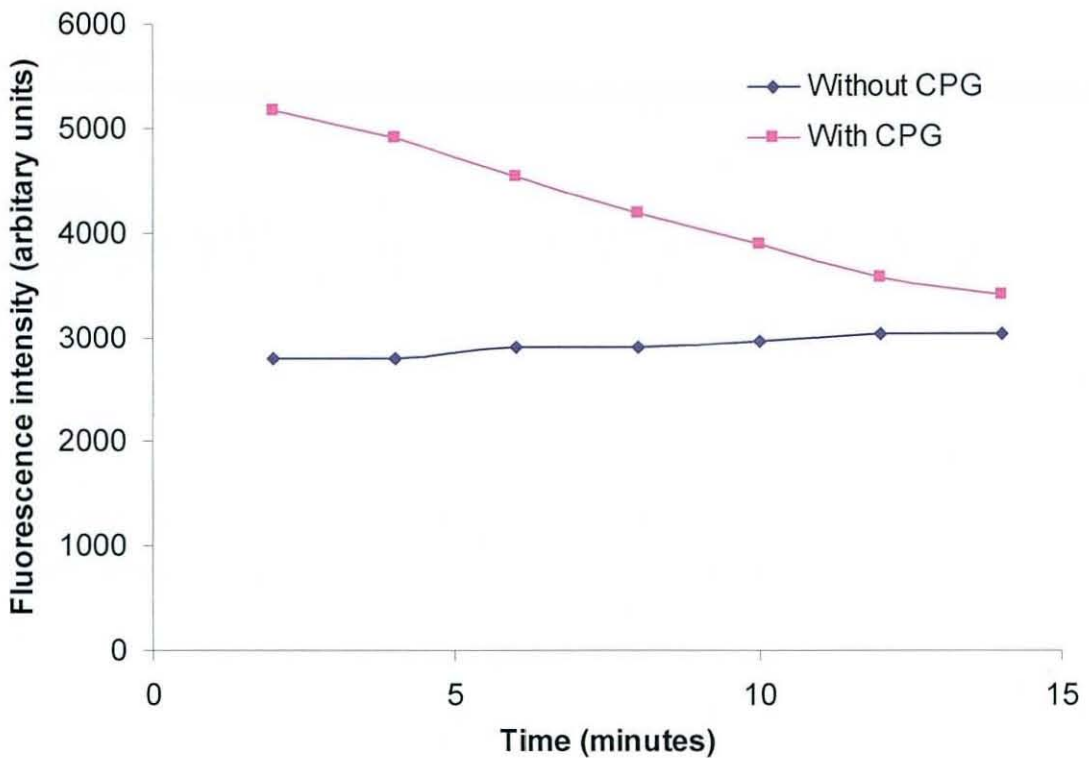
6.2.2 Results

Injections of BODIPY: α -casein and naphthofluorescein were made through a column packed with 12.0 mg proteinase K - CPG. A high concentration of BODIPY: α -casein was used to give sufficient fluorescence intensity, and naphthofluorescein was also used as it is extremely fluorescent and therefore easy to detect whilst being cheap. When each of these was injected, nothing was detected i.e. these dyes appeared to be retained on the column.

As a control experiment therefore, BODIPY: α -casein was mixed in a standard 3 ml fluorescence cuvette with a random amount of proteinase K – CPG by inverting the cuvette five times. The cuvette was incubated at 35 °C and at time intervals the liquid was decanted off and the fluorescence measured. The liquid was then again mixed with the beads and returned to the water bath for a further time before the liquid was again decanted off and the fluorescence measured. This was repeated over time.

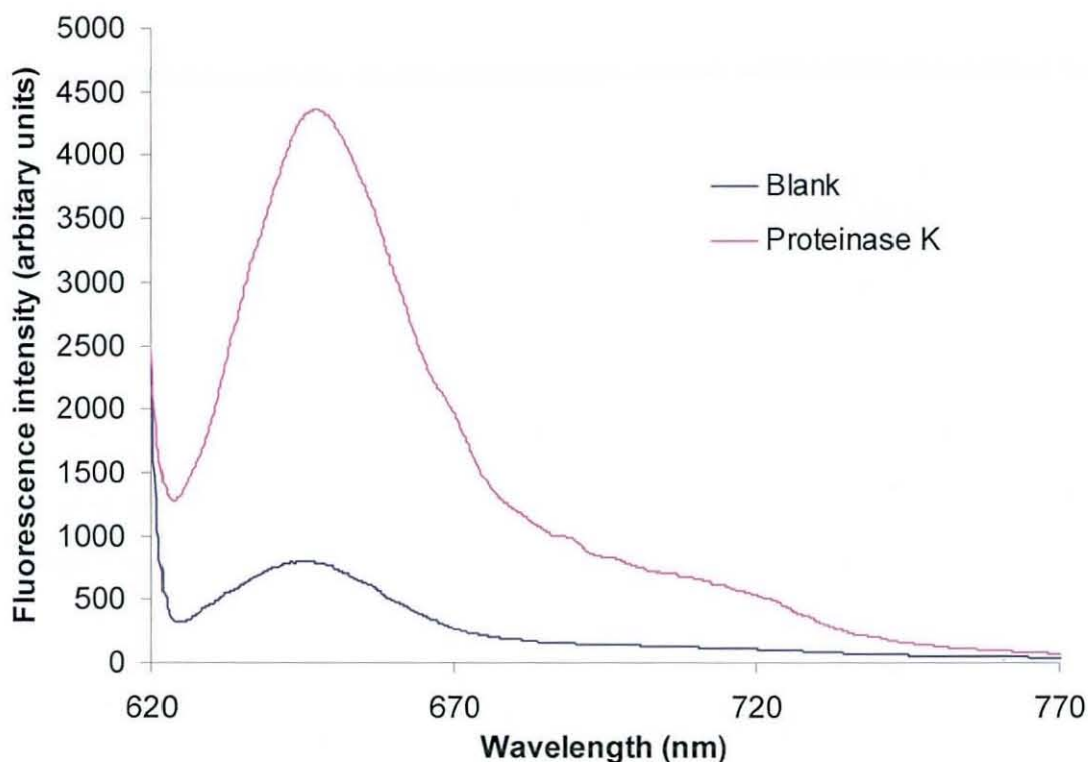
As shown by figure 6.6, it was observed that over time the fluorescence of BODIPY: α -casein decreased in the presence of the proteinase K – CPG when compared to the fluorescence of BODIPY: α -casein in the absence of CPG.

Figure 6.6 – Fluorescence intensity of BODIPY: α -casein over time when incubated in the presence and absence of proteinase K - CPG



Interestingly it was found that incubation of BODIPY: α -casein for 2 minutes with proteinase K – CPG produced an enhancement of fluorescence when compared to BODIPY: α -casein incubated with CPG with no proteinase K immobilised (figure 6.7). This suggested that proteinase K was definitely successfully immobilised onto the CPG as the fluorescence enhancement indicated that the substrate was subjected to proteolytic cleavage.

Figure 6.7 – Enhancement of fluorescence seen when BODIPY: α -casein was incubated with proteinase K – CPG compared with CPG

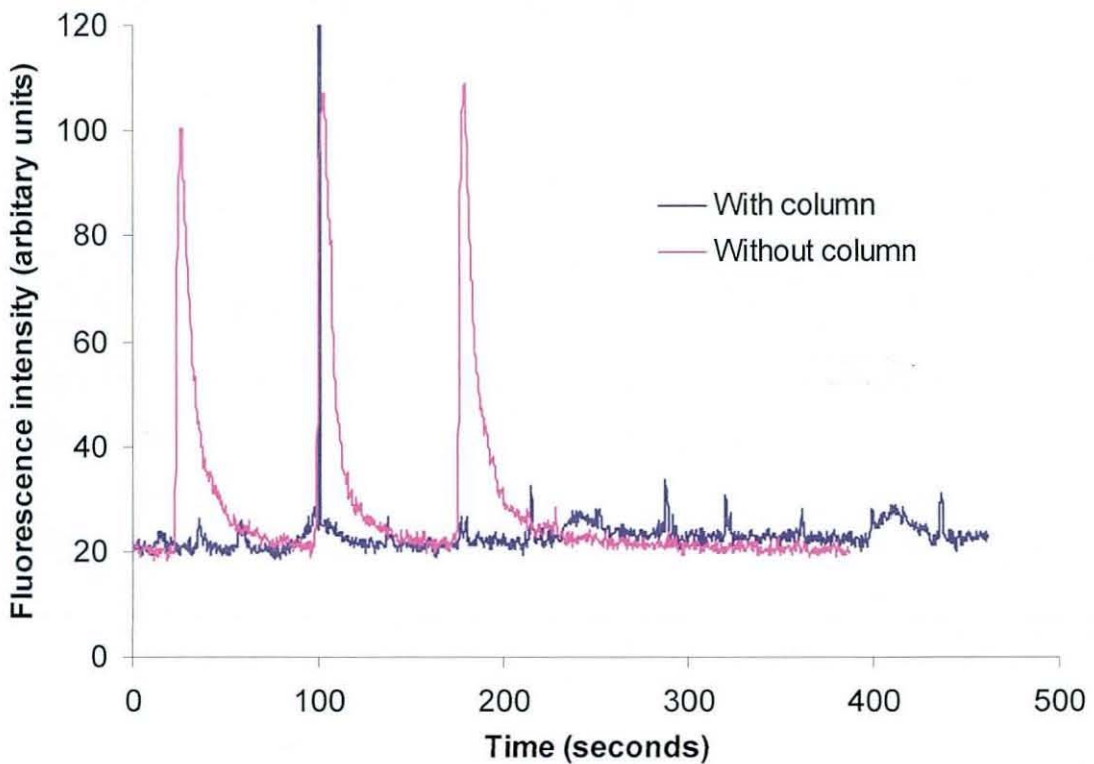


Conversely it was found that when naphthofluorescein was incubated with the proteinase K – CPG the fluorescence intensity remained relatively constant over time. (It is shown later in section 6.2.2.4 that naphthofluorescein does not appear out of the column either. However, this is due to column length, not due to sticking to the CPG, which explains the fact that when naphthofluorescein was incubated with proteinase K – CPG the fluorescence intensity remained constant).

The column was re-packed with 7.5 mg alkaline protease – CPG to see whether a change in the immobilised enzyme would alter the flow of BODIPY: α -casein and naphthofluorescein through the column. However, both were also retained in this column.

Injections of BODIPY: α -casein were then made through a column containing raw aminopropyl CPG i.e. without activation or immobilisation of enzyme. From the results shown in figure 6.8, it was observed that some BODIPY: α -casein was able to flow through this column, although most was retained.

Figure 6.8 – Fluorescent peaks of BODIPY: α -casein injected into a buffer stream with and without a raw CPG column



On the basis of the results that had been found it was decided that a number of possibilities could account for the fact that the enzyme reactor retained both BODIPY: α -casein and naphthofluorescein.

It was seen that BODIPY: α -casein was stable with respect to its fluorescence intensity over time whereas in the presence of proteinase K – CPG the fluorescence dropped. This could be because the stability of the product was different to that of the substrate, perhaps due to the fluorophore molecules being more exposed in the product. It could also be explained by sticking of the substrate or product to the proteinase K – CPG chips, or by the CPG somehow quenching the fluorescence of BODIPY: α -casein.

Another possibility was product inhibition. Product binds to the active site by the same bonds that bind the substrate. As a result, enzyme-product complexes are formed which prevent the substrate from binding the enzyme. As both substrate and product compete for the active site and both cannot bind simultaneously, this is a type of competitive inhibition.

One explanation is that substrate was cleaved into peptides and so enhancement of fluorescence was seen. However, with increasing time these peptides may have been attracted to each other so that the fluorophore molecules again came into close contact and the internal energy transfer effects were again seen.

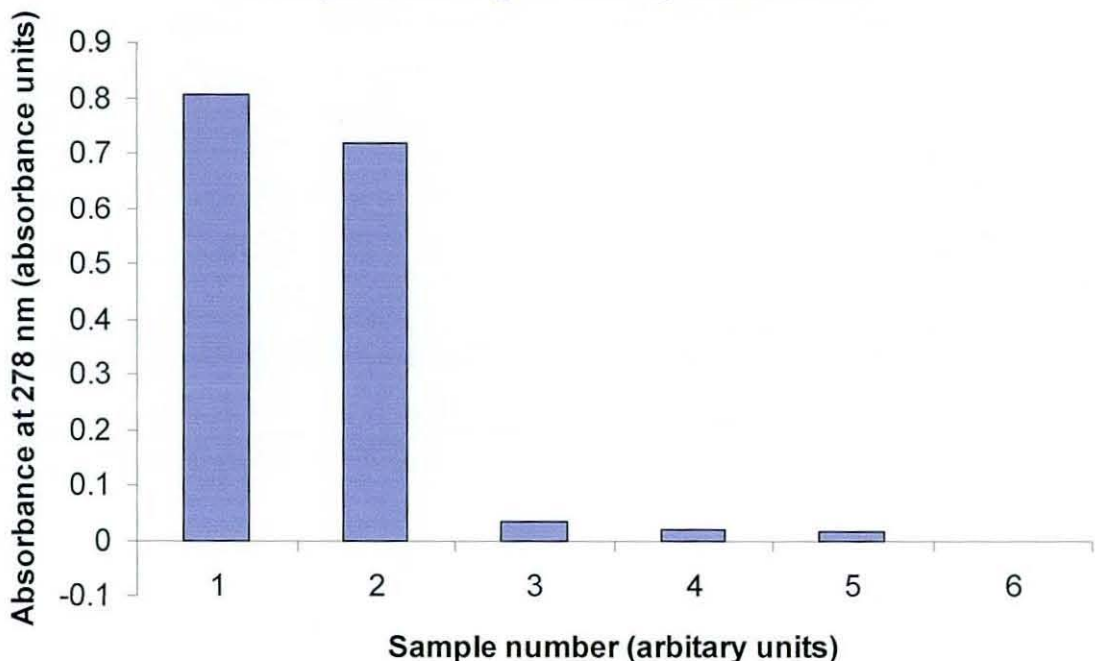
One other potential reason was that the pore sizes were too small and therefore became blocked. The average pore size was 182 Å and if it is assumed that an enzyme has a rough size of 60 Å, then actual space for the substrate to flow through becomes limited.

A combination of any of these possibilities may also have been true.

6.2.2.1 Absorbance experiments to investigate sticking of α -casein to glutaraldehyde activated CPG

An experiment was conducted to determine whether α -casein would stick to glutaraldehyde-activated CPG. Absorbance measurements were made at 278 nm using an Unicam 8700 UV / Visible Spectrometer. A standard 3 ml quartz absorbance cuvette was used and baselines were taken with buffer prior to measurements. A 0.1 % α -casein solution was made up in 0.1 M phosphate buffer pH 6.0 and a random amount of glutaraldehyde-activated beads were then added to this solution in a centrifuge tube, the mixture agitated, and then spun down using a Clandon MLW T52.1 centrifuge for 2 minutes. The liquid was then decanted off and an absorbance reading was taken. A fresh solution of 0.1 M phosphate buffer pH 6.0 was then added to the beads and the method repeated. Repetitions were made until protein was no longer detected in the buffer. The results are given in figure 6.9.

Figure 6.9 – Absorbance of 0.1 % α -casein solution before (1) and after (2) mixing with glutaraldehyde-activated CPG and subsequent readings of freshly added buffer



Comparing samples 1 and 2, it appeared that 11.28 % of the α -casein was lost. However, when fresh buffer was added and then the absorbance measured, traces of α -casein were detected, indicating that the α -casein was getting caught up in the CPG chips. After fresh buffer was added until α -casein was no longer detected, a loss of 2.85 % was found which could be attributed to experimental error. This experiment therefore showed that α -casein was not sticking to the CPG.

6.2.2.2 Blocking of free aldehyde groups using ethanolamine

Free aldehyde groups on the proteinase K – CPG without attached enzyme were blocked using ethanolamine as described in section 2.2.11. This was carried out to make sure all aldehyde groups were blocked so that substrate could not bind to any free groups. Ethanolamine was used as an alternative to Bovine Serum Albumin (BSA) or α -casein that commonly are used for blocking because these proteins would be used as a substrate by the immobilised enzyme. Tris (hydroxymethyl)amino methane (TRIS) could also have been used, but this is more bulky and so 3.0 M ethanolamine pH 9.0 was used. Sodium cyanoborohydride (NaCNBH_3) at a concentration of 10 mM was used to make the linkage permanent.

It was found, however, that blocking had little effect and BODIPY: α -casein was still retained in the enzyme reactor.

6.2.2.3 Evaluation of CPG with a larger pore size of 972 Å

CPG with a larger mean pore diameter was evaluated as a support to investigate whether pore size was limiting the flow of BODIPY: α -casein through the enzyme column. Using a larger pore size it was found that whilst 2.95 % naphthofluorescein was detected, which was still very poor, both BODIPY and BODIPY: α -casein were still retained within the column. This suggested that something other than pore size was responsible, although the result with naphthofluorescein was a slight improvement.

6.2.2.4 Evaluation of shorter enzyme reactors

The 5 cm long glass columns that had been used up to this point were replaced with 2.5 cm long columns to see whether this would affect the amount of material that was retained. Columns were packed with blocked 8 mg proteinase K – 972 Å CPG and injections of the dyes naphthofluorescein, Cy5, and BODIPY were made as well as injections of BODIPY: α -casein and α -casein.

As shown in figures 6.10 to 6.14, the use of a shorter column made a noticeable difference. When naphthofluorescein, Cy 5, and α -casein were injected, all were detected. When BODIPY and BODIPY: α -casein were injected, nothing was detected.

Figure 6.10 – Injection of naphthofluorescein with and without 2.5 cm column

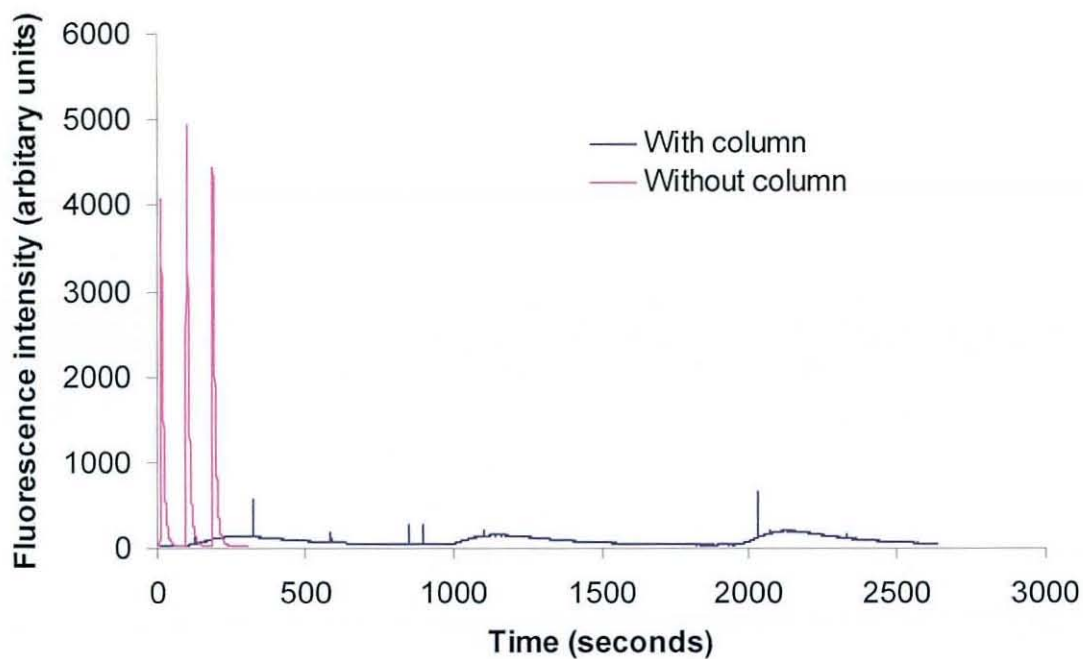


Figure 6.11 – Injection of Cy5 with and without 2.5 cm column

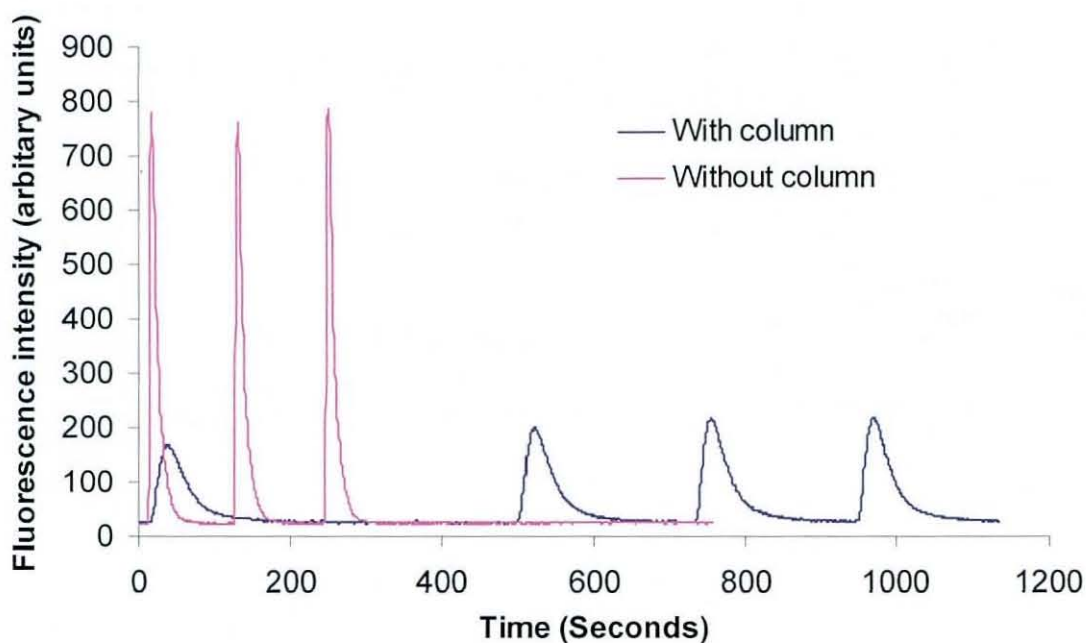


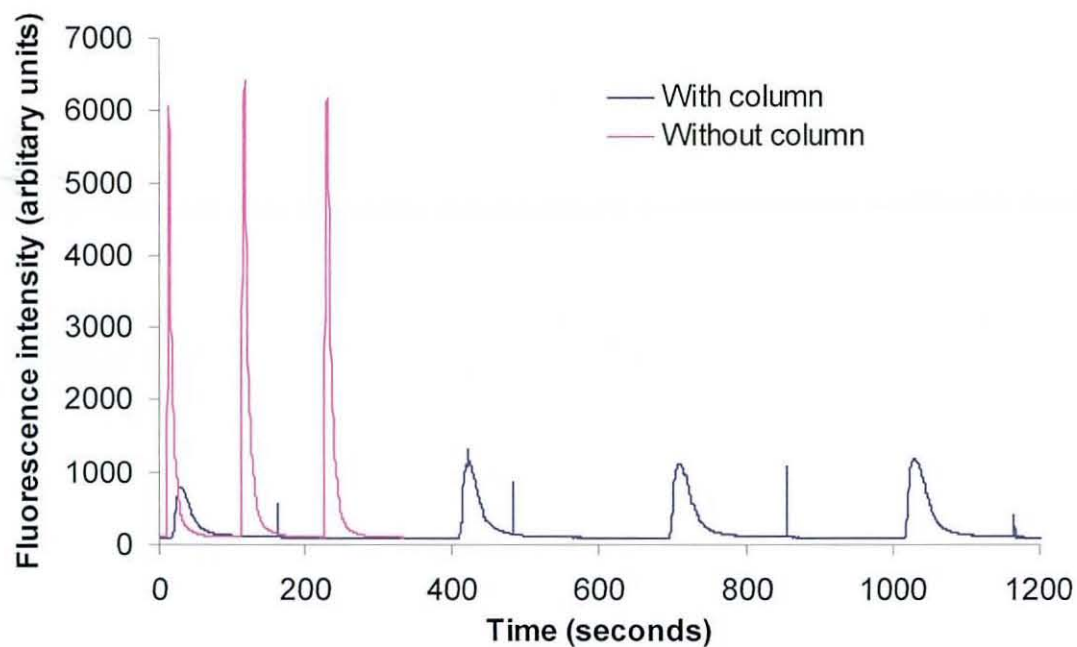
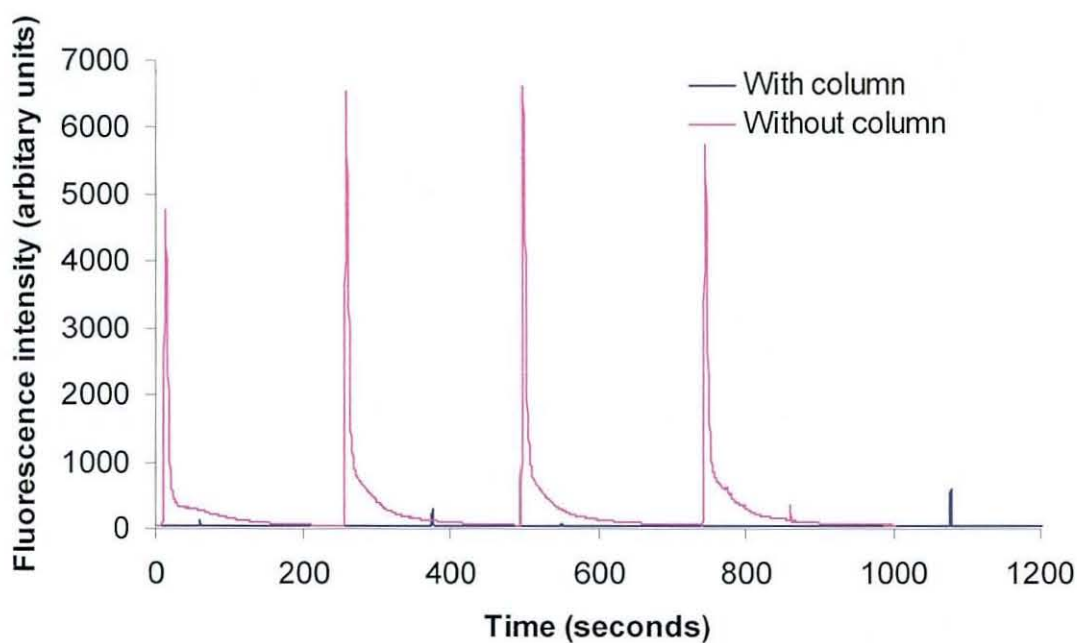
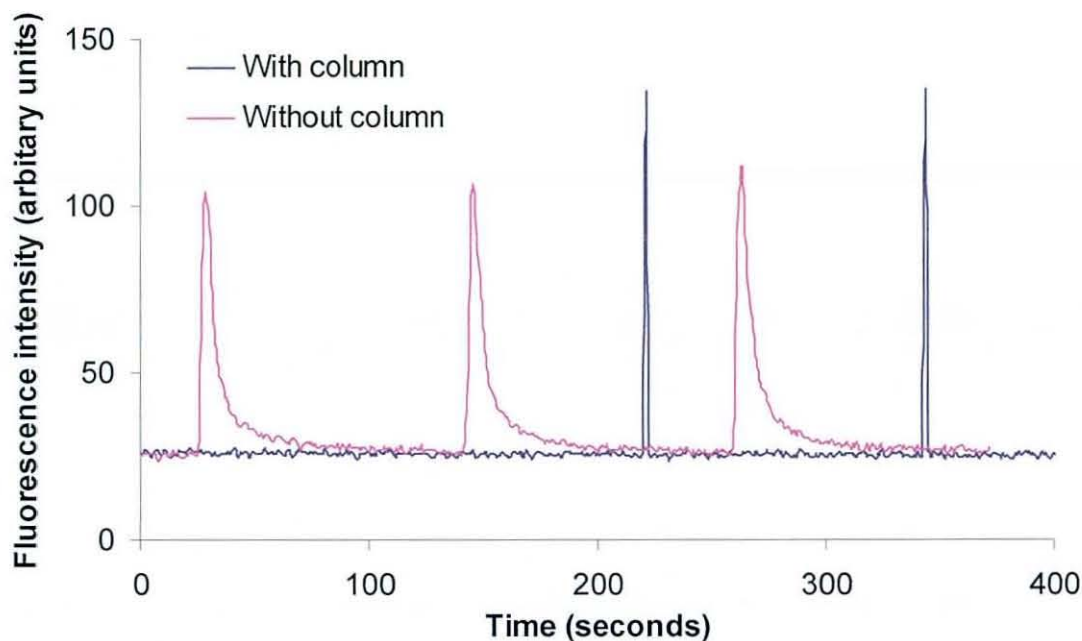
Figure 6.12 – Injection of α -casein with and without 2.5 cm column**Figure 6.13 – Injection of BODIPY with and without 2.5 cm column**

Figure 6.14 – Injection of BODIPY: α -casein with and without 2.5 cm column



These results suggested that for some reason the BODIPY fluorophore was sticking to the CPG retaining both the fluorophore and the conjugate, since α -casein flowed through freely. BODIPY, as described in section 1.1.4.3, is relatively non-polar whereas both naphthofluorescein and Cy 5 are polar molecules. It is therefore possible that the polarity of the molecule influences its ability to flow through the CPG. One possibility is that polar molecules dissolve in the buffer more easily and are pulled through the column in solution. However, BODIPY with no ionic charge may stick to the CPG instead of being pulled along by the buffer.

The use of CPG as a support in these assays has proved to be incompatible with the BODIPY fluorophore. CPG was therefore not pursued as a support for immobilisation.

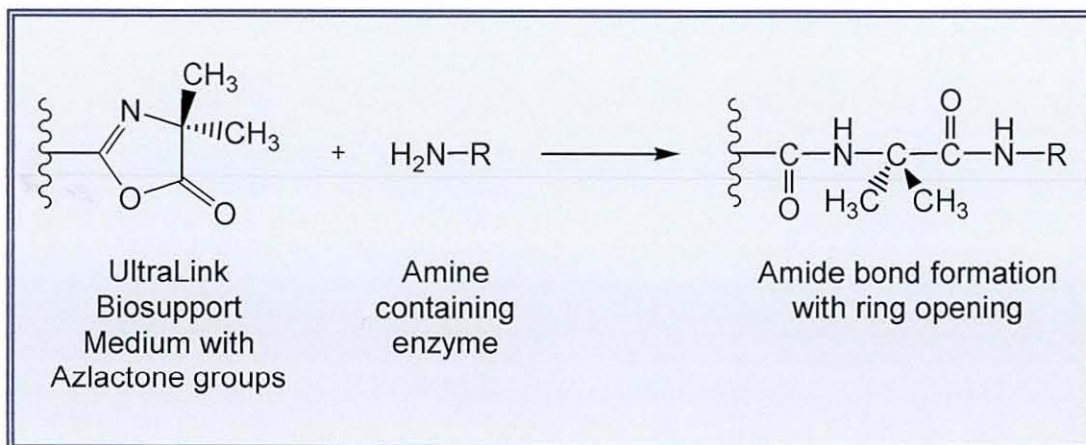
6.3 MULTI-DETERMINATION PROTEASE INHIBITION ASSAYS USING PIERCE ULTRALINK MEDIUM AS A SUPPORT

6.3.1 Procedures

UltraLink™ Biosupport Medium, 0.25 g (approximately 2 ml of gel), was purchased from Pierce Chemical Company to investigate whether this medium could be used as an alternative to controlled-pore glass for immobilisation of proteolytic enzymes and incorporation into a glass column. The immobilisation of enzyme onto the UltraLink Medium was carried out according to the manufacturer's instructions and is described in section 2.2.13. An 8 mg / ml solution of proteinase K (lot 90K8604, 11.3 Units / mg) was used for immobilisation onto 0.1254 g UltraLink Medium. A quench solution of 3.0 M ethanolamine pH 9.0 was used to block unreacted azlactone sites.

Pierce UltraLink Biosupport Medium is a preactivated, ready-to-use bead with a bis-acrylamide / azlactone copolymer composition. The copolymer is slightly hydrophobic and highly crosslinked and because the azlactone functionality is copolymerised with the matrix material, the binding capacity is an integral part of the bead. This results in a high functionality throughout the porous bead matrix. The support rapidly reacts with nucleophiles, allowing coupling to a wide range of proteins. The beads have an average particle size of 50 – 80 µm in diameter with a pore size of 1000 Å and a functionality of 250 µmoles per g of beads. The matrix is stable over a pH range of 1 – 13 and due to the rigid polymeric nature of the support, it has excellent utility in medium to low pressure applications. This support was thus chosen for these attractive features and the ease of use. The reaction scheme for enzyme immobilisation is given below in figure 6.15.

Figure 6.15 – Reaction scheme for the immobilisation of enzyme onto UltraLink Biosupport Medium



The amount of proteinase K actually immobilised onto the Biosupport Medium was determined by measuring the amount of proteinase K left in the filtrate using the Bicinchoninic Acid (BCA) protein assay, as described in section 2.2.14. The use of Triton® X-100 surfactant in the bead production could have interfered with an assay using absorbance at 280 nm for the measurement of uncoupled enzyme. Hence, the BCA protein assay was used. By subtracting the amount of proteinase K left in the filtrate from the total amount of proteinase K added, the amount of proteinase K actually immobilised onto the support could be deduced.

Evaluation of the proteinase K immobilised onto UltraLink was carried out in a flow injection analysis system similar to that depicted in figure 6.1, but using a single stream with one packed column. A flow rate of 1.06 ml / min was used with 20 mM carbonate pH 9.5 as the buffer system at room temperature. An Omnifit glass column 5 cm in length with a reactor volume of 350 μl (figure 6.5) was used with 2 μm aluminium frits. Columns were packed as described above in section 6.2.1.

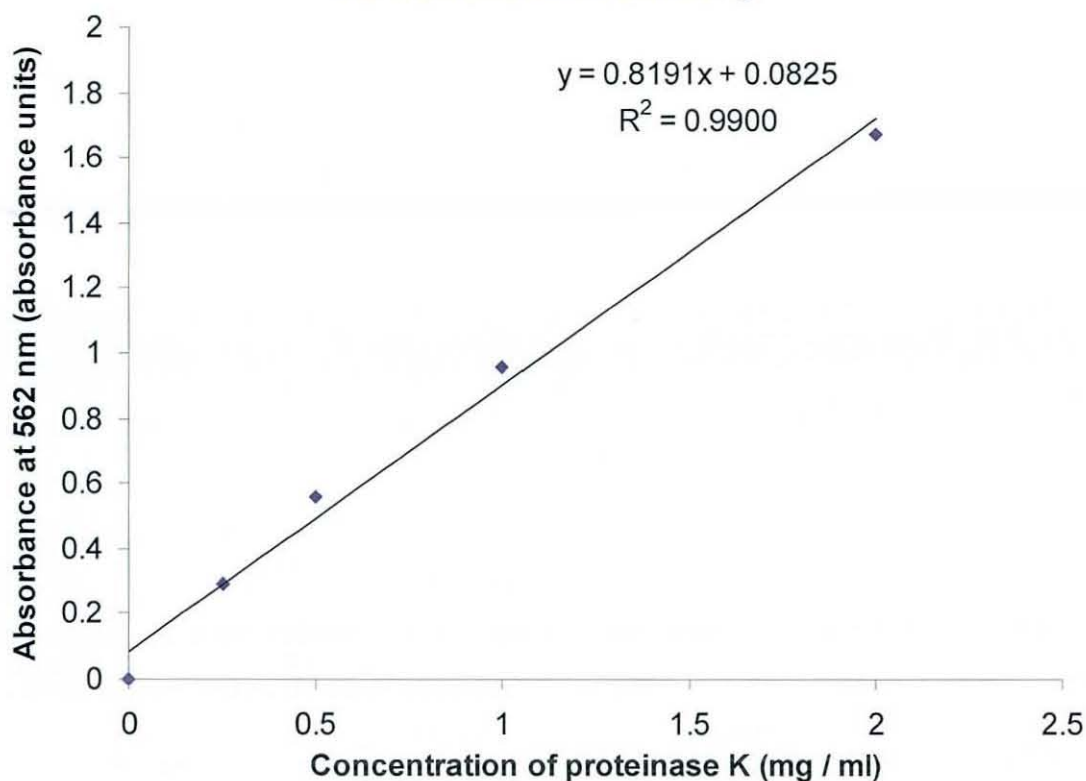
All fluorescence measurements were made using an F-4500 fluorescence spectrophotometer with excitation and emission bandwidths of 10nm. Time scans were performed with excitation and emission wavelengths of 605 nm and 644 nm for BODIPY solution and BODIPY: α -casein conjugate, and 280 nm and 350 nm for α -casein solution.

6.3.2 Results

6.3.2.1 Determination of amount of proteinase K immobilised onto UltraLink Biosupport Medium

The BCA assay was used to calculate the amount of proteinase K left in the filtrate after the immobilisation procedure had been carried out. A set of standard proteinase K solutions of concentrations 0.25 mg / ml, 0.50 mg / ml, 1.00 mg / ml, and 2.00 mg / ml was used to generate a calibration graph. As shown in figure 6.16, the calibration graph showed good linearity over this concentration range with an R^2 of 0.9900. The absorbance of the unknown proteinase K solution was 1.064 absorbance units. By substituting this absorbance into the equation of the calibration graph, the amount of proteinase K left in the filtrate was determined. Hence by deduction from the total amount of proteinase K used, the amount of proteinase K immobilised onto the UltraLink Biosupport Medium was deduced. This was 13.603 mg proteinase K, which corresponds to a coupling efficiency of 85 %.

Figure 6.16 – Calibration graph of proteinase K concentration using the Bicinchoninic Acid assay



6.3.2.2 Evaluation of UltraLink as a support for multi-determination protease inhibition assays in a flowing system

Injections of BODIPY, BODIPY: α -casein, and α -casein were made through the column containing proteinase K immobilised onto UltraLink in order to determine whether these materials would flow through the column. Injections were also made through an empty column as a blank to compare the results. As shown in figures 6.17 to 6.19, all three compounds were able to flow through the column freely.

Figure 6.17 – Injection of BODIPY through both an empty column and a column packed with proteinase K - UltraLink

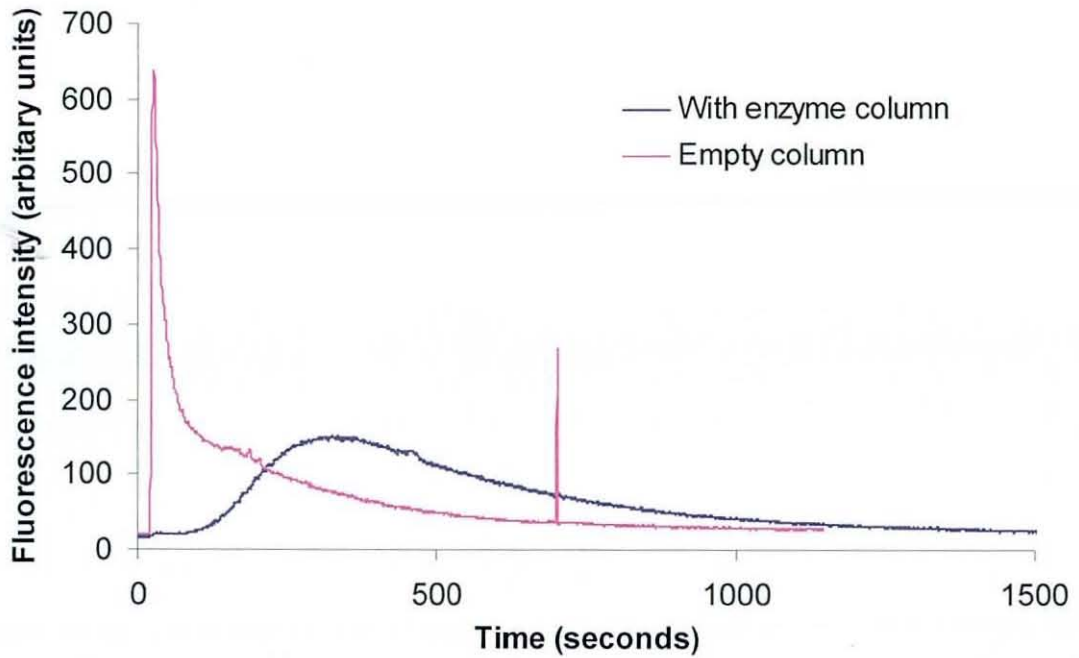


Figure 6.18 – Injection of BODIPY: α -casein through both an empty column and a column packed with proteinase K - UltraLink

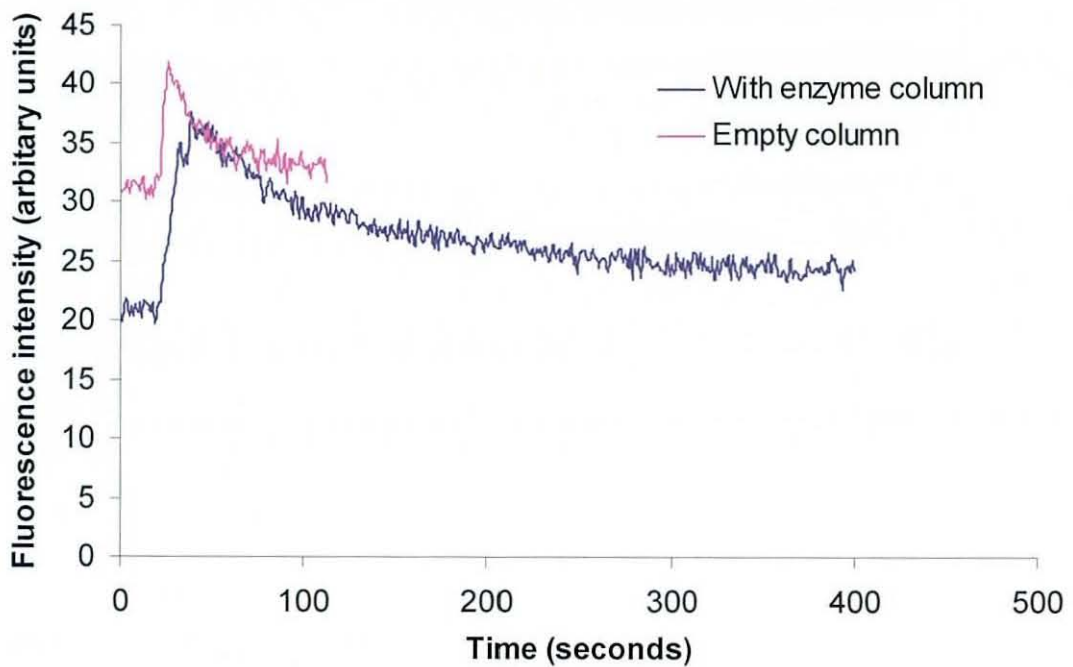
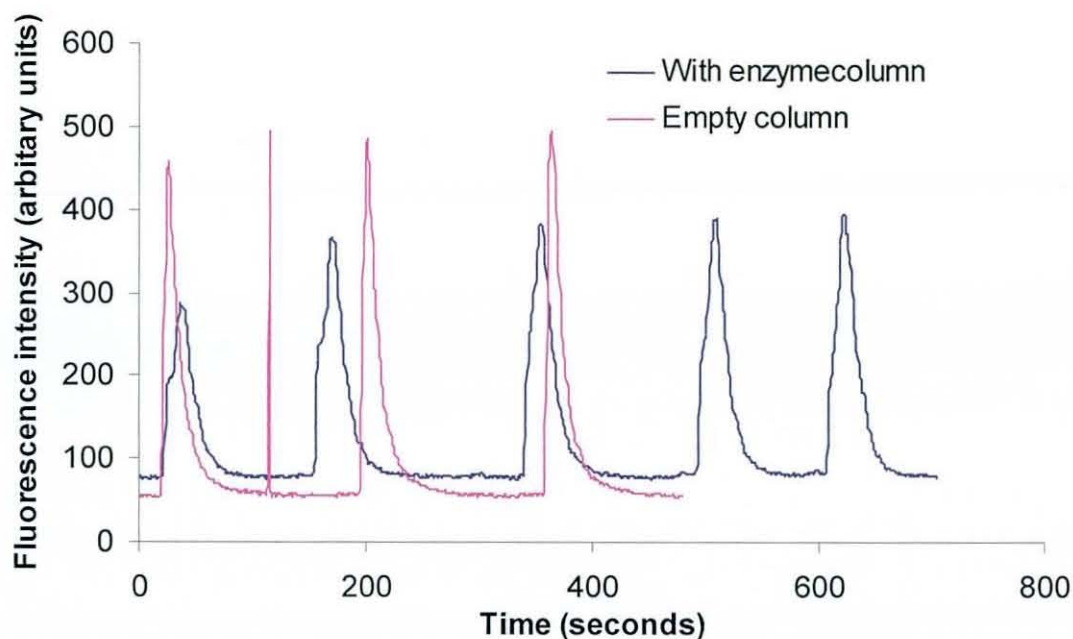


Figure 6.19 – Injection of α -casein through both an empty column and a column packed with proteinase K - UltraLink



As peak broadening occurred, the peak areas were compared in order to calculate the percentage of fluorescent material that was flowing through the UltraLink Medium compared with that flowing through the empty column. These percentages are given in table 6.1.

MATERIAL	AMOUNT FLOWING THROUGH ULTRALINK COLUMN (%)
BODIPY	118
BODIPY: α -CASEIN	369
α -CASEIN	107

Table 6.1 – Amount of different materials flowing through a packed proteinase-UltraLink column compared with an empty column

It is apparent from table 6.1 that BODIPY, BODIPY: α -casein, and α -casein were all capable of flowing through the column containing proteinase K immobilised onto UltraLink Biosupport Medium. This was a huge improvement when compared to controlled-pore glass and these initial results with UltraLink were extremely promising. It also certainly confirms that a BODIPY-CPG interaction was responsible for the problems that were found when using CPG.

It can be seen that both BODIPY and α -casein flow through the medium freely with all the material being detected. The percentage of material flowing through the packed column was derived by comparing the peak areas obtained through packed and empty columns. However, peak areas were calculated using the F-4500 software, which does not give an absolutely accurate value. This may account for the fact that the percentages for BODIPY and α -casein are slightly above 100 % when in theory a maximum of 100 % should have been recorded.

The fact that 369 % BODIPY: α -casein appeared to flow through the packed column is very encouraging. This suggested that not only was BODIPY: α -casein able to flow through this support, unlike controlled-pore glass, but that the conjugate was being digested by the immobilised proteinase K into highly fluorescent peptides.

These preliminary results with UltraLink Biosupport Medium indicate that this support is more conducive than controlled pore glass for use with BODIPY: α -casein. No sticking of either dye or protein to UltraLink was observed.

Unfortunately, as time did not permit, these experiments were not continued and would therefore constitute further work. Nevertheless, on the basis of the preliminary results presented here, it seems logical to suggest that the application of this medium to the set-up shown in figure 6.1 would produce an effective multi-determination protease inhibition assay for high throughput screening.

6.4 MULTI-DETERMINATION PROTEASE INHIBITION ASSAYS USING SOLUBLE ENZYME

As lack of time prevented further investigation into the use of UltraLink Medium in flow-based multi-determination protease inhibition assays, the use of a soluble enzyme system was very simply demonstrated in order to show the principle behind these multi-determination assays. On this kind of macro-system, the soluble method would not be preferred due to the high amounts of enzyme required and the level of complexity of the flow system when compared to that of the flow system incorporating immobilised enzymes.

6.4.1 Procedures

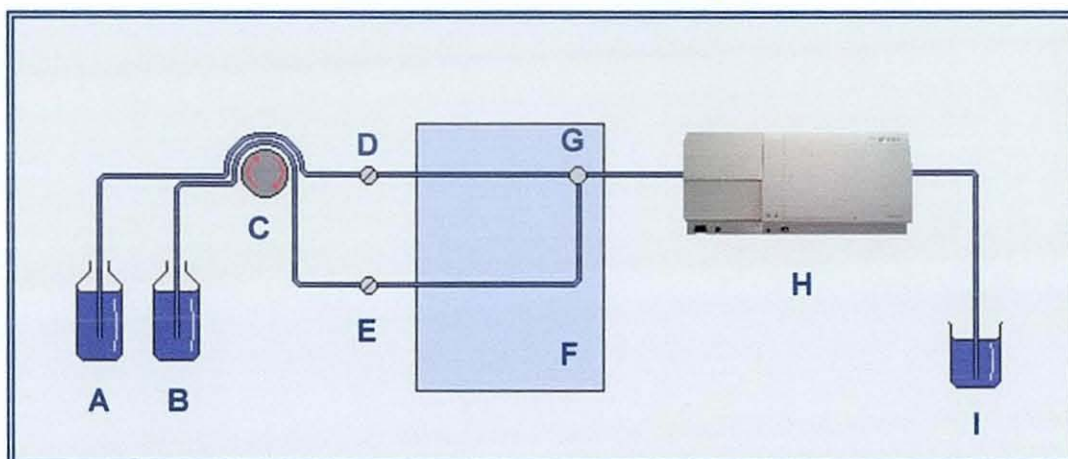
All fluorescence measurements were made using an F-4500 fluorescence spectrophotometer with excitation and emission bandwidths of 10 nm. Time scans were performed with excitation and emission wavelengths of 605 nm and 644 nm respectively.

For these assays pepsin and proteinase K were chosen to represent two enzymes with pH extremes and from different classes: proteinase K is a serine protease working at pH 9.5, whereas pepsin is an aspartate protease working at 2.0. Pepstatin A was chosen as the model inhibitor as it is active over a wide pH range and so would work in either buffer system.

Enzyme solutions were made up by dissolving enzyme into 500 ml of the optimised buffer systems for each enzyme. 0.50 mg / ml pepsin was made up in 20 mM glycine pH 2.0, and 0.02 mg / ml proteinase K in 20 mM carbonate buffer pH 9.5. N₂ was then bubbled through each of the enzyme solutions to remove O₂. Assays were carried out at 35 °C using a thermostatted water bath.

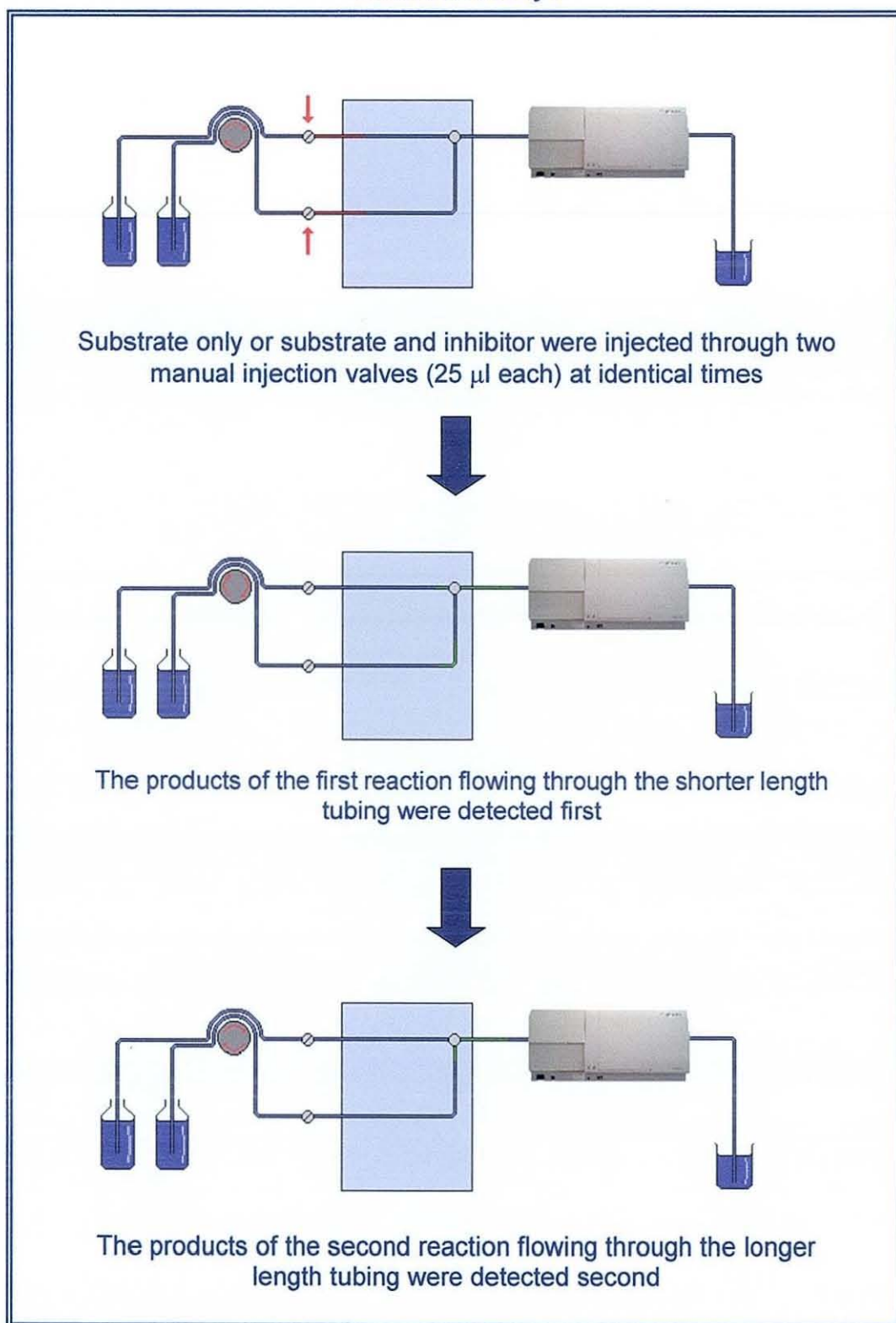
The flow set-up that was used for these experiments is shown in figure 6.20 and the individual steps that were carried out are depicted in figure 6.21.

Figure 6.20 – Schematic of flow system used for soluble multi-determination assays



(A = enzyme solution 1, B = enzyme solution 2, C = peristaltic pump, D = substrate or substrate / inhibitor injection valve 1, E = substrate or substrate / inhibitor injection valve 2, F = thermostatted water bath, G = T-junction, H = fluorescence detector, I = waste)

Figure 6.21 – Sequence of events for multi-determination protease inhibition assays



6.4.2 Results

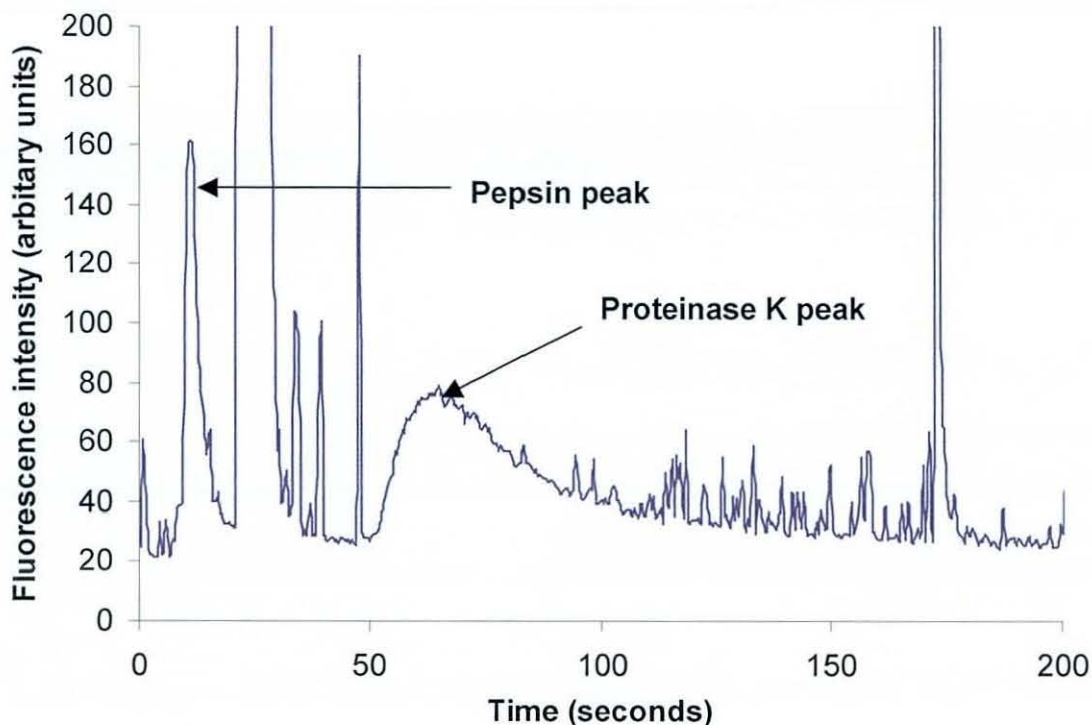
6.4.2.1 Determination of length of flow tubing required for separation of peaks

As the principle behind this multi-determination assay was to inject two lots of substrate with or without inhibitor and detect the two lots of product formed in each of the enzyme streams by time-dependent separation, it was important to determine the length of tubing required to cause such a separation. The pepsin enzyme solution was flowed through the short length of tubing whereas proteinase K was flowed through the longer length of tubing. The consequence of this was that the first detected peak was due to the pepsin component and the second peak was due to the proteinase K component. The tubing that was used for these experiments was Omnifit Teflon tubing with an internal diameter of 0.8 mm. As a minimum length of tubing was required for the shorter length pepsin section, a length of 25 cm was used between the injection valve connector and the T-junction. This was chosen so that the assay components would flow through the thermostatted water bath before detection and to cover the physical distance between the injection valve and T-junction. Using this length of tubing, the time between injection and detection was 9 seconds.

A longer length of tubing of 65 cm for the proteinase K part was firstly added. However, using this length a time of 16 seconds was observed between injection and detection, which was too short for peak separation. In order for a separation of the peaks to occur the first peak has to return to the baseline first, so sufficient time must be allowed for this to occur. A length of 355 cm was then added which equated to a time of 45 seconds between injection and detection. This length was enough to separate the two peaks, but the first peak had not quite reached the baseline properly. This length was therefore increased to 399 cm, which allowed a 50 second separation, and the two peaks were properly resolved as

demonstrated by figure 6.22. For further experiments therefore a shorter length of 25 cm and a longer length of 399 cm were used.

Figure 6.22 – Separation of two enzyme peaks using 25 cm and 399 cm length tubing

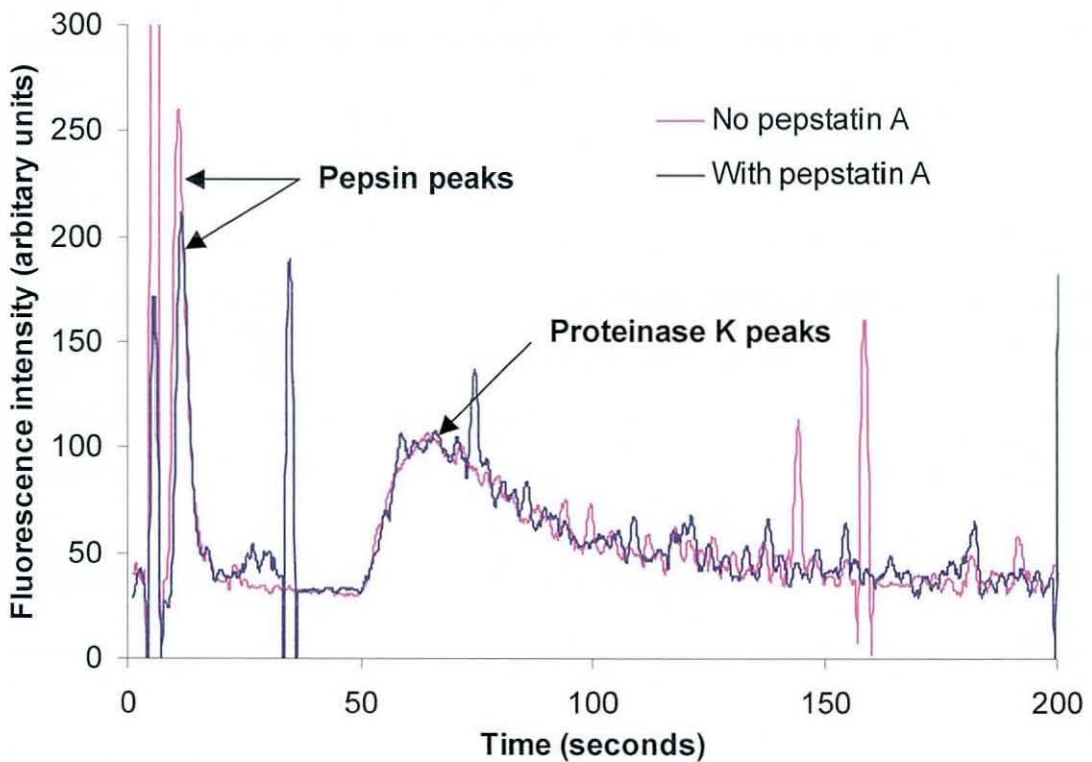


The length of tubing that was required for separation of the two peaks was not ideal. The length of 399 cm had to be coiled for placement in the water bath. This soluble set-up would be much more applicable to a miniaturised system where such long lengths would not be needed.

6.4.2.2 Multi-determination protease inhibition assay

As time was very short the idea of this investigation was to demonstrate the principle, so the results that are presented here are of a qualitative nature rather than quantitative. Triplicate injections of substrate in the absence and presence of pepstatin A were made using the system shown in figure 6.20 with tubing lengths as described in section 6.4.2.1. The time scans showing an example of these injections are given in figure 6.23.

Figure 6.23 – Multi-determination using two proteolytic enzymes – pepsin and proteinase K – and the selective inhibition of these enzymes by pepstatin A



From figure 6.23 it can be seen that the effect of an inhibitor on two proteolytic enzymes has been determined in the same assay. From the pepsin peaks it is evident that inclusion of pepstatin A inhibited pepsin, signified by the reduction in fluorescence intensity. However, if the proteinase K peaks are examined, these are identical whether pepstatin A was present or not. This indicated that pepstatin A did not inhibit proteinase K. This was as expected as pepstatin A is an aspartate protease inhibitor.

Using the substrate BODIPY: α -casein it has been shown that multi-determination assays using the described system were possible and these assays have been used to show the selective inhibition of two proteolytic enzymes by pepstatin A. Not only two proteolytic enzymes, but two enzymes that operate at opposite pH values – pH 2.0 for pepsin and pH 9.5 for proteinase K. In addition these enzymes represented two different classes of protease – pepsin being an aspartate protease and proteinase K being a serine protease. This system would be suitable for the high throughput screening of compounds against more than one target enzyme.

6.5 DISCUSSION

The suitability of applying the long-wavelength fluorogenic substrate BODIPY: α -casein to multiplexed protease and protease inhibition assays has been investigated. The use of immobilised enzymes in these assays much simplifies the flow system needed, and due to the advantages of immobilised enzymes as discussed in section 1.7.1, it was decided that immobilisation of proteolytic enzymes to a suitable support was required. However, studies on the use of controlled-pore glass as a support were unsuccessful due to an incompatibility with the BODIPY fluorophore. It appeared that BODIPY, which has no ionic charge, stuck to the support thereby retaining the fluorophore within the enzyme reactor. One hypothesis on the cause of this sticking was based on polarity. It was found that polar dyes flowed through the CPG column freely, and it was thus postulated that the more polar the fluorophore, the less sticking would occur. This possibly may have been due to polar molecules dissolving into solution with more ease.

An alternative support was explored for immobilisation of enzymes and subsequent use with BODIPY: α -casein. Pierce UltraLink Biosupport Medium was chosen for the reasons discussed and was shown to be extremely promising for use in these assays. Enzyme was successfully immobilised onto this medium and UltraLink was shown to support the flow of BODIPY: α -casein. Initial results also suggested that as BODIPY: α -casein flowed through the enzyme reactor, the immobilised enzyme cleaved the substrate into fluorescent peptides. These results were extremely encouraging and promising and with more time this system could potentially be developed for multi-determination. This would constitute future work.

Soluble enzyme experiments were carried out as a proof of principle determination and it was shown that BODIPY: α -casein could be used as a pH-independent substrate for use in flow-based multiplexed screening programmes. However, this format was less favourable than that using immobilised proteases, as the system was more complex. In addition, due to the high concentrations of enzyme needed and the long lengths of tubing that were required for peak separation, this soluble format would be much more suited to a miniaturised system. The application of these assays to a chip-based system would comprise future work and will therefore be discussed as such. However, further work on the effects of flow rate ought to be carefully studied. At a lower flow rate the dispersion of the zones will be less and they will overlap less, so it may be possible to have shorter tubes for the second enzyme. In that case it might be possible to get more samples per hour through even though the flow rate has been reduced.

CHAPTER 7

Conclusions and future work

7.1 CONCLUSIONS

The objective of this investigation was to develop new fluorescence methods for the rapid and sensitive determination of proteolytic enzymes and their inhibitors. In addition, the possibilities of applying these enzyme and inhibition assays to a flow injection analysis system and the development of multi-determination assays were also to be examined. These assays were aimed at a high throughput screening application.

To a large extent these objectives have been successfully fulfilled. Fluorescence polarisation and energy transfer methods have both been explored for use in protease and protease inhibition assays. Of these two approaches, energy transfer methods have proven superior. As discussed in section 3.4, this is of no great surprise as inherent problems in polarisation methods include (a) polarisation filters reducing exciting and emitted light intensities; (b) polarised light pre-selecting only a fraction of the potentially excitable molecules; and (c) polarisation changes being small and temperature sensitive. It was found that for fluorescence polarisation assays a lightly labelled substrate was required, and these offer an advantage due to less steric hindrance of the proteolytic enzyme over those heavily labelled substrates used in energy transfer methods. These substrates using fluorescence polarisation have successfully been used to analyse different proteases with broad specificity and fluorescence polarisation has the additional benefits of simplicity and relative immunity to the inner filter effect.

However, these pros of fluorescence polarisation are outweighed by the fact that the fluorescence polarisation changes are very small which greatly limits the sensitivity of the assay. The pH sensitivity of fluorescein as a label was also extremely limiting as easy analysis of acid proteases was prevented. The use of

long wavelength fluorophores, which are usually preferred due to the decrease in background fluorescence found within the long wavelength region (ca. 600 – 900 nm), was also unsuccessful.

The usefulness of this technique was further limited by the requirement of a cuvette-based approach, with the associated low throughput and high reagent usage

Energy transfer methods have been investigated in two different formats (a) a single fluorophore used as an excess label attached to a protein substrate, with proteolytic action fragmenting it and producing enhanced fluorescence through abolition of internal energy transfer, and (b) use of two fluorophores attached to one substrate, with energy transfer from one to another and proteolytic cleavage causing a fluorescence enhancement through disruption of the energy transfer.

Double-labelled energy transfer methods have been investigated using the donor / acceptor pairs of FITC / TMRITC and Cy 5 / Cy 5.5. One major advantage that this method possesses over the single-labelled energy transfer methods is that intensity ratio measurements are made using double-labelled substrates, and so instrument fluctuations will largely cancel out. The use of two fluorophores should also produce a larger Stokes shift, helping to solve a real problem in long wavelength measurements.

For reasons discussed in chapter 4, it was found in the double-labelled section of work that lightly labelled donor and heavily labelled acceptor conjugates were required for use in FRET protease assays.

FITC / TMRITC, although promising in theory, were found not to be perfect. The phenomenon of energy transfer was shown to exist in these conjugates, however the results were extremely difficult to interpret and by no means ideal for use in protease assays. Initial results with Cy 5 and Cy 5.5 as donor and acceptor

respectively were unsuccessful as the two emission peaks were unable to be resolved. However, Cy 5 and Cy 5.5 have previously been shown to be a good donor / acceptor pair [287, 288].

It is obvious that the synthesis of double-labelled substrates is more complex both to investigate and to achieve in routine practice than single-labelled substrates, although with more work spent this energy transfer method may give advantages over other energy transfer methods.

The use of a single excess-labelled substrate has, by far, been the most successful fluorescence method for the determination of proteolytic enzymes and inhibitors. A substrate utilising α -casein labelled with a long wavelength BODIPY dye has been synthesised. The relatively low molar ratio of dye to protein used in this work provides an ideal substrate for proteolytic enzymes as worries that the use of highly derivatised molecules might sterically hinder the activity of some proteases are minimised. In addition, BODIPY dyes have no overall charge, so dye conjugation should not greatly affect the protein substrate conformation and enzyme-active site interactions.

This substrate has been shown to be a suitable intramolecularly quenched conjugate for the determination of proteolytic enzymes and their inhibitors in flow-based screening programmes. The application of BODIPY: α -casein to the determination of different proteolytic enzymes with varying pH optima (ranging from pH 2.0 to 9.5) has been demonstrated. The suitability of BODIPY: α -casein as a substrate in enzyme inhibition assays has also been investigated. Limits of detection of 2.50 μ M, 0.14 μ M, 7.20 nM, and 0.161 nM were obtained for 3-nitrophenylboronic acid, leupeptin, pepstatin A, and aprotinin respectively. The assays are rapid, taking at most 2 minutes for the enzyme assay and 5 minutes for the inhibition assays. The flow-based procedure that has been described is particularly well suited to the high throughput screening of candidate drug molecules.

The progression of this work was to aim towards the adaptation of the flow-based procedure to a set-up that would allow the multi-determination of proteolytic enzymes and their inhibitors. This would further improve the throughput of the system – an essential requirement of high throughput screening. As time was short, only the feasibility of this set-up was explored. The use of immobilised enzymes was investigated, as this far simplified the set-up required. Two different supports were studied – controlled pore glass (CPG) and Pierce UltraLink Biosupport Medium. It was found that CPG was unsuitable for use with the BODIPY fluorophore, thereby preventing its use in these assays. BODIPY was found to stick to the CPG resulting in the fluorophore being retained within the enzyme column. This was possibly related to the ionic charge as polar dyes were found to flow freely through the column whereas the non-polar BODIPY molecule did not.

Pierce UltraLink Biosupport Medium was therefore investigated as an alternative to CPG and was shown to be extremely promising as a support in these assays. Enzyme was successfully immobilised onto this medium and UltraLink was shown to support the flow of BODIPY: α -casein. Initial results also suggested that as BODIPY: α -casein flowed through the enzyme reactor, the immobilised enzyme cleaved the substrate into fluorescent peptides. With more time this system could potentially be developed for multi-determination.

For proof of principle soluble enzyme experiments were carried out. However, this format was less favourable than that using immobilised proteases, as the system was more complex. In addition, due to the high concentrations of enzyme needed and the long lengths of tubing that were required for peak separation, this soluble format was much more suited to a miniaturised system.

7.2 FUTURE WORK

7.2.1 Fluorescence polarisation

Although fluorescence polarisation was found to be less sensitive than energy transfer methods, polarisation does possess several attractive features. The cuvette-based approach hampered the usefulness of this technique due to the associated low throughput and high reagent usage. A flow-based system would improve this throughput significantly. It would therefore be extremely useful to transfer the assay to a flow injection analysis system similar to that described for the quenched substrate, if only to test the sensitivity of polarisation measurements in systems where small volumes are illuminated and observed in the instrument.

Another area that would be worth pursuing is the use of BODIPY and other long wavelength fluorophores. Experiments using BODIPY: α -casein with fluorescence polarisation were unsuccessful. However, these substrates were highly quenched, causing significant depolarisation through non-radiative energy transfer and thus severely limiting the assay span and sensitivity. A study of much more lightly labelled substrates containing little or no quenching and their applicability to fluorescence polarisation may prove to be more promising. In addition assessment of other long wavelength fluorophores would be useful. For use in fluorescence polarisation the dye requires a suitable fluorescence lifetime for appreciable Brownian rotation to occur. There are worries about the extent of polarisation effects with short-lifetime long-wavelength fluorophore molecules (section 1.2.1.3). This may be one of the few areas where long-wavelength fluorophores are not very useful. However, if a suitable long-wavelength fluorophore could be found this could prove valuable.

7.2.2 Double-labelled substrate

As double-labelled substrates possess potential advantages over other energy transfer methods, more work in this area could be fruitful. It is evident that much more work is required to determine the optimum donor and acceptor ratios required for the substrate to be used as a protease substrate. The use of Cy 5 and Cy 5.5 as donor and acceptor respectively would be worth further study. The effect of bandwidth is one experimental parameter that could be investigated. The use of narrower bandwidths would result in less direct acceptor excitation, although presumably at the expense of sensitivity. Laser excitation may therefore be more promising as presumably laser excitation would allow less direct excitation of the acceptor along with more sensitivity.

7.2.3 Single-labelled substrate

This type of assay has been most suited for the determination of proteolytic enzymes and their inhibitors for use in flow-based screening programmes. In addition to the further work on multi-determination that needs to be carried out, which is described in section 7.2.4, the enhancement of the operation of the flow-based procedure described should be considered. Computer-controlled valves could replace the manually operated switching and injection valves, thereby providing a semi-automated system.

7.2.4 Multi-determination

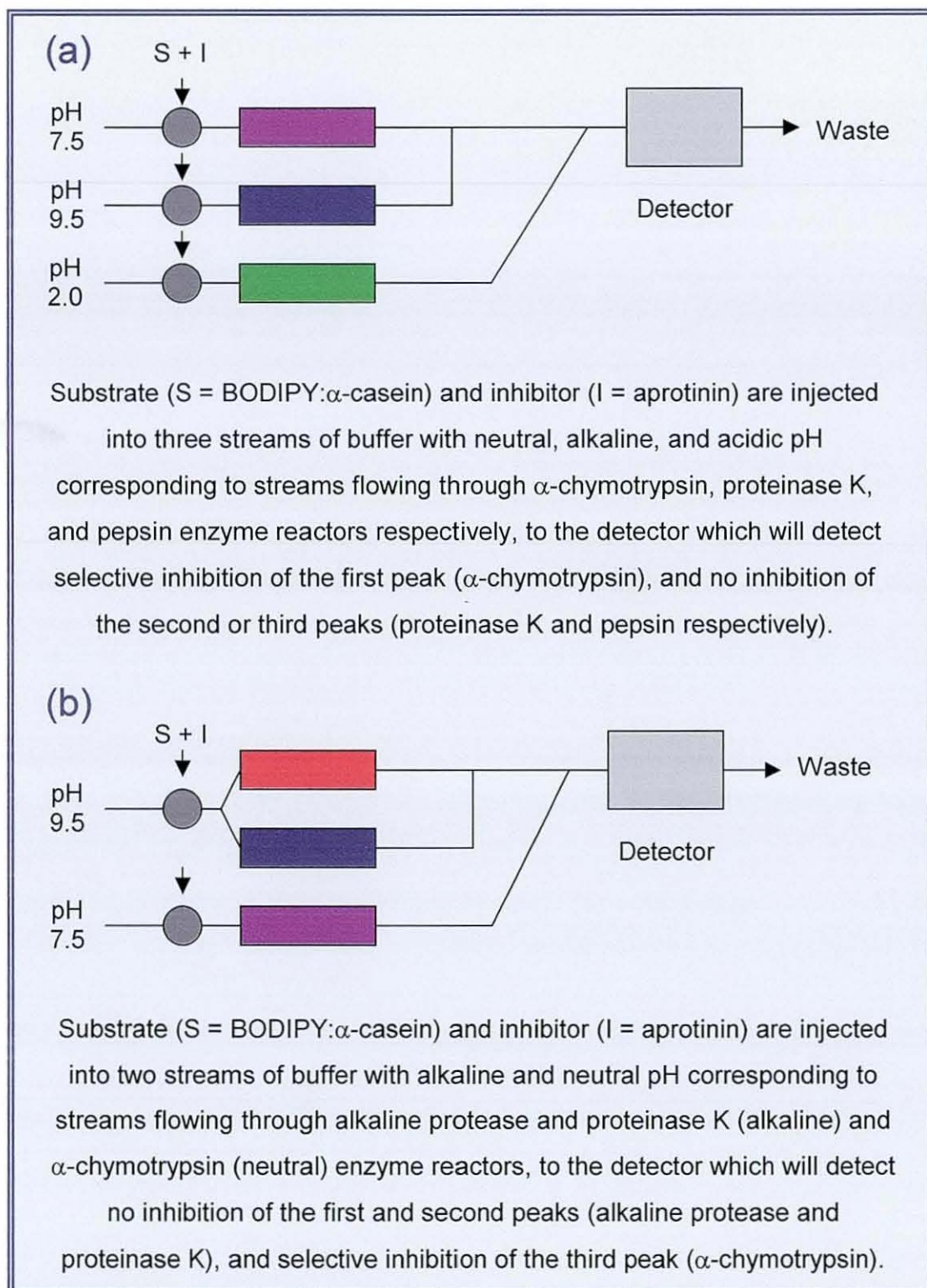
Multi-determination allows a higher throughput and thus is extremely important in the future development of the single-labelled substrates for high throughput screening. The work that was carried out showed that Pierce UltraLink

Biosupport Medium was extremely promising as a support for protease immobilisation. To begin, enzyme columns containing this support could be integrated into the proposed dual inhibition assay described in section 6.2.1 and depicted in figure 6.1. Using the proteolytic enzymes and inhibitors from the assays described in chapter 5, numerous set-ups are possible. Figure 7.1 illustrates just a couple of examples of possible set-ups out of the many combinations that are possible.

Numerous other set-ups are possible for multi-determination and various other supports are available for enzyme immobilisation. Much more work can therefore be carried out in the area of multi-determination.

However, although parallel channels allow multi-determination, which in turn produces a greater throughput of samples, the current macro-system has a limit. It would be impractical to keep paralleling up because of the amount of tubing that would be required and the number of injection valves needed. This is particularly problematic when carrying out the assays manually. Space requirements would also increase, as well as the cost associated with high reagent consumption. This macro system therefore becomes limiting. The designs of such assays are much more suited to miniaturised assay formats, and hence a discussion of the transferral of these protease and protease inhibition assays to a miniaturised system is given.

Figure 7.1 – Possible set-ups for multi-determination assays: (a) one inhibitor, three enzymes with three different pH optima, (b) one inhibitor, three enzymes with two different pH optima



7.2.5 Miniaturised protease and protease inhibition assays

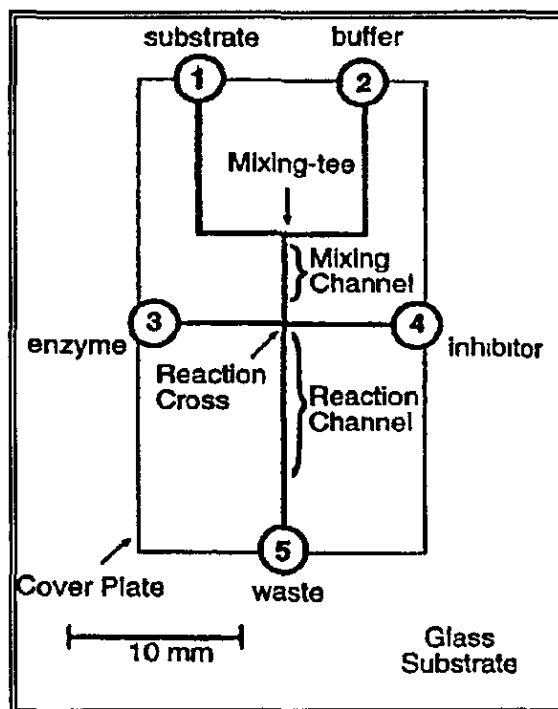
Section 1.5.1 discussed the pressures that currently face drug discovery companies and the role of HTS to harness the discovery potential of large numbers of compounds efficiently, by reducing time and cost and increasing information gained. The need to minimise the cost of screening has been addressed primarily by reducing the volume of sample to be screened, which, in turn, has resulted in the miniaturisation of HTS technology as a whole.

The benefits of the miniaturisation of HTS were discussed in section 1.5.4, and due to the potential improvements that could be achieved by miniaturising the protease and protease inhibition assays, this seems an obvious step in their continuing development.

Many discussions on the general concepts and techniques, and the background of the emergence of this technology can be found [298 - 306].

Chip-based systems have been described previously for enzyme assays including β -galactosidase [307]. Its inhibition by various inhibitors, performed in a microfabricated channel network using electrokinetic flow to precisely mix substrate, enzyme and inhibitor, was also illustrated. A schematic of the enzyme analysis chip taken from the paper is shown in figure 7.2.

Figure 7.2 – Schematic diagram of the chip designed for enzyme assays in [307]



Microchip-based assays have also been described for protein kinase A [308] and ACLARA BioSciences, Inc., have reported the design, fabrication and characterisation of plastic microfluidic devices for conducting highly parallel enzyme-based assays [309]. Caliper Technologies Corporation have commercialised lab-on-a-chip technology [310] and have fabricated high throughput systems to perform on the order of tens of thousands of experiments per day per chip, using nanoliter volumes of reagents [311]. Chips have been designed for assays with fluorogenic substrates, e.g. protease assays, for biochemical assays, e.g. kinase assays, and for cellular assays, e.g. G-protein coupled receptor assays [312].

Using a chip-based system, the ability to manufacture a set-up with many parallel channels is much simpler than with a macro system. The soluble enzyme multi-determination system that was described in section 6.4 would be much better suited to a miniaturised system. Many parallel channels of different lengths would be easily manufactured in such a system, allowing the determination of two or more activities in nanoliter volumes using time separation. The need for such small volumes of reagents means that the soluble enzyme set-up could be used without the huge expenses found with such a set-up on a macro scale. The small amounts of sample required in miniaturised systems are also much better suited to the small amounts of compounds that are synthesised by combinatorial chemistry methods, allowing a better connection of combinatorial chemistry to HTS.

Alternatively, the immobilised enzyme format could also be used. The fabrication of micro-porous silica structures providing an efficient method of retaining a catalyst within a reactor system has been demonstrated by researchers at the University of Hull [313].

One area that needs to be considered in the design of chip-based protease and protease inhibition assays is the method of heating the assay components. From an examination of the literature, it appears that several methods are suitable, although the following overview is by no means exhaustive. A simple and common method to heat fluids in a chip is by the use of heating blocks [314 - 316]. This is an example of contact heating.

Another commonly used method is that of resistive heating [317, 318]. Heating accomplished using a resistive heater and thermocouple for temperature monitoring has been used for DNA analysis where the heater and thermocouple are mounted to the bottom of the chip [317], and for the polymerase chain reaction (PCR) mounted on the top [318]. An integrated microchip device for the

digestion, separation and postcolumn labelling of protein and peptides [319] has made use of a small detachable resistive heater. A thin Thermofoil™ Heater (Minco Products, Inc.) was used to control the temperature in the reaction channel. The heater was attached to the glass chip using a C-clamp and thermal contact between the heater and the microchip was provided using a piece of thermally conductive silicone elastomer. The heater temperature and the glass temperature on top of the microchip were measured using thermocouples, the temperatures of which did not differ more than 2 °C in the temperature range 20 to 80 °C down the length of the reaction channel. Simplified calculations showed that heating less than 2 mm length of the reaction channel (11.4 μm deep and 43.6 μm wide) is necessary to heat the fluid inside to the desired temperature.

Microfabricated devices constructed from glass and PDMS with integral heaters have been described [320], which were used for heterogeneous catalysis reactions. The *in situ* heater was fabricated from Nichrome wire (0.25 mm diameter) carefully formed into a serpentine shape and immobilised in the PDMS top plate and covering the channels. In the finished chips, the heating element was 2 mm from the catalyst surface and was able to heat 15 mm of channel length. Heating was achieved by a potentiostat (0 – 270 V) and monitored via a digital thermometer with the temperature probe located close to the reaction channel.

The methods already discussed have all been examples of contact heating. However, non-contact heating allows for fast switching of temperature and reactions, as opposed to the conventional contact devices.

A non-contact heating method has been demonstrated for control of a chemical reaction in a microchip by utilising the photothermal effect produced by a compact diode laser [321]. A light absorbing target on top of a glass microchip above a microchannel through which reactants flowed was utilised to heat the reaction media. In order to increase the temperature locally in the microchannel,

a 10mW diode laser was used to heat an absorbing target (black ink point, produced by felt tip marker) Heat flux was conducted through the glass cover plate and an increase in the temperature of the mixed solution in the main microchannel was noted. The reaction media were heated in a stopped flow mode. Reaction volume was estimated to be 10 nl and with laser powers of 2, 4, 7, 8, and 10 mW, the temperature change induced was estimated to be 1.9, 3.2, 5.0, 5.6, and 7 °C, respectively for 5 s of irradiation. The small heat capacity of the heated reaction space allowed the equilibrium temperature to be reached within 2 s

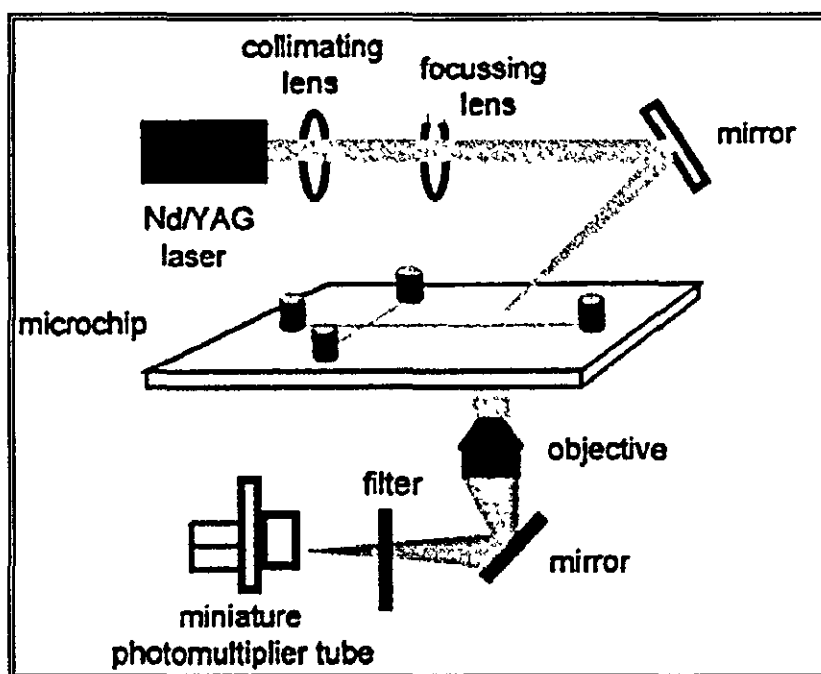
Non contact heating was also applied to PCR, which was carried out in capillary tubes rather than on a microchip [322]. Infrared light was produced by a tungsten lamp, which was focussed on the optically transparent PCR reaction chamber where the sample was heated due to the absorption of radiation. The temperature was sensed in a reference capillary with a thermocouple. A mirror placed behind the capillaries enhanced the effect of heating. A workable solution is provided by the construction of a reference probe that exhibits identical thermal properties as the sample. Using this method, a heating rate of 65 °C / s was seen.

Three distinct methods of heating are apparent: heating blocks (contact), resistive heating (contact), and using a light source (non-contact). Heating blocks are simple, but bulky. Resistive heating seems promising, with the advantage of different combinations. The Integral heaters immobilised in the PDMS top plate used in [320] are easy to produce and the Thermofoil™ heater (Minco Products, Inc.) that was used as a detachable heater [319] also appears promising. The non-contact methods, although faster, are expensive However, the speed obtained with these methods is not crucial for protease and protease inhibition assays.

A potentially tricky part of the design of a miniaturised system would be the excitation of the fluorophore and subsequent detection. Detection of fluorescence on a chip is described briefly in many papers [323 - 330], which possess a general scheme of using laser excitation by focusing the laser with a lens into the reaction channel and collection of fluorescence with a microscope objective and detection using a photo-multiplier tube (PMT).

Ocvirk, G. *et al.*, [331] reported a detailed confocal detection scheme for sub-picomolar detection. Excitation from an air-cooled argon ion laser (488 nm) passed through a 485 nm bandpass filter, was reflected by a dichroic mirror and was focused onto the glass device using a microscope objective. Fluorescence emission was collected, passed through a dichroic mirror and measured with a PMT. A schematic of this set-up is shown in figure 7.3, taken from the paper.

Figure 7.3 – Schematic diagram showing the set-up for a confocal fluorescence detection on a chip, taken from [331]



A completely integrated microfluidic / micro-optic device has been described [332], in which refractive microlens arrays and aluminium aperture arrays were integrated on both sides of a chemical chip.

A disposable sensor chip and optical readout device well suited for medical point-of-care diagnostics used an evanescent field generated by total internal reflection of a laser beam [333]. This field was then used for excitation of fluorophore markers. The sensor chip was optically connected to a prism of the optical readout device by a refractive-index-matching fluid.

Two different ways to get light into and fluorescence out of a chip seem to be promising. Firstly, the use of lasers (LEDs etc.), focusing lenses / microscope objectives, and filters in order to accurately focus the excitation light into the channel and then detect using a PMT. Secondly, the fabrication of waveguides in the chip coupled to fiber optics to get the light both in and out. These methods would require much thought and planning in order to develop a realistic miniaturised system for the determination of proteolytic enzymes and their inhibitors.

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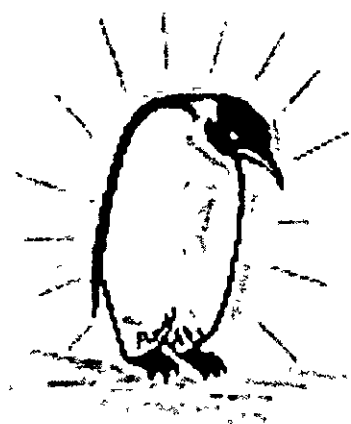
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Appendices

APPENDIX A**Supplier names and addresses**

SUPPLIER	ADDRESS
AD Instruments, Ltd.	Grove House Grove Road Hastings TN35 4JS, UK
Amersham Pharmacia Biotech UK, Ltd.	Amersham Place Little Chalfont Buckinghamshire HP7 9NA, UK
Anachem, Ltd.	20 Charles Street Luton Bedfordshire LU2 0EBG, UK
Cambio, Ltd.	34 Newnham Road Cambridge CB3 9EY, UK
Elkay Laboratory Products (UK), Ltd.	Unit 4, Marlborough Mews Crockford Lane Basingstoke, Hampshire RG24 8NA, UK

Eppendorf Scientific, Inc	1 Cantiague Road Westbury New York, USA
Fisher Scientific UK	Bishop Meadow Road Loughborough Leicestershire LE11 5RG, UK
Grant Instruments (Cambridge), Ltd.	29 Station Road Shepreth Royston Hertfordshire SG8 6PZ, UK
Hellma (England), Ltd.	Cumberland House 24 – 28 Baxter Avenue Southend-on-Sea Essex SS2 6HZ, UK
Hitachi Scientific Instruments	7 Ivanhoe Road Hogwood Industrial Estate Finchampstead Berkshire RG40 4QQ, UK
Molecular Probes, Inc.	Cambridge Bioscience 24 – 25 Signet Court Newmarket Road Cambridge CB5 8LA, UK

Omnifit, Ltd.	2 College Park Coldhams Lane Cambridge CB1 3HD, UK
Perkin-Elmer, Ltd.	Post Office Lane Beaconsfield Buckinghamshire HP9 1QA, UK
Pierce (Perbio Science UK, Ltd.)	Century House Tettenhall Cheshire CH3 9RG, UK
Precisa Balances Ltd.	4 Vermont Place Tongwell Milton Keynes, Buckinghamshire MK15 8JA, UK
Sigma-Aldrich Company, Ltd.	Fancy Road Poole Dorset BH12 4QH, UK
USF Elga Ltd.	High Street Lane End High Wycombe Bucks. HP14 35H, UK

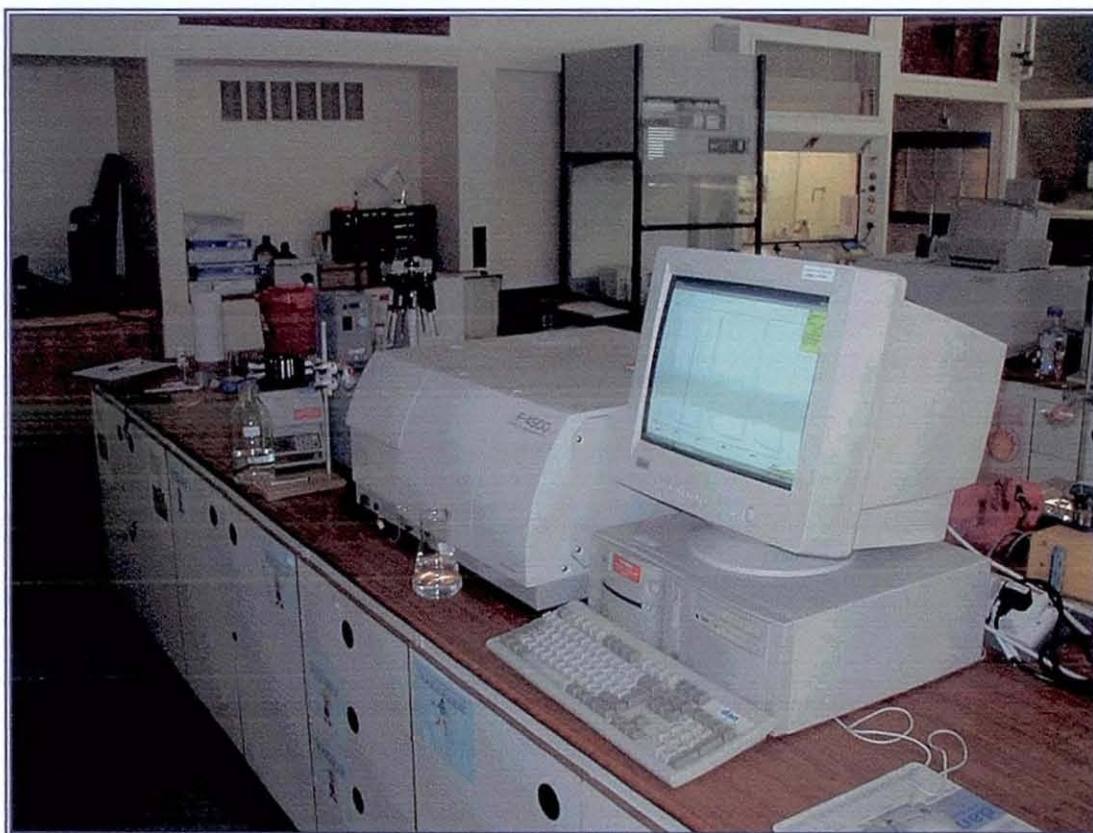
<p>Unicam Limited</p>	<p>York Street Cambridge CB1 2PX, UK</p>
<p>Whatman International Ltd.</p>	<p>St Leonard's Road 20 / 20 Maidstone Kent ME16 0LS, UK</p>

Table A - Supplier names and addresses

APPENDIX B

Appearance of the model F-4500 fluorescence spectrophotometer (Hitachi) located in our laboratory

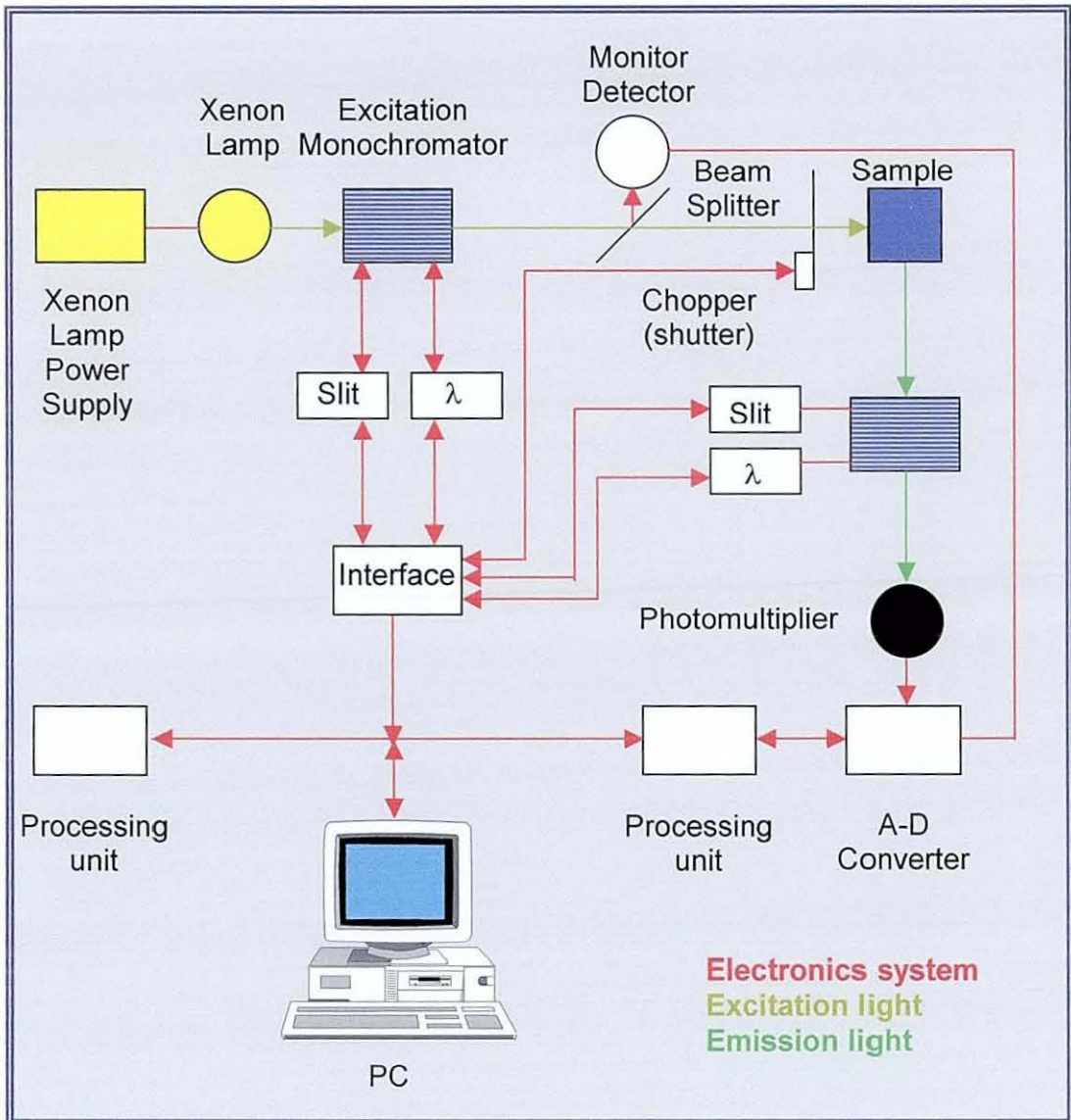
Figure B – Appearance of the F-4500 in our laboratory



APPENDIX C

Block diagram of the layout of the model F-4500 fluorescence spectrophotometer (Hitachi)

Figure C – Internal block diagram of the F-4500



APPENDIX D

Instrument specifications for the Hitachi F-4500 and Perkin-Elmer LS 50B fluorescence spectrometers

PARAMETERS	F-4500	LS 50B
Light source	150 W Xenon lamp	Xenon flash lamp, pulsed at line frequency (50 or 60 Hz)
Monochromators	Excitation: 200 – 600 nm Emission: 200 – 900 nm	Excitation: 200 – 800 nm Emission: 200 – 900 nm
Excitation slit width	1.0, 2.5, 5.0, 10.0 nm	2.5 – 15 nm in 0.1 nm increments
Emission slit width	1.0, 2.5, 5.0, 10.0, 20.0 nm	2.5 – 20 nm in 0.1 nm increments
Scanning speed	15, 60, 240, 1200, 2400, 12000, 30000 nm / min	10 – 1500 nm / min in 1 nm increments
Wavelength accuracy	± 2.0 nm	± 1.0 nm
Response	0.004, 0.01, 0.05, 0.1, 0.5, 2.0, 8.0 seconds	0.02 – 9.98 seconds in 0.01 s increments
PMT Voltage	400, 700, 950 V	Auto

Table D – Instrument parameters of the F-4500 and LS 50B

APPENDIX E

Appearance of the LS 50B fluorescence spectrophotometer (Perkin-Elmer) located in our laboratory

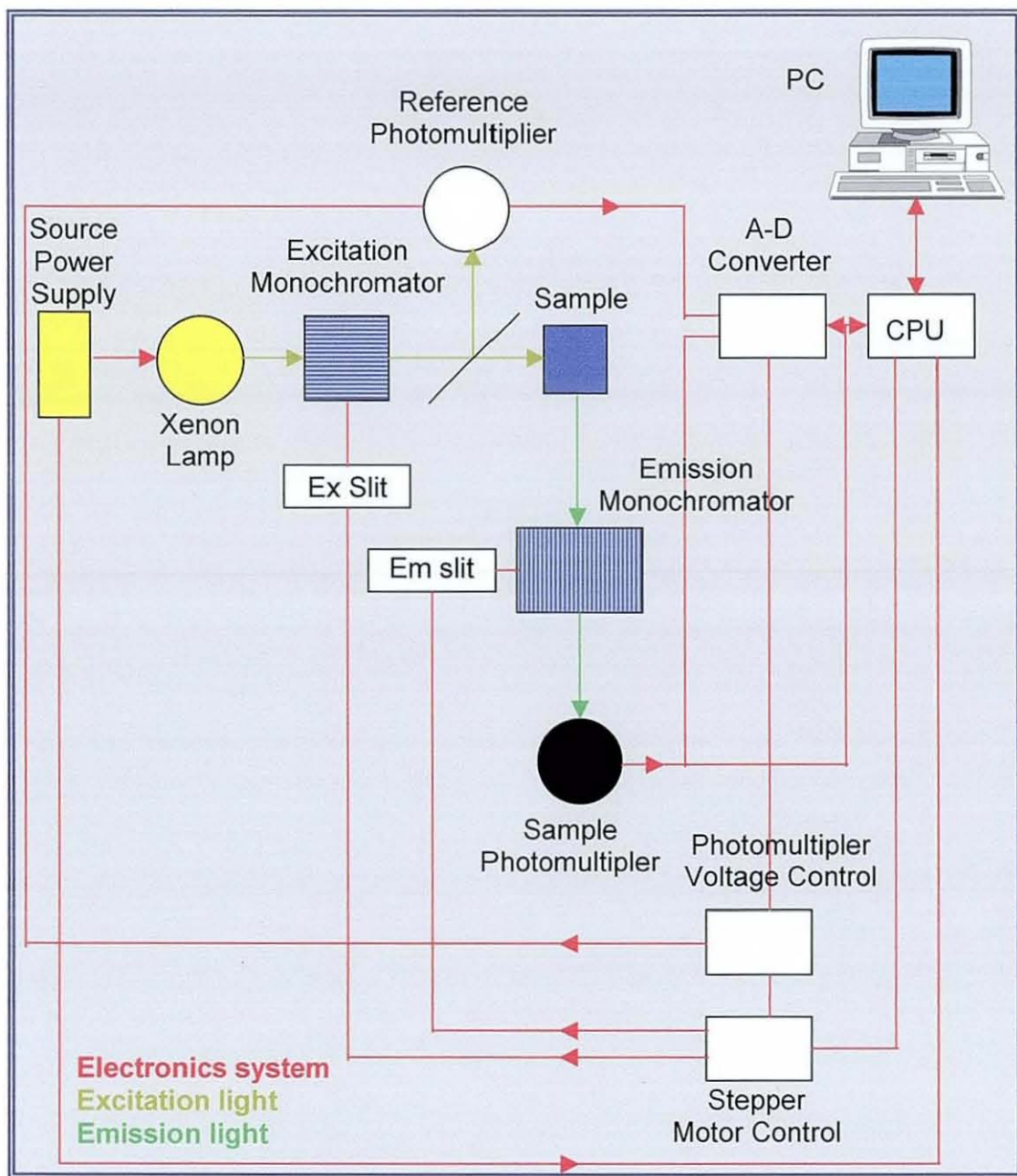
Figure E – Appearance of the LS 50B



APPENDIX F

Block diagram of the layout of the Perkin-Elmer LS 50B fluorescence spectrophotometer

Figure F - Optical layout of the LS 50B fluorescence spectrophotometer



APPENDIX G**Calculation of fluorescein absorbance at 280 nm as a percentage of that at 494 nm**

Fluorescein $A_{280 \text{ nm}} = 1.023$, and $A_{494 \text{ nm}} = 2.545$. Therefore, absorbance at 280 nm as a percentage of the absorbance at 494 nm = $(1.023 / 2.545) \times 100 = 40 \%$.

APPENDIX H**Calculation of BODIPY absorbance at 280 nm as a percentage of that at 630 nm**

BODIPY $A_{280 \text{ nm}} = 0.196$, and BODIPY $A_{630 \text{ nm}} = 2.073$. Therefore, absorbance at 280 nm as a percentage of the absorbance at 630 nm = $(0.196 / 2.073) \times 100 = 9.5 \%$.

APPENDIX I**Calculation of α -casein molar extinction coefficient (ϵ) at 280 nm**

According to the Beer Lambert Law absorbance is related to the molar extinction coefficient ϵ (in $\text{cm}^{-1} \text{M}^{-1}$), concentration c (in M), and path length l (in cm) by: $A = \epsilon cl$. A 0.1% solution of α -casein (0.01 g per 10 ml) was made up and the absorbance at 280 nm measured ($A_{280 \text{ nm}} = 0.882$).

$$\begin{aligned} \text{Molar concentration} &= (\text{weight (g)} / \text{RMM (g)}) \times (1000 \text{ (ml)} / \text{volume (ml)}) \\ &= (0.01 / 24\,000) \times (1000 / 10) = 4.17 \times 10^{-5} \text{ M} \end{aligned}$$

Substituting into the Beer Lambert Law:

$$\epsilon = A / c = 0.882 / 4.17 \times 10^{-5} = 21,151 \text{ cm}^{-1} \text{ M}^{-1}.$$

APPENDIX J**Calculation of α -casein absorbance at 350 nm as a percentage of that at 280 nm**

α -casein $A_{280 \text{ nm}} = 0.417$, and α -casein $A_{350 \text{ nm}} = 0.046$. Therefore, absorbance at 350 nm as a percentage of the absorbance at 280 nm = $(0.046 / 0.417) \times 100 = 11\%$.

APPENDIX K**Calculation of BODIPY absorbance at 280 nm as a percentage of that at 350 nm**

BODIPY $A_{280 \text{ nm}} = 0.117$, and BODIPY $A_{350 \text{ nm}} = 0.338$. Therefore, absorbance at 280 nm as a percentage of that at 350 nm = $(0.117 / 0.338) \times 100 = 35 \%$.

APPENDIX L**Calculation of BODIPY molar extinction coefficient (ϵ) at 350 nm**

According to the Beer Lambert Law absorbance is related to the molar extinction coefficient ϵ (in $\text{cm}^{-1} \text{M}^{-1}$), concentration c (in M), and path length l (in cm) by: $A = \epsilon cl$.

A $1.66 \times 10^{-5} \text{ g / ml}$ solution of BODIPY was made up and the absorbance at 350 nm measured ($A_{350 \text{ nm}} = 1.049$).

$$\begin{aligned} \text{Molar concentration} &= (\text{weight (g)} / \text{RMM (g)}) \times (1000 \text{ (ml)} / \text{volume (ml)}) \\ &= (1.66 \times 10^{-5} / 660.5) \times (1000 / 1) = 2.52 \times 10^{-5} \text{ M} \end{aligned}$$

Substituting into the Beer Lambert Law:

$$\epsilon = A / c = 1.049 / 2.52 \times 10^{-5} = 41,627 \text{ cm}^{-1} \text{M}^{-1}$$

APPENDIX M**Calculation of flow rate**

Flowing buffer through the flow system and collecting the buffer for 5 minutes in a glass vial that had been used to zero the analytical balance was used to determine the flow rate for use in protease and protease inhibition assays. The weight of the vial was then taken and the weight recorded. This was carried out in triplicate. This calculation makes the assumption that 1 g = 1 ml buffer.

Triplicate weights for 5 minutes: 9.2536, 9.2417, 9.2561. Therefore, the average of weights = 9.250467 with a standard deviation (SD) = 0.007694, and percentage SD = 0.083.

9.250467 ml was collected over 5 minutes, therefore for 1 minute $9.250467 / 5 = 1.85$ ml was collected. Therefore, flow rate = 1.85 ml / min.

APPENDIX N

Determination of incubation loop volume

Buffer was flowed through the incubation loop and captured by closing the valve so that the incubation loop was segregated from the rest of the flow system. Air was then flowed through the rest of the system to remove any traces of buffer. A glass vial was used to zero the analytical balance after which the buffer captured in the incubation loop was flowed out into the glass vial. The glass vial was then re-weighed. This measurement was repeated 6 times. Determination of loop volume assumes that 1 g = 1 ml buffer.

Weight of six readings (mg): 660.6, 667.0, 667.6, 671.0, 675.5, 678.0 This gives an average = 669.95, with a standard deviation (SD) = 6.298 and a percentage SD = 0.94 %. Therefore, loop volume = 670 μ l.

