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Date March 28, 1995

Monitoring Enzymatic Hydrolyses of Small Peptides Using Electrohydrodynamic Mass Spectrometry

A Thesis

Presented for the

Master of Science

Degree

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Abstract

This work involves the development and testing of a new sample introduction technique to allow enzymatic hydrolyses of peptides to be monitored in real time using Electrohydrodynamic Mass Spectrometry (EHMS). The hydrolyses were carried out in aqueous acetate buffer. Because EHMS requires an organic cosolvent, the aqueous reaction mixture was mixed with a thioglycerol (TG) solution in a low dead volume tee before entering the mass spectrometer. The enzyme used in this work was carboxypeptidase-Y because of its ability to cleave most amino acids.

Hydrolyses were performed on two carbobenzyloxy-protected dipeptides, and initial velocities were determined at different substrate concentrations. Kinetics information, such as the Michaelis constant (K_m) and maximum rate of the reaction (V_{max}), was obtained from the mass spectrometric data for one of the protected dipeptides and compared with that obtained using a well established method: the ninhydrin method with UV-Visible detection. The results revealed that systematic errors were present in the EHMS experiments. Possible causes for the systematic errors are contamination of the reaction mixture by TG and/or imperfect temperature regulation of the reaction. A pentapeptide, leucine-enkephalin, was hydrolyzed and monitored for 24 hours by EHMS (continuously) and the ninhydrin method (at timed intervals). Rate constants for the release of the first two amino acids were determined from the EHMS data and compared with those obtained through deconvoluting the optical data. Again, systematic errors were found to be present in the MS experiments.

The results of this study prove for the first time that biochemical reactions

can be monitored continuously using EHMS. Despite the systematic errors present, this work showed that EHMS can serve as a useful qualitative probe.

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Chapter 1

Introduction

1.1 Carboxypeptidase-Y

The importance of enzymes in biological processes cannot be overstated. The complex array of biochemical reactions involving specific enzymes controls life. Enzymes are very powerful catalysts that can accelerate reactions by up to six orders of magnitude [1]. Therefore, the chemistry of enzymes is intrinsically important in biochemical processes. Enzymes also play a vital role as analytical reagents in assays of the enzymes themselves and of other species, such as various substrates. Enzymes can be exploited as an analytical tool to sequence peptides, for example [2,3]. In any context, the chemistry of enzyme reactions can be complex. Its study constitutes a challenging analytical problem and is the subject of this thesis. The work described here focuses on obtaining kinetics information from enzymatic hydrolyses of small peptides. The reactions will be monitored by electrohydrodynamic mass spectrometry (EHMS) [4]. The kinetics information obtained will be compared to that received from monitoring the reactions using a classical method, the ninhydrin method of Moore and Stein [5], in order to evaluate the EHMS method. Not only kinetics but also sequence information will be obtained for a substrate in this study; therefore, a suitable enzyme must be chosen for this purpose.

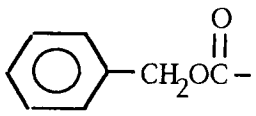
There are many different classes of enzymes to choose from. For purposes of sequencing, an enzyme that hydrolyzes peptides or proteins (called a peptidase) is particularly useful [6]. Endopeptidases are enzymes that cleave peptide bonds at some position within a peptide chain other than at a terminal

amino acid. Most endopeptidases target a specific peptide bond (e.g., that adjacent to a specific target amino acid). Exopeptidases are enzymes that cleave peptide bonds at either the N-terminal or C-terminal amino acid in a peptide [6]. Like all catalysts, the peptidase is not consumed, and can proceed to cleave amino acids sequentially; therefore, an exopeptidase was chosen for use in this study.

Carboxypeptidases are examples of exopeptidases. They hydrolyze the peptide bond at the C-terminus amino acid in peptides and proteins. There are different types of carboxypeptidases, each differing in amino acid specificity [6]. One example, carboxypeptidase-Y (CPY), is characterized by its ability to cleave most amino acid residues, including proline (an imino acid), at an optimum pH of 5.5 to 6.5 [2]. CPY is very soluble in aqueous solution at low ionic strength. The molecular weight of CPY is approximately 61,000 [7,8]. Barring fragmentation or multiple charging, its high molecular weight makes it mass spectrometrically "transparent." It is relatively stable in the presence of denaturants, such as 6 M urea [5]. In addition to these properties, it is readily available. For these reasons, CPY was chosen for these studies.

It has been observed that catalysis by CPY is effective regardless of whether the penultimate and/or terminal amino acids in the peptide have aromatic or aliphatic side chains [9]. Although the release of a terminal lysine, arginine, or histidine is rather slow, the release of the terminal amino acid is extremely slow only if glycine is located in the penultimate position [10]. Dipeptides are not hydrolyzed, and cleavage of tripeptides is minimal [2,11]. The steriospecificty of CPY has been shown by its failure to hydrolyze substrates containing D-amino acids [9]. The hydrolysis stops when a D-amino acid is in the C-terminal or penultimate position.

Although dipeptides are not hydrolyzed by CPY, the addition of a protecting group, such as benzyloxycarbonyl ("carbobenzoxy" or CBZ) (see Structure 1.1), to



Structure 1.1

the amino group of a dipeptide allows hydrolysis to proceed [9]. In fact, an accepted method of assaying CPY activity involves derivatizing the amino group of a dipeptide, then monitoring the catalytic liberation of the C-terminal residue [2,11]. The progress of the reaction can be followed by measuring the absorbance (at 570 nm) of a purple ninhydrin complex formed with the amino acid cleaved by the CPY [5]. Complex formation is slow and requires heating, but full color development is obtained within 15 minutes.

The ninhydrin method does not distinguish between individual amino acids; most amino acids (except for proline, an imino acid) react with essentially equal efficiency to form complexes of similar spectroscopy [12]. Thus, if a hydrolysis is monitored which involves the liberation of more than one amino acid from a peptide, the absorbance measured represents the sum of the absorbances due to each amino acid. Mass spectrometry, however, can distinguish between individual amino acids (except for the isomeric pair leucine/isoleucine).

The purpose of this work is to try to determine if EHMS can be used to accurately monitor the rates of CPY-catalyzed reactions. This will be accomplished by measuring kinetics parameters using EHMS and comparing those parameters

to ones obtained by the ninhydrin method. The kinetics parameters are from a near century-old model that predicts the behavior of enzyme-catalyzed reactions.

1.2 Michaelis-Menten Kinetics

For most enzyme-catalyzed reactions, the rate of product formation will decrease with time [6], as demonstrated by the decreasing slope of the progress curve in Figure 1.1. Different causes may contribute to this decrease, such as reactant depletion or enzyme inhibition due to product formation, for example [6]. Therefore, the concept of the *initial* velocity has been adopted for use in the study of enzymes. The initial velocity is obtained from the linear portion of the curve in Figure 1.1. The curve is practically a straight line as long as the amount of product formation or substrate depletion is not high enough to start slowing the rate of reaction [6] (typically, up to a point corresponding to about 30% reduction of the initial substrate concentration).

For many enzymes, including CPY, the initial rate of reaction (v) varies with the substrate concentration ([S]) in a manner represented in Figure 1.2. (The initial rate (v) is defined as the slope of the initial, roughly linear, part of Figure 1.1.) At low substrate concentrations, v is roughly proportional to [S]. However, at high substrate concentrations, v becomes independent of [S]. This assumes a fixed enzyme concentration.

In 1913, Michaelis and Menten proposed a model to account for the kinetics properties represented in Figure 1.2 [13]. Essential to their model is the formation of an enzyme-substrate complex as an intermediate, according to the following reaction scheme:

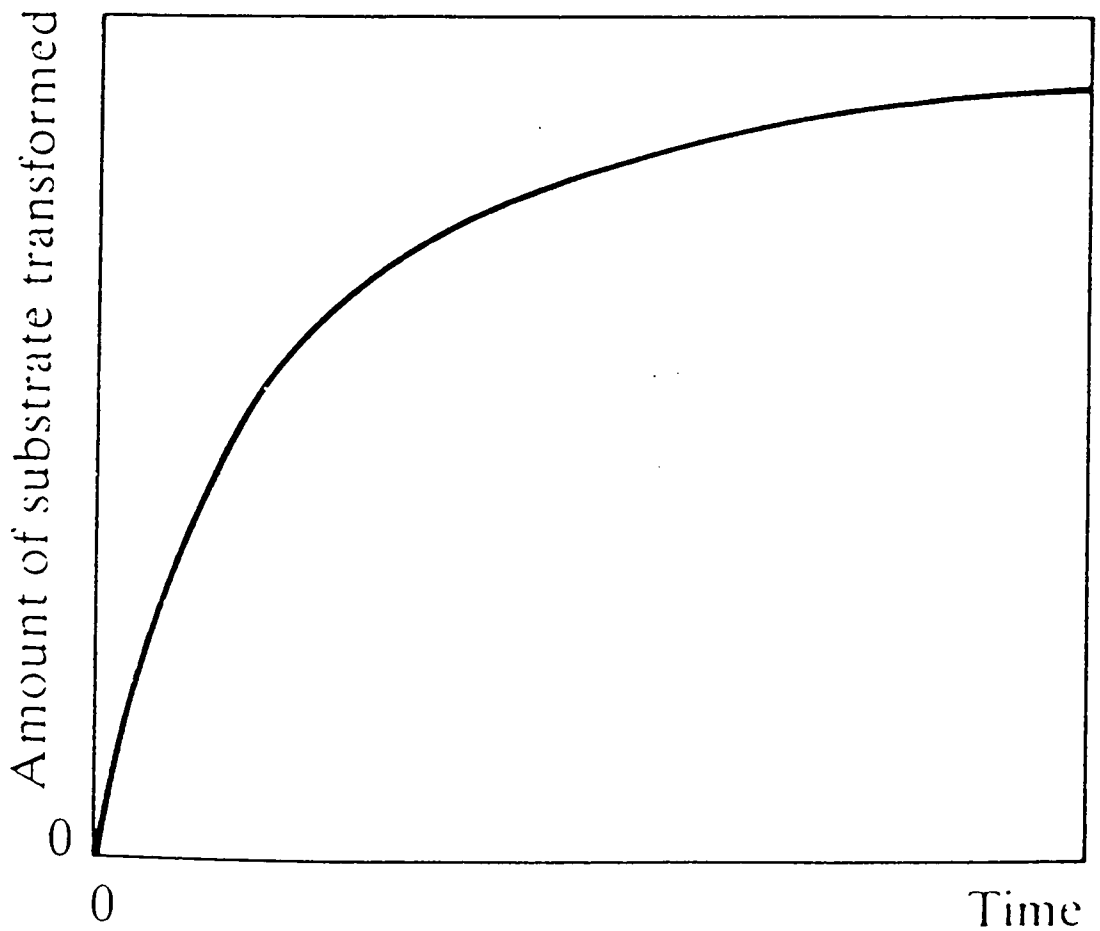


Figure 1.1. A typical reaction progress curve showing the amount of substrate transformed to product versus time. [Taken from ref. 6]

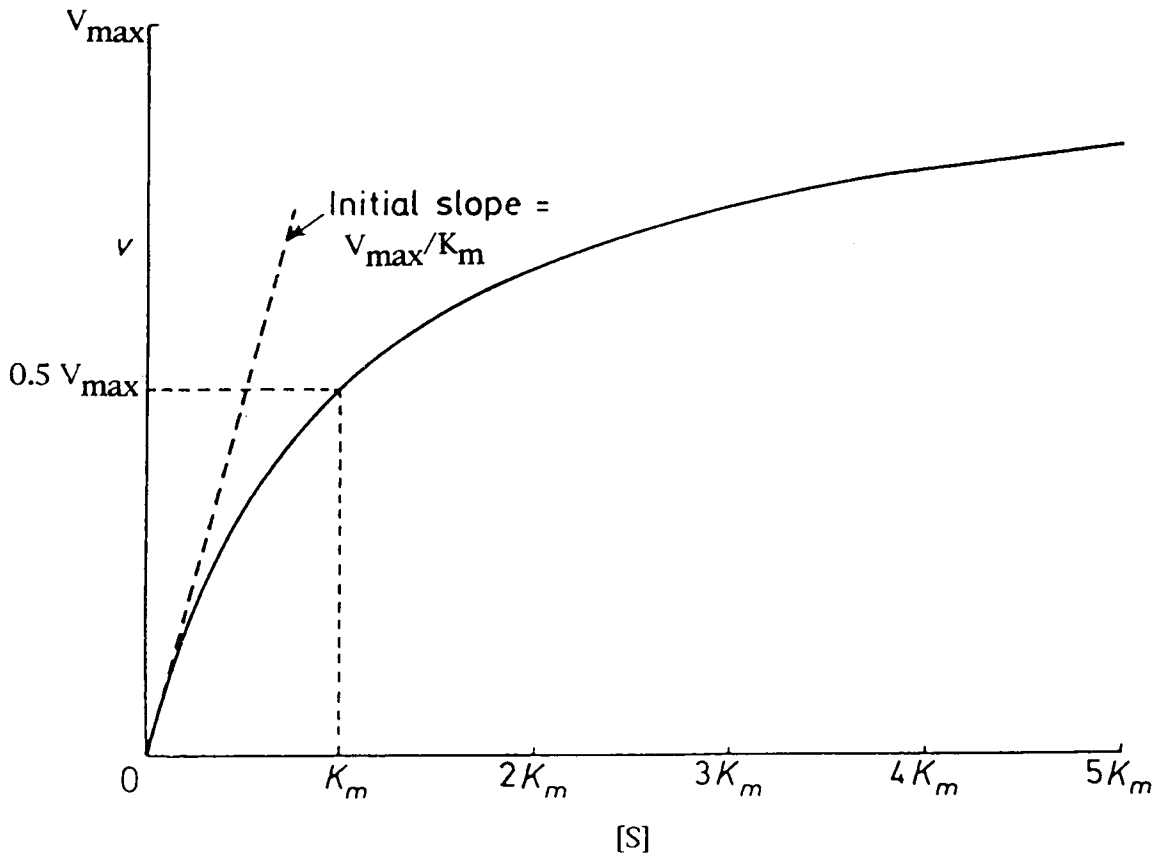


Figure 1.2. Plot of initial velocity, *v*, against substrate concentration, [S], for a reaction obeying the Michaelis-Menten equation. [Taken from ref. 17]

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E + P \qquad (1)$$

where E denotes the enzyme; S, the substrate; ES, the enzyme-substrate complex; and P, the product(s). k_1, k_2, and k_3 are the temperature-dependent rate constants for the formation of the ES complex, the dissociation of the ES complex, and the formation of product, respectively. It is assumed that none of P reverts to ES or S.

For this mechanism, at a given temperature, the rate of product formation, *v*, is given by the following equation [1]:

$$v = k_3[E]_T[S]/(K_m + [S]) \qquad (2)$$

where $[E]_T$ is the total enzyme concentration and K_m, the Michaelis constant, is defined as:

$$K_m = (k_2 + k_3)/k_1 \qquad (3)$$

The maximal rate, V_{max}, occurs at high substrate concentration, when the active sites on the enzyme are saturated with substrate. When this is true, $[S] >> K_m$, and Equation (2) reduces to:

$$V_{max} = k_3[E]_T \qquad (4)$$

Substituting for $k_3[E]_T$ in Equation (2) gives:

$$v = V_{max}[S]/(K_m + [S]) \quad (5)$$

Refer back to Figure 1.2. From Equation (5), it can be seen that at low substrate concentration, when [S] << K_m, then $v = V_{max}[S]/K_m$; the rate of reaction is proportional to [S]. Under these circumstances, "V_{max}/K_m has the fundamental meaning of the first order rate constant for the reaction E + S ----> E + P" [17]. At very high substrate concentrations, when [S] >> K_m, then $v = V_{max}$, and the rate is independent of [S]. K_m can be determined by finding the substrate concentration at which the rate of product formation is half of its maximal value. From Equation (5), when [S] = K_m, then $v = V_{max}/2$. Equation (5) is generally referred to as the Michaelis-Menten Equation [1].

Besides K_m and V_{max}, another useful kinetic parameter is the turnover number of the enzyme, which is defined as "the number of substrate molecules converted into product by an enzyme molecule in a unit of time when the enzyme is fully saturated with substrate" (i.e., at V_{max}) [1]. The turnover number is numerically equal to the rate constant k_3. If the total enzyme concentration is known, the maximal rate, V_{max}, reveals the turnover number of the enzyme using Equation (4).

If an enzyme operates according to Equation (1), then K_m and V_{max} can be derived from data like that of Figure 1.2 for initial rates of reaction measured at different substrate concentrations. To facilitate determinations of K_m and V_{max}, the Michaelis-Menten equation can be rewritten in a form to allow the results to be plotted as a straight line [6]. This is routinely performed in one of three ways:

$$[S]/v = (K_m/V_{max}) + ([S]/V_{max}) \quad (6)$$

$$v = V_{max} - (K_m v/[S]) \quad (7)$$

$$1/v = (1/V_{max}) + (K_m/(V_{max}[S])) \quad (8)$$

A plot of $[S]/v$ against [S] (Equation (6)) yields a straight line of slope $1/V_{max}$ and intercepts of K_m/V_{max} on the $[S]/v$ axis and $-K_m$ on the [S] axis (see Figure 1.3). This type of plot is referred to as a Hanes plot [14]. Likewise, straight-line plots are obtained from Equations (7) and (8) by plotting v against $v/[S]$ and $1/v$ against $1/[S]$, respectively (see Figures 1.4 and 1.5). These plots are referred to as Eadie-Hofstee [15] and Lineweaver-Burk [16] plots, respectively. In Figures 1.3, 1.4, and 1.5, the error bars were derived from a fixed uncertainty ($\pm 0.05 V_{max}$) in the experimental determination of the initial velocity, given assumed values of K_m and V_{max}.

According to Cornish-Bowden, the Hanes plot "is the most satisfactory of the three" [17] for the following reasons. With the Lineweaver-Burk and Eadie-Hofstee plots, there are very large deviations of some of the points at low substrate concentration; thus, the use of these plots should be discouraged, even though the Lineweaver-Burk plot is by far the most popular and widely used of the three [17]. The Eadie-Hofstee plot has the added disadvantage that v, which is usually regarded as the dependent variable, appears in both coordinates (accounting for the odd error bars in Figure 1.4). Therefore, the Hanes plot will be used for data treatment in the present studies.

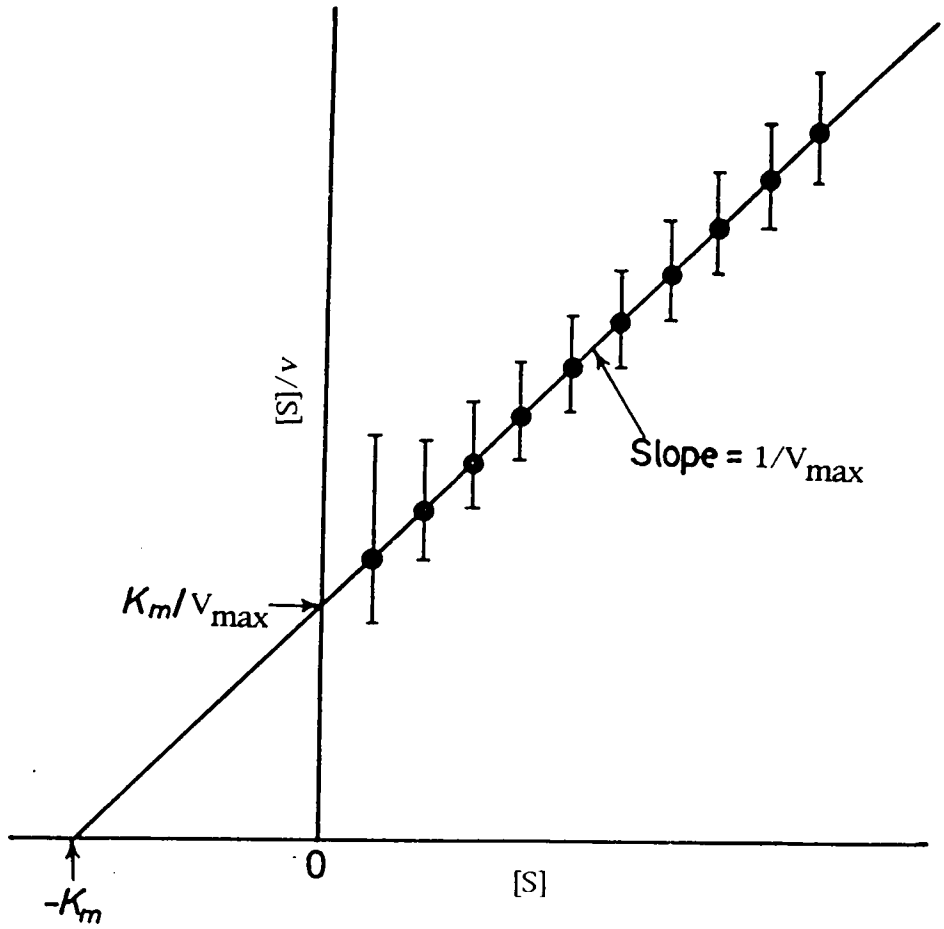


Figure 1.3. Plot of [S]/v against [S], with error bars of $\pm 0.05 V_{max}$ in v (Hanes plot). [Taken from ref. 17]

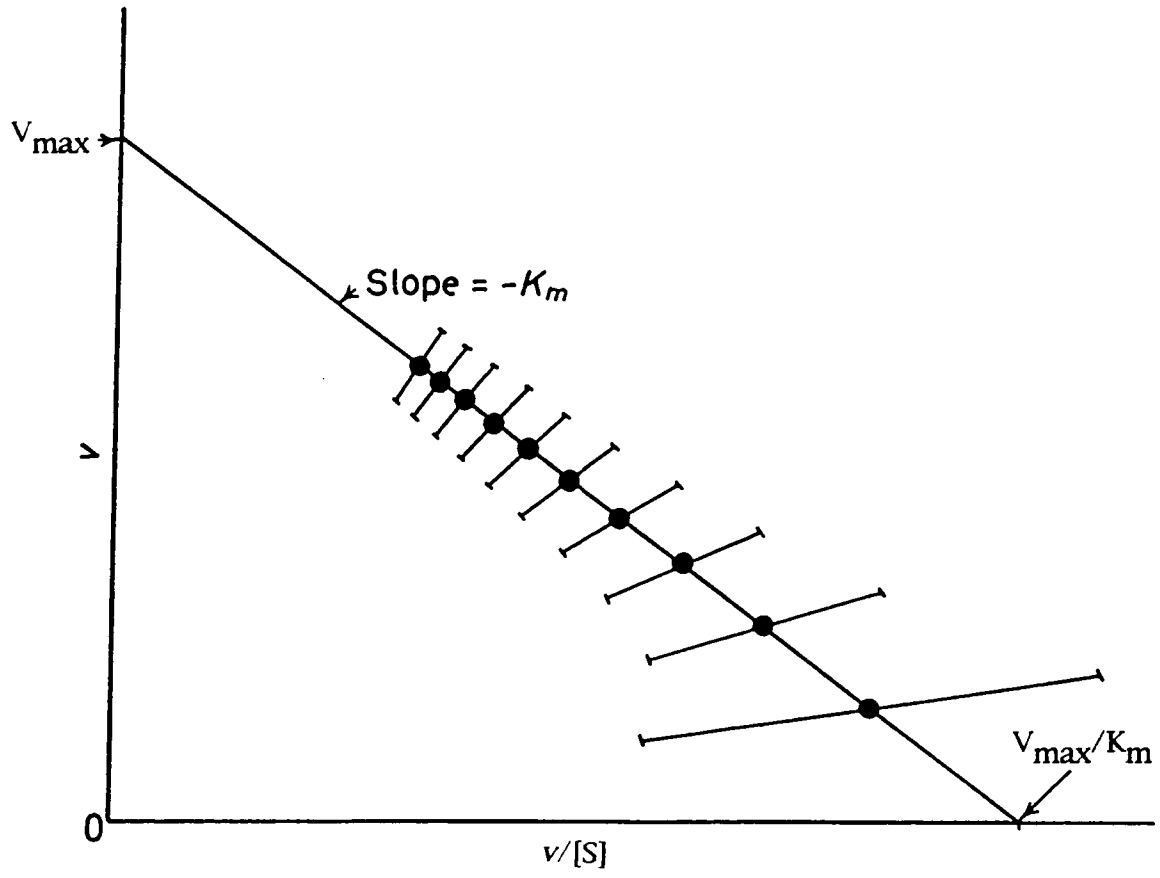


Figure 1.4. Plot of v against $v/[S]$, with error bars of $\pm 0.05 V_{max}$ in v (Eadie-Hofstee plot). [Taken from ref. 17]

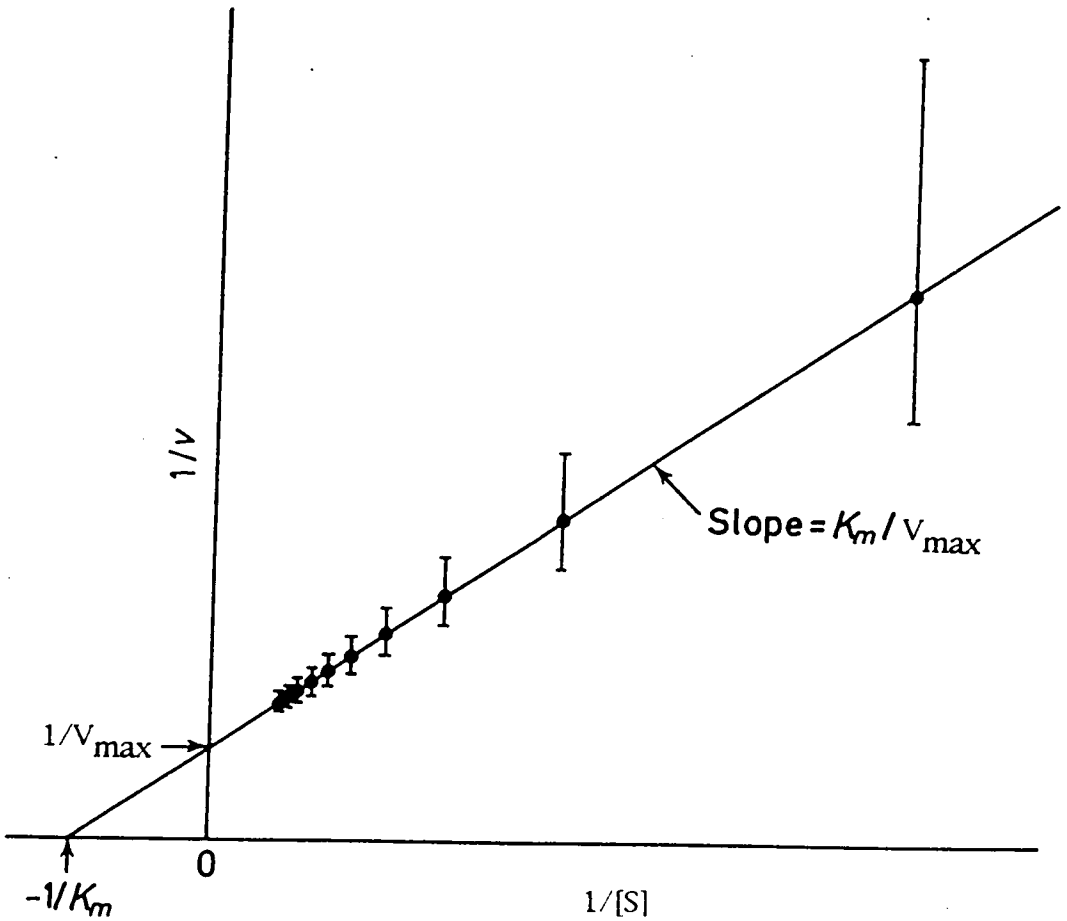


Figure 1.5. Plot of $1/v$ against 1/[S], with error bars of $\pm 0.05 V_{max}$ in v (Lineweaver-Burk or double reciprocal plot). [Taken from ref. 17]

In order to determine K_m, an appropriate substrate concentration range must be chosen (i.e. one at which the enzyme is not saturated). Table 1.1 reveals the predictions of Michaelis-Menten kinetics for the relative initial velocity of hydrolyses at different substrate concentrations [18]. It can be seen that if K_m is to be determined, it is desirable for the substrate concentration to be at least lower than 10 K_m because, at higher substrate concentrations, there is not much change in the initial velocity. Cleland has recommended that a suitable substrate concentration range for the determination of K_m and V_{max} is from 0.2 K_m to 5 K_m [19]. For purposes of this study, the concentration range used was from K_m to 4 K_m (limited on the low end by mass spectrometric limits of detection and on the high end by substrate solubility).

1.3 Mass Spectrometry and Enzymes

The coupling of mass spectrometry and enzymes provides very powerful methods of studying biological systems [20]. Several different mass spectrometric ionization methods have been employed to monitor reaction mixtures, including Fast Atom Bombardment (FAB) [21-30], thermospray [31-33], plasma desorption [34], laser desorption [35], electrospray [36,37], and electrohydrodynamic mass spectrometry (EHMS) [38,39].

Often, mass spectrometry is used with periodic batch sampling to monitor a reaction that is proceeding "ex situ." For instance, Caprioli and Smith [24] used FAB to obtain information about the rates of the hydrolysis of a tetrapeptide by trypsin (an endopeptidase which hydrolyzes the peptide bond on the carboxy-side of lysine and arginine residues). At timed intervals, aliquots of the reaction

Table 1.1. Properties of the Michaelis-Menten equation. Listed below are initial velocities (in terms of the maximal rate) at different substrate concentrations (in terms of the Michaelis constant) for an enzyme obeying the Michaelis-Menten equation.

Substrate Concentration [S]	Initial Velocity *v*
1000 K_m	0.999 V_{max}
100 K_m	0.99 V_{max}
10 K_m	0.91 V_{max}
3 K_m	0.75 V_{max}
K_m	0.50 V_{max}
0.33 K_m	0.25 V_{max}
0.10 K_m	0.091 V_{max}
0.01 K_m	0.01 V_{max}

Taken from reference 18.

mixture were removed, mixed with glycerol (a low-volatility FAB matrix), and analyzed by FAB. K_m and V_{max} were determined mass spectrometrically by determining the reaction rate at different substrate concentrations. The authors verified their results by comparing the rates obtained by FAB to those obtained using well-established GC/MS methods [40]. They showed that FAB can be used to quantitatively measure compounds which are part of active enzyme systems.

Caprioli and Fan [25] also used FAB to obtain partial sequence information from the digestion of the peptides ribonuclease S, substance P, and (Ile^5) angiotensin II by carboxypeptidase-Y (CPY). These peptides contain 20, 11, and 8 amino acid residues, respectively. Each substrate was reacted with CPY separately. FAB was used to analyze aliquots of the reaction mixtures removed at timed intervals and again dissolved in glycerol (the FAB matrix). The $[M + H]^+$ ion of the intact substrate, as well as the $[M + H]^+$ ions of the resulting hydrolysis products were monitored. The first eight residues of ribonuclease S were confirmed, as well as the first four of substance P and the first six of (Ile^5) angiotensin II after a reaction time of two hours for each substrate. Using a similar approach, Kim *et al.* [26] used FAB and CPY to confirm the first five residues of bradykinin (a peptide containing nine amino acids) after a reaction time of 17 hours, and the first four residues of angiotensin II after a reaction time of 40 minutes. Spectral complexity prevented confirmation of the sequence of all amino acids.

Tandem mass spectrometry (MS/MS) can be used in combination with FAB and enzymes to obtain sequence information of oligopeptides. Basically, in MS/MS, ions of a certain m/z are isolated and further fragmented before being mass analyzed [41]. In this way, one can observe "fragments of fragments." The

use of ex situ enzymes coupled with MS/MS is probably the single most widely used and important mass spectrometric tool for sequencing [28, 41]. Usually, 4-sector instruments are used for high energy collisions and reasonable product and precursor resolution.

To circumvent the obstacle of a limited instrumental mass range, large proteins can be hydrolyzed by an enzyme (usually an endopeptidase), products are then separated by HPLC, and then analyzed by FAB to obtain molecular weight (and some structural) information for each. For example, trypsin and/or chymotrypsin are commonly used endopeptidases in this context [27]. V8 protease, an endopeptidase that cleaves the peptide bond after glutamic acid and aspartic acid residues, has been used as well [27]. Another enzyme, such as an exopeptidase, can then be added to the tryptic product (for example) directly on the FAB probe tip, and further sequence information can be obtained [27]. This method is useful if the molecular weight of the initial substrate exceeds the mass range of the instrument being used.

C-terminal sequencing of peptides has also been accomplished by electrospray ionization mass spectrometry (ESIMS) in conjunction with batch sampling [36]. In ESIMS, multiple charge states of large molecular weight compounds, such as proteins, can be produced; therefore, the mass-to-charge ratio is reduced so that it falls within the mass range of ordinary mass analyzers. Duffin and Smith [36] confirmed the last six amino acid residues of the peptide neurotensin by performing ESIMS on aliquots taken from a carboxypeptidase digestion of the peptide.

Because the rate of release of amino acids by carboxypeptidase enzymes can vary depending on the penultimate and carboxy-terminal amino acid, it is

difficult to know when to take a batch sample. As a result, continuous monitoring of the reaction is often favored over batch sampling. In this context, Rosnack and Stroh [37] used an on-line reactor for continuous ESIMS monitoring of a peptide being digested by carboxypeptidase enzymes. The first 19 amino acids from glucagon were successfully sequenced using this method.

An early attempt at monitoring reactions proceeding "in situ" (i.e. within the mass spectrometer) was that of Smith and Caprioli [22] who used FAB with a glycerol/water matrix to monitor enzymatic hydrolyses of substrates in real time. Both an endo- (trypsin) and exopeptidase (dipeptidyl aminopeptidase I (DAP I)) were used on their common assay substrates (p-toluenesulfonyl-L-arginylmethylester and glycyl-L-phenylalanine-β-naphthylamide, respectively). DAP I cleaves two amino acids at a time (as dipeptides) sequentially from the amino terminus of a peptide. Also, the exopeptidase CPY was used to hydrolyze (val^4) angiotensin III (a heptapeptide). A decrease in the $[M + H]^+$ ion signal of each substrate and an increase in that of the product(s) were measured during the reaction. Because FAB involves bombarding the sample in the evacuated ion source with high energy xenon or argon atoms (2-10 keV of translational energy), it was found that continuous beam bombardment longer than 2 minutes resulted in substantial loss of enzyme activity. However, if the sample was exposed to bombardment for consecutive 0.5 minute periods with 6 minute intervals between exposures, the enzyme did not suffer any loss of activity for up to 20 minutes. Enzyme activity was eventually lost due to bombardment, which both induced fragmentation and accelerated solvent evaporation.

In order to remedy the problem of sample drying in FAB, Caprioli *et al.* [42] developed a continuous-flow sample probe which allowed for reaction mixtures to

be introduced directly and continuously into the mass spectrometer. With continuous-flow FAB (CF-FAB) or "flow-FAB," the samples analyzed can be essentially aqueous, containing as little as 10% glycerol. This is a definite advantage over the standard FAB technique if reaction mixtures are to be monitored. A lower glycerol content is favorable to sustaining enzyme activity. Reactions can be monitored over longer time periods without batch sampling. Flow rates are typically 5-10 μL/min [29]; however, sample requirements can be reduced by operation in a "flow injection mode" in which typically 0.5 μL sample aliquots are introduced into the flowing stream [29, 30]. Caprioli has used flow injection CF-FAB with selected ion monitoring to measure reaction rates and other kinetics parameters for the hydrolysis of a tetrapeptide by trypsin [29]. van Breeman and Davis [30] used CF-FAB to measure the rates of hydrolysis of angiotensin II and [Arg^8]vasopressin by the enzymes chymotrypsin (an endopeptidase which cleaves peptide bonds on the carboxy- side of aromatic amino acids) and trypsin, respectively.

One disadvantage of FAB and CF-FAB is that each can result in rather complex mass spectra, representing a superposition of the fragmentation spectrum of the substrate, the products, the intermediates, and the matrix background. This is a result of the excess internal energy imparted to the sample during the ionization process.

When using enzymes to sequence peptides, often sample consumption is a concern due to the rather high cost of enzymes. The use of immobilized enzymes offers one solution to this problem. Although immobilized enzymes have not yet been used with FAB or CF-FAB, they have been used with another continuous stream mass spectrometric technique: thermospray mass spectrometry [31-33].

Thermospray liquid chromatography/mass spectrometry (LC/MS) allows direct analysis of an LC eluent. Briefly, the eluent (usually an aqueous solution containing a volatile buffer) is introduced to the mass spectrometer through a heated capillary, from which it emerges as a supersonic jet containing vaporized solvent and fine droplets. This high-velocity stream then passes through a heated ion source into a vacuum line. According to one model [31], ions are evaporated from the surface of the droplets and are subsequently mass analyzed. In peptide sequencing applications, a solution of substrate can be passed through a column containing an immobilized endopeptidase, such as trypsin or chymotrypsin. The sample is then passed through an LC column to separate the reaction products. At this point, the sample can be analyzed by thermospray MS [31,33]. Alternatively, the sample can pass through an additional column containing immobilized exopeptidases before being mass analyzed [31-33]. Columns containing different immobilized enzymes can be exchanged with others in order to provide the best means of sequencing a peptide. In these applications, unless a stopped flow is used, the substrate exposure to the enzyme depends on the flow rate. In some cases, this can lead to incomplete sequence data.

Electrohydrodynamic mass spectrometry (EHMS) has also been used to monitor enzymatic hydrolyses of peptides [38,39]. EH is a very "soft" ionization technique in which no fragmentation is observed [4]. This makes EH an attractive tool to monitor enzymatic hydrolyses of peptides because there is less cluttering of the mass spectrum due to fragment ions (as in FAB). EH ionization relies upon a high electric field (10^6V cm^{-1}) to desorb ions from a solution. Experimental details will be shown in Chapter 2. Basically, a liquid sample flows into a metal capillary emitter which is biased at high voltage (typically 8kV). Historically,

vacuum constraints required the use of non-volatile solvents (such as glycerol). This constraint was relieved partly by Cook and Murawski [38] and Bel and Cook [39] and has been further relieved in the work to be described here. The tip of the emitter is positioned in the plane of an extractor electrode which is biased at about -2kV. Ions are essentially "pulled" from the solution to the gas phase and are then mass analyzed. Cook and Murawski used EHMS to monitor the hydrolysis of N-CBZ-Phe-Leu (carbobenzoxy-phenylalanyl-leucine) by CPY using a matrix of 70/30 (v/v) glycerol/water with NaI as a supporting electrolyte [38]. Six mass spectra were recorded over 4 hours. The intact substrate was not detected; however, despite appreciable scatter in the data, ions due to the products (N-CBZ-Phe and Leu) were detected. Their apparatus required the placement of a syringe (containing the reaction mixture) within the ion source. The presence of the glycerol slowed the kinetics of the reaction.

Bel and Cook [39] improved on the system of Cook and Murawski by changing the MS matrix in order to increase the sampling efficiencies of the substrate and hydrolysis products. They used EHMS to monitor a CPY-catalyzed hydrolysis of a pentapeptide using a 60/40 (v/v) thiodiethanol (TDE)/water matrix with NaI as supporting electrolyte. Mass spectra were recorded at roughly 90 minute intervals (with gaps) over a 20 hour period. Molecular ions of the intact peptide and hydrolysis products were detected; however, Bel and Cook noted that the amino acids appeared in the mass spectra before the corresponding co-products, indicating that sensitivity was better for the amino acids than for the peptides. Bel and Cook's apparatus was similar to Murawski and Cook's and thus required the TDE to be present in the reaction mixture, which slowed the hydrolysis. Increasing the water content above 40% led to

unstable emission of ions.

The goal of this research is to improve on the system of Bel and Cook in monitoring enzymatic hydrolyses of peptides using EHMS. In order to accomplish this, a new sample introduction technique was developed that allows for the biochemical reaction to occur in a totally aqueous environment, thus avoiding deactivation of the enzyme due to the organic cosolvent. The MS matrix is added post-reaction prior to entering the mass spectrometer. Continuous monitoring of the reaction mixture should allow for the determination of rate constants and other kinetic parameters as well as sequence information.

Chapter 2

Experimental

All mass spectrometric and optical rate determinations were obtained in triplicate. Unless otherwise noted, standard deviations for rates are pooled standard deviations of the replicate rate measurements. The pooled standard deviations were used with corresponding "t" values [43] to calculate 95% confidence limits. For hydrolyses of protected dipeptides, initial velocities were determined under conditions in which the amount of peptide hydrolyzed was not more than approximately 30%. Reactions were not followed to completion.

IUPAC standard three-letter abbreviations for amino acids are used throughout this work. The following abbreviations are used: Ala = alanine; Gly = glycine; Leu = leucine; Phe = phenylalanine; Tyr = tyrosine; Val = valine. For oligopeptides, the first amino acid listed corresponds to the amino terminus.

2.1 Mass Spectrometry

Electrohydrodynamic ionization (EH) mass spectrometry (MS) was performed using an AEI MS-902 mass spectrometer. A block diagram of this double focusing instrument is shown in Figure 2.1. A VG Update electronics console and an off-axis conversion dynode (-5 kV) and electron multiplier detector had been added prior to these studies as equipment upgrades. Data acquisition, data processing, and instrument control were provided by a Vector 2 data system from Teknivent running on a Zenith 386-25 personal computer.

Data was acquired using magnet scans in the "histogram mode" at a scan

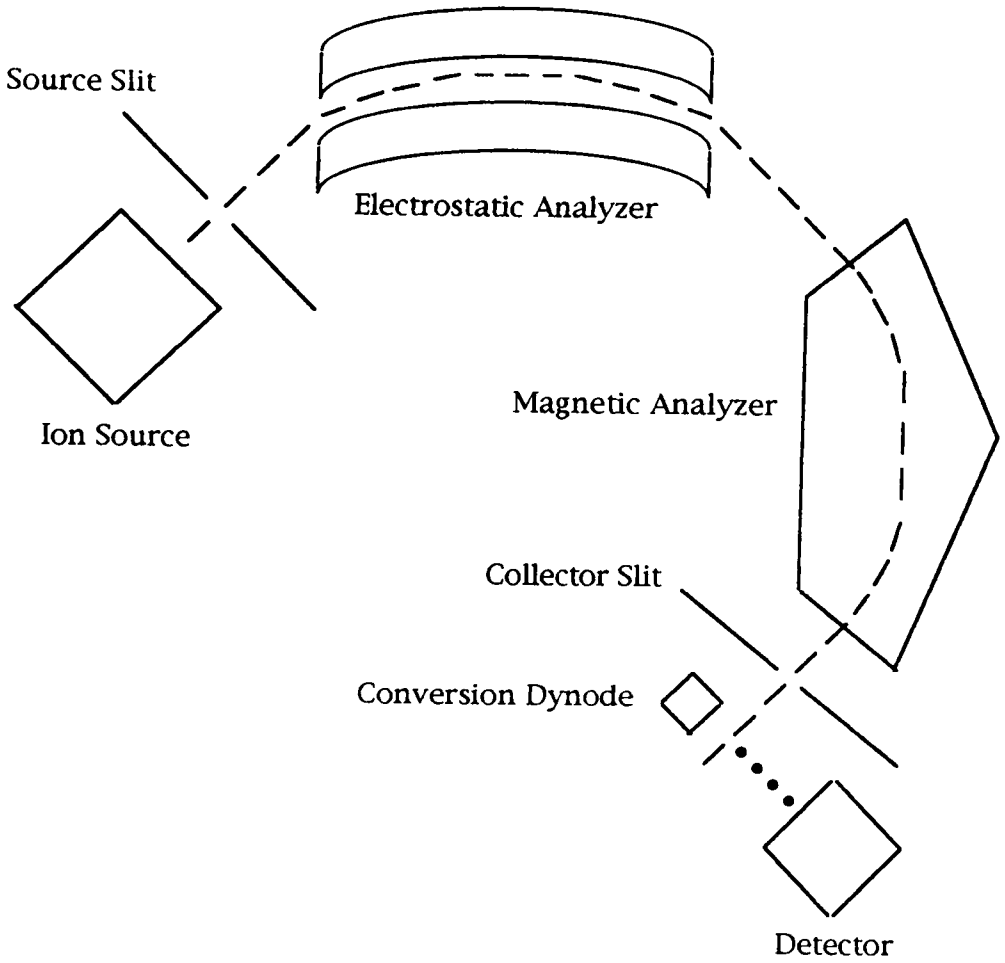


Figure 2.1. Block diagram of the AEI MS-902.

rate of about 30 seconds/decade over a mass range of 600-50 daltons. In the histogram mode, mass-to-charge ratio (m/z) centroids from raw data peak profiles are calculated by the data system. A mass spectrum for each scan is recorded as a bar graph (histogram) of peak intensity versus the m/z centroid. Maximum sensitivity was achieved by fully opening the source and detector slits on the MS902, giving a resolution of ca. 300. All mass spectrometric data was smoothed (10 scans at a time) using a sliding Savitzky-Golay smoothing routine in the following fashion: A plot of the intensity of each analyte (amino acid) peak versus time was smoothed. A plot of the intensity of the internal standard (alanine) peak versus time was also smoothed. The smoothed intensity of the analyte peak at a given time was then divided by the smoothed intensity of the internal standard peak at the same time. The data were treated in this manner, instead of averaging the ratio of the analyte peak to the internal standard peak from all scans prior to smoothing, in order to lessen the effect of variations in the signal of both peaks within each scan. Hydrolyses of protected dipeptides and leucine-enkephalin were performed in triplicate.

Figure 2.2 depicts the EH ion source (refer to Table 2.1 for parts list). This represents a modification of the EH source described previously [44,45]. It was changed in order to facilitate introduction of aqueous samples. Source voltages of 8.0 kV emitter, -1.5 to -2 kV extractor, and 0 V collector were used. A sample flowing through a polyimide-coated fused silica capillary (25 μm i.d., 144 μm o.d., typically 50 cm long; Polymicro Technologies) reaches the platinum emitter (200 μm i.d., 400 μm o.d., 1 in. length; Hamilton). Electrical connection from the Pt emitter to the sample occurs from sample overflow from the fused silica capillary. This creates a "sample pool" in the tip of the Pt emitter (see Figure

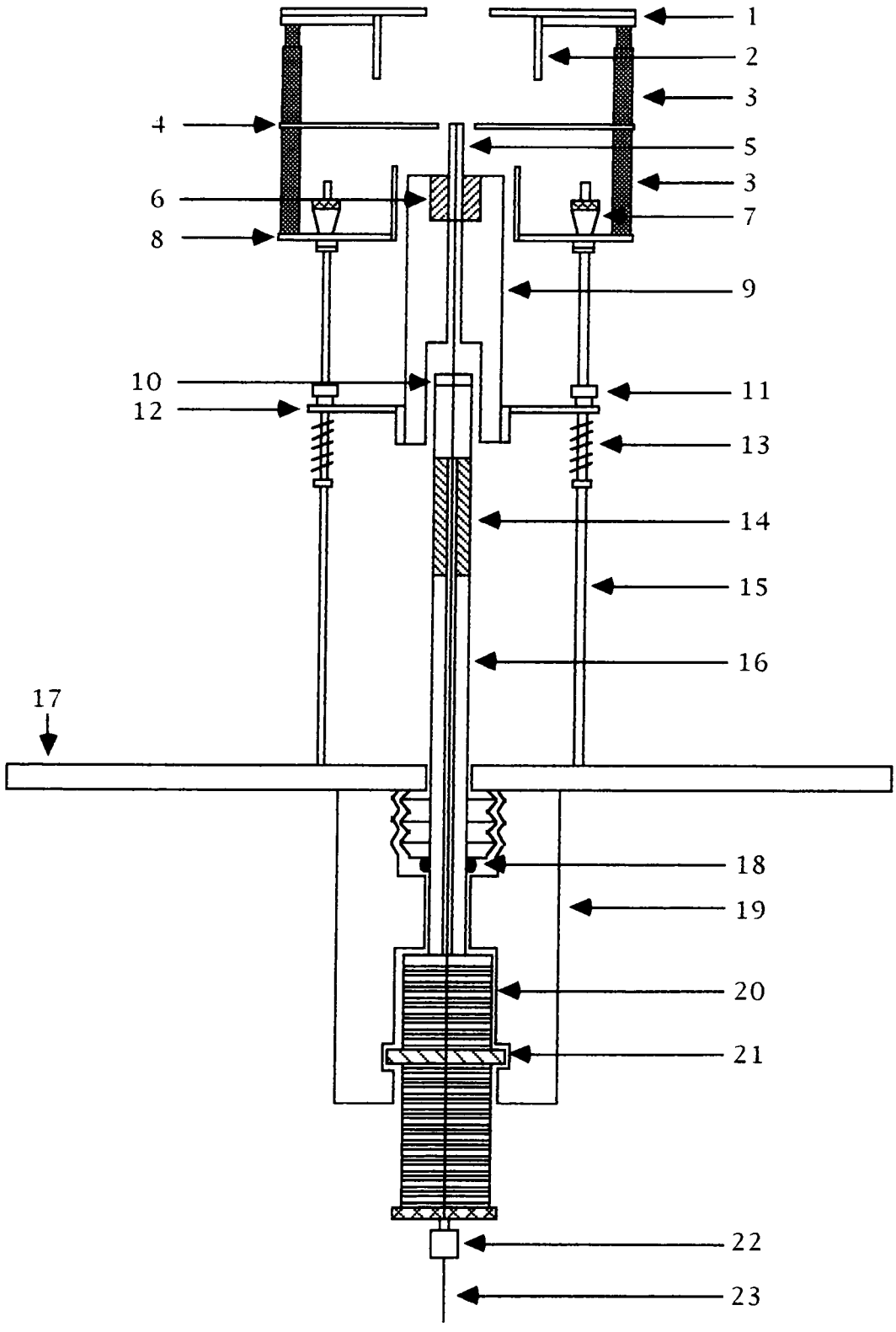


Figure 2.2. EH ion source. Refer to Table 2.1 for parts list.

Table 2.1. Parts list for EH ion source shown in Figure 2.2.

Part Number	Description
1	stainless steel (ss) collector electrode
2	ss sputter shield
3	delrin insulators
4	ss extractor electrode
5	200 μm i.d. Pt emitter
6	brass cylinder
7	ss mounting nuts
8	ss mounting plate
9	teflon support sleeve
10	1/16" Swagelok ss union containing 0.3 mm vespel-graphite ferrule to hold silica capillary
11	ss alignment nuts
12	ss support sleeve holder
13	spring for tilt adjustment
14	delrin insulator
15	ss threaded support rod
16	brass shaft
17	ss vacuum flange
18	rubber o-ring
19	aluminum capillary positioner body
20	threaded portion of brass capillary positioner shaft (40 threads/in.)

Table 2.1 (continued).

Part Number	Description
21	brass knurled nut
22	1/8" Swagelok ss fitting containing 0.3 mm teflon ferrule to provide vacuum seal
23	25 μm i.d., 144 μm o.d. fused silica capillary

2.3). A small amount of low-volatility co-solvent, such as thioglycerol (TG) or glycerol, is required for operation of this modified EH source, presumably in order to maintain a small reservoir (of a few nL) and thus electrical connection between emitter and sample. The positioning of the fused silica capillary inside the Pt emitter is extremely important in order to achieve stable emission (Figure 2.3). Positioning is attained by use of the capillary positioner [46] shown at the bottom of Figure 2.2. Analyzer and source pressures were typically 7×10^{-7} torr and 1×10^{-5} torr, respectively. Analyzer pressures were measured with a Fil-Tech ionization gauge (using an HPS Division Model 919 gauge controller) and maintained using a Balzers TPH 270 turbomolecular pump. Source pressures were also measured with a Fil-Tech ionization gauge (using a Series 260 Granville Phillips gauge controller) and were maintained with an Edwards Series MK2 Diffstak diffusion pump with a liquid nitrogen cold trap.

In order to use EHMS to monitor the progress of a hydrolysis reaction taking place in a totally aqueous environment, a method was developed in which the aqueous reaction mixture was combined "post-reaction" with a solution containing an organic co-solvent (MS matrix) just before entering the mass spectrometer (see Figure 2.4 and Table 2.2). The buffered reaction mixture was mixed with a water/thioglycerol (TG) solution containing para-toluenesulfonic acid (PTSA) in a stainless steel low dead volume tee (Swagelok; SS-1F0-3GC). For studies involving catalysis by the enzyme carboxypeptidase-Y (CPY), reaction ceased at this point because the TG completely deactivates the enzyme [12]. The PTSA served as supporting electrolyte and as a source of protons. This new sample introduction technique is described in detail below.

Two syringe pumps (Harvard Apparatus, Model 11), each accommodating a

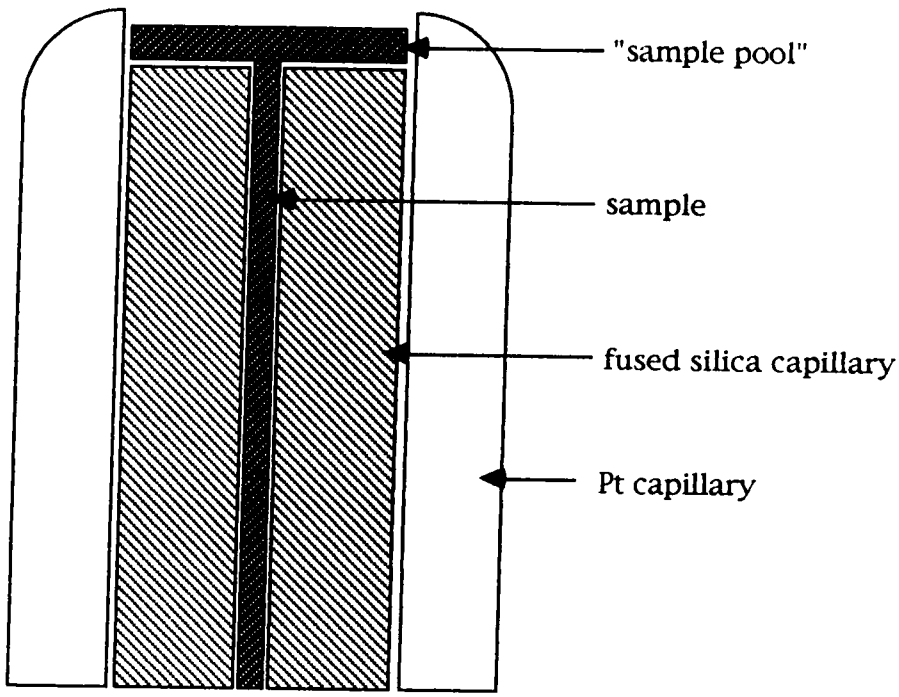


Figure 2.3. "Sample pool" at the tip of the Pt emitter.

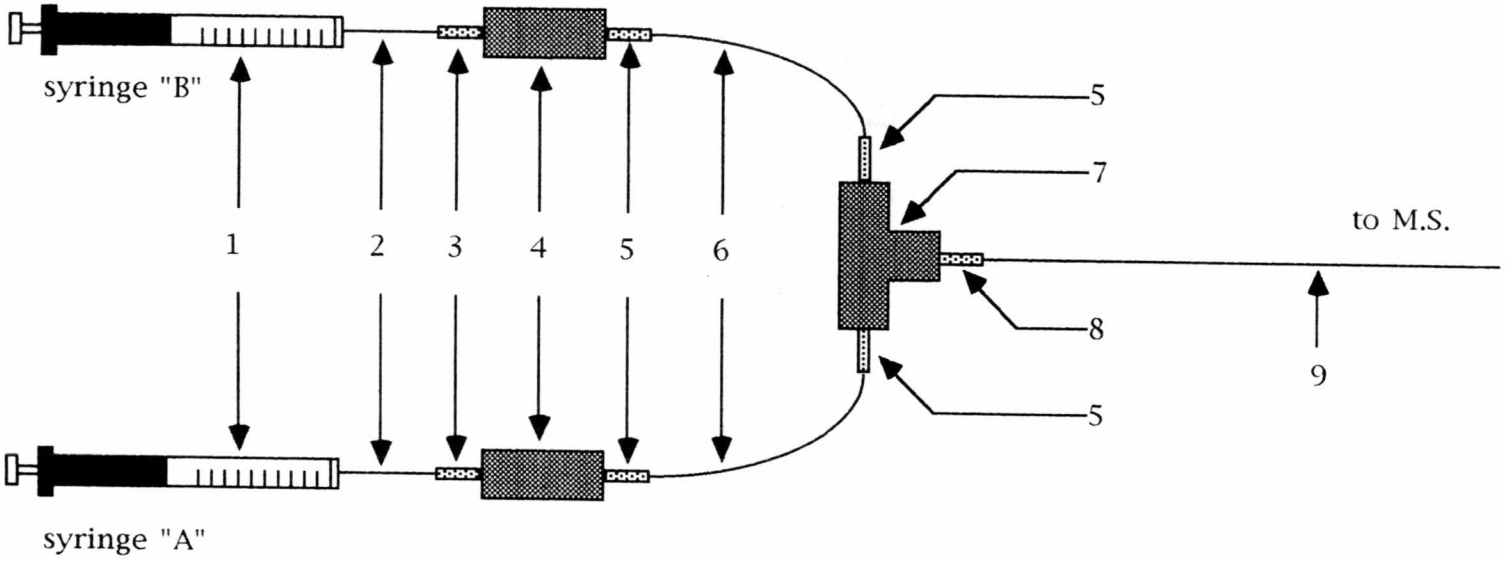


Figure 2.4. Tee assembly (not to scale). Refer to Table 2.2 for parts list.

Table 2.2. Parts list for tee assembly shown in Figure 2.4.

Part Number	Description
1	25 μL gastight syringe
2	syringe needle (0.006" i.d., 0.028" o.d.)
3	polyetheretherketone (PEEK) tubing (0.030" i.d., 0.0625" o.d.)
4	stainless steel (ss) low dead volume union
5	PEEK tubing (0.020" i.d., 0.0625" o.d.)
6	15 cm of fused silica capillary (25 μm i.d., 365 μm o.d.)
7	ss low dead volume tee
8	PEEK tubing (0.007" i.d., 0.0625" o.d.)
9	50 cm of fused silica capillary (25 μm i.d., 144 μm o.d.)

25 μL gastight syringe (Hamilton, Model 84880), were employed. The flow rate of each syringe pump was typically 2.7 μL/hr. The removable stainless steel syringe needles (Hamilton, RN 80464; 0.006 in. i.d., 0.028 in. o.d.) used were square cut at 90°. Each syringe needle fit into a 1/16" stainless steel low dead volume (0.066 μL) union (Swagelok; SS-1F0-6GC). Because these unions were designed for 1/16" tubing, each syringe needle was threaded through a one inch long square cut piece of polyetheretherketone (PEEK) tubing (Alltech; 0.030 in. i.d., 0.0625 in. o.d.) which, in turn, fit into the union. The needle and PEEK sheath were both inserted as far as possible into the union to minimize dead volume. Stainless steel ferrules were used in all fittings to seal to the PEEK tubing, which in turn held the syringe needles (or fused silica capillaries, as described below) in place. From each low dead volume union ran 15 cm of fused silica capillary (25 μm i.d., 365 μm o.d.; Polymicro Technologies) threaded at each end through one inch pieces of PEEK tubing (Alltech; 0.020 in. i.d., 0.0625 in. o.d.). These capillaries ran to a 1/16" stainless steel low dead volume (0.28 μL) tee (Swagelok; SS-1F0-3GC). Finally, from the tee emerged a 50 cm piece of fused silica capillary (25 μm i.d., 144 μm o.d.; Polymicro Technologies) that was sealed to the tee using a one inch piece of PEEK tubing (Alltech; 0.007 in. i.d., 0.0625 in. o.d.) in the same way as described above. This capillary ran from the tee through the positioner to the emitter inside the mass spectrometer (Figure 2.2).

For temperature control of the reaction, the following modification was made. Between the syringe containing the buffered, aqueous reaction mixture and the low dead volume tee was attached a 10 μL "reaction reservoir" which consisted of 5.09 m of fused silica capillary (50 μm i.d., 144 μm o.d.; Polymicro Technologies) wrapped into a coil and submerged in a constant temperature bath

(Haake Model FS) maintained at 25 ($\pm$ 0.1)^{O}C (see Figure 2.5 and Table 2.3). This experimental design gave rise to two periods during which the reaction solution was not temperature-regulated. Refer to Figure 2.5. The reaction mixture in syringe A was not at constant temperature. The capillary from the syringe to the constant temperature bath (approximately 0.39 μL corresponding to 8.5 minutes at the flowrate employed) and from the bath to the tee (approximately 0.36 μL corresponding to 8 minutes) was not temperature-regulated. Of the 509 cm of capillary used for the reaction reservoir, approximately 475 cm (corresponding to 9.3 μL) were actually submerged in the constant temperature bath. Hydrolyses of the protected dipeptides typically lasted 120-150 minutes, which required 5.4-6.8 μL of the reaction mixture. During hydrolyses of protected dipeptides, the reaction mixture was therefore temperature-regulated 93-95% of the time. Hydrolyses of leucine-enkephalin lasted 24 hours, which required about 65 μL of the reaction mixture (syringes had to be refilled twice). During the hydrolyses of leucine-enkephalin, the reaction mixture was temperature-regulated only 27% of the time. This may have caused a systematic error.

The syringes had metal handles (see Figure 2.6) and therefore had to be insulated to prevent electrical leakage through the solution to the handles and then to the syringe pumps. Electrical insulation was accomplished by threading the barrel and handle of each syringe through pieces of rubber stoppers that had holes cut out of them. The pieces of rubber stoppers were placed at points on the metal handles where the syringes came into contact with the pumps. The metal plungers were isolated from the pumps using 1/8" pieces of plexiglass placed between the end of the plunger (see Figure 2.6) and the syringe pump.

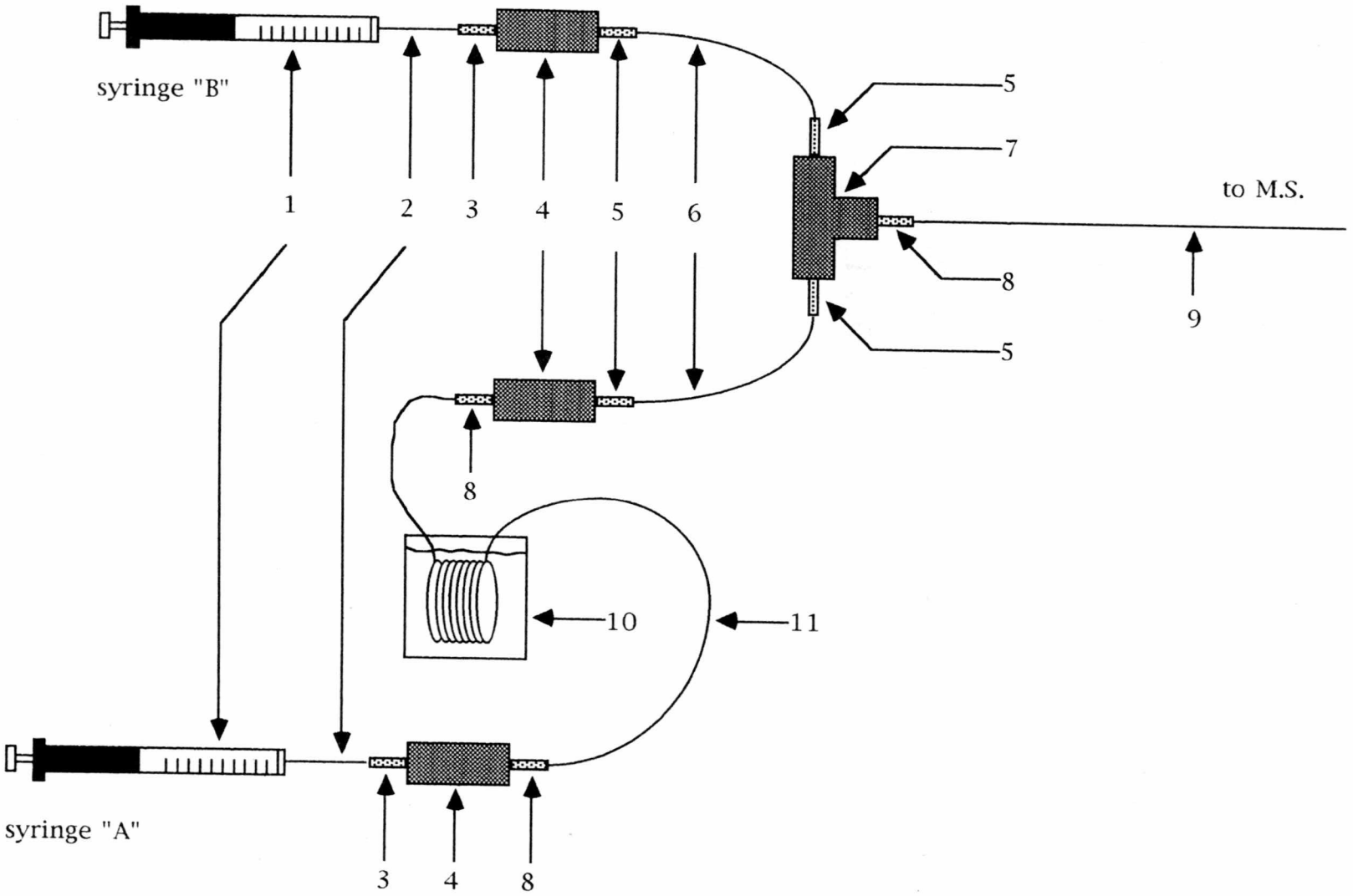


Figure 2.5. Tee assembly with temperature bath (not to scale). Refer to Table 2.3 for parts list.

Table 2.3. Parts list for tee assembly shown in Figure 2.5.

Part Number	Description
1	25 μL gastight syringe
2	syringe needle (0.006" i.d., 0.028" o.d.)
3	polyetheretherketone (PEEK) tubing (0.030" i.d., 0.0625" o.d.)
4	stainless steel (ss) low dead volume union
5	PEEK tubing (0.020" i.d., 0.0625" o.d.)
6	15 cm of fused silica capillary (25 μm i.d., 365 μm o.d.)
7	ss low dead volume tee
8	PEEK tubing (0.007" i.d., 0.0625" o.d.)
9	50 cm of fused silica capillary (25 μm i.d., 144 μm o.d.)
10	constant temperature water bath
11	5.09 m of fused silica capillary (25 μm i.d., 144 μm o.d.)

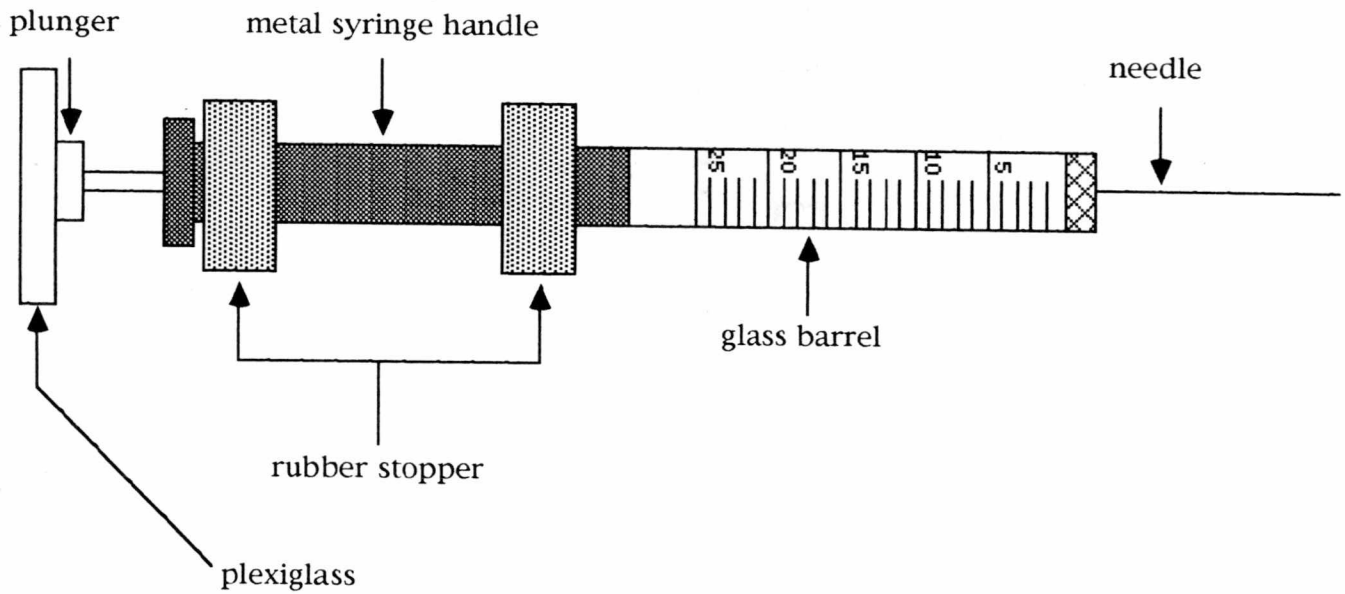


Figure 2.6. 25 microliter gastight syringe.

2.2 Optical Spectroscopy

A Hewlett Packard model HP8452 photodiode array UV/Vis spectrophotometer was used for absorbance measurements. This is a single beam instrument. The absorbance was determined at a wavelength of 570 nm, which is the observed λ_{max} for the complex of ninhydrin with amino acids or peptides (see Figure 2.7). The manufacturer-reported precision for the absorbance agrees with the experimental precision (determined by reading the absorbance of a ninhydrin-amino acid complex several times while refilling the cuvette each time) of $\sigma = \pm 0.002$ absorbance units. The specified precision for the wavelength was $\pm$ 2 nm. A quartz cuvette with a 1 cm pathlength was employed. Water served as the blank.

2.3 Additional Instruments

An Orion Research Model 701A pH meter with a combination glass electrode (Corning Model 476531) was used for all pH measurements. This instrument was calibrated with pH 4.0 and pH 7.0 standard buffer solutions.

2.4 Preparation of Solutions

Distilled water passed through a Milli-Q Continental Water Systems Corporation (Model ZD20 115 84) purification system to obtain a resistance of at least 18 MΩ cm^{-1} was used to prepare all aqueous solutions. A list of all other reagents used can be found in Table 2.4.

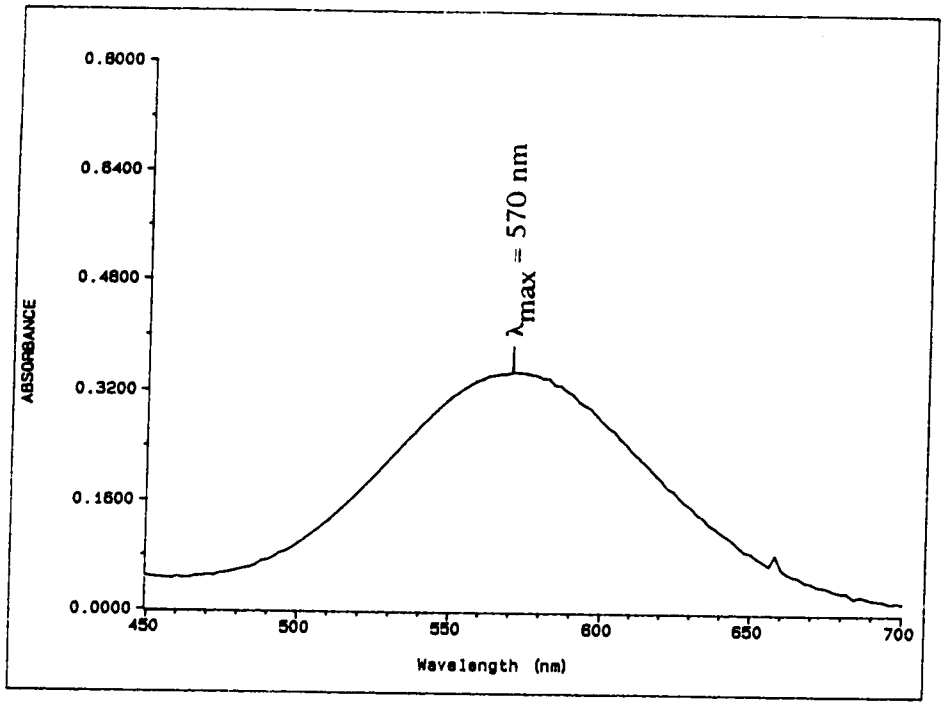


Figure 2.7. Absorption spectrum of the leucine-ninhydrin complex in 0.29 mM acetate buffer showing λ_{max} at 570 nm. The concentrations of leucine and ninhydrin are approximately 0.016 mM and 1.7 mM, respectively.

Table 2.4. List of reagents used.

Reagent	Supplier	Purity	Lot #
L-alanine	Sigma	98%	101F-0534
acetic acid	Mallinckrodt	Reagent Grade	2504 KHSR
carboxypeptidase-Y (lyophilized powder)	Sigma	20%	92H8070
N-carbobenzoxy-phenyl-alanyl-glycine	Sigma	98%	11H0715
N-carbobenzoxy-phenyl-alanyl-leucine	Sigma	98%	94F-0545
N-carbobenzoxy-valyl-leucine	Sigma	98%	84F-0007
ethylene glycol monomethyl ether	Mallinckrodt	Reagent Grade	5003 KJVS
glycine	Sigma	99%	101F-0406
hydrindantin	Sigma	99%	79F0943
L-leucine	Sigma	98%	36F-0765
leucine-enkephalin (acetate salt)	Sigma	99%	16F-58201
ninhydrin	Sigma	Sigma Grade	39F0692
pH 4 standard buffer solution	Baxter	Reagent Grade	M028
pH 7 standard buffer solution	Baxter	Reagent Grade	D028
L-phenylalanine	Sigma	98%	32H0521

Table 2.4 (continued).

Reagent	Supplier	Purity	Lot #
potassium acetate	Mallinckrodt	Reagent Grade	6700 KHTT
potassium hydroxide	Mallinckrodt	Reagent Grade	6984 KLKE
2-propanol	Mallinckrodt	Reagent Grade	3032 KLNE
sodium acetate trihydrate	Mallinckrodt	Reagent Grade	7364 KMDZ
sodium iodide	Mallinckrodt	Reagent Grade	1141 KBPP
thiodiethanol	Aldrich	99%	07602HW
thioglycerol	Sigma	98%	33H1116
p-toluenesulfonic acid monohydrate	Aldrich	98.5%	4603HE
Tyrosylglycylglycine	Sigma	97%	125F01471

Sodium Acetate Buffer: Glacial acetic acid was added dropwise, with stirring, to 60 mL of 2 M sodium acetate until a pH of 5.5 was reached. This solution was diluted to 100 mL with water in a volumetric flask. The final concentration of the buffer was approximately 1.2 M. This buffer was stored in the refrigerator ($4^{o}C$) and used to prepare the ninhydrin reagent solution described below [5].

Ninhydrin Reagent: 25 mL of ethylene glycol monomethyl ether was pipetted into a 500 mL dark amber glass bottle. Nitrogen gas was slowly bubbled through the solvent for 10 minutes before approximately 0.05 g of hydrindantin and 0.66 g of ninhydrin were added to the bottle. The contents were stirred until dissolved. Nitrogen was bubbled through this solution continuously. Next, 8.3 mL of the sodium acetate buffer solution was added. The solution at this stage turned red. The solution was stored in the amber bottle to avoid exposure to light, which decomposes the ninhydrin reagent and causes the red color to fade to a pale yellow. The concentration of this ninhydrin solution was approximately 0.120 M. Ninhydrin solution was used only on the day of its preparation due to its unstable nature [5].

Potassium Acetate Buffer: 25 mM acetic acid was added dropwise, with stirring, to 25 mM potassium acetate solution until a pH of 6 was obtained. For mass spectrometric experiments, sufficient alanine was added to make the buffer 6.7 mM in the amino acid, and the solution was filtered through a Gelman Metricel filter (0.45 μm). A 20 mM potassium acetate buffer solution containing 5.4 mM alanine was prepared by diluting the buffer solution containing alanine with water. These were also stored in the refrigerator.

Water/Thioglycerol Solution: A solution that was about 100 mM p-toluenesulfonic acid (PTSA) and 5.4 mM alanine (internal standard) in 60/40 (v/v) water/thioglycerol (TG) was prepared. This solution was filtered as described above and stored in the refrigerator.

"Blank" Solution: The blank solution was prepared by mixing equal volumes of the water/thioglycerol solution and the 20 mM potassium acetate buffer solution containing 5.4 mM alanine. The final concentrations of the components in the blank solution were 10 mM acetate, 5.4 mM alanine, and 50 mM PTSA in 80/20 (v/v) water/TG.

Amino Acid Stock Solutions: 5 mM leucine was prepared in 20 mM potassium acetate buffer. Dilutions of this solution were made with 20 mM potassium acetate buffer to obtain solutions that were 1-4 mM leucine. These solutions were used to obtain a leucine calibration curve for the optical experiments involving protected dipeptides. A 5 mM glycine stock solution and subsequent dilutions with potassium acetate buffer were prepared in the same manner and were used to obtain an optical calibration curve for these dipeptide experiments.

A second leucine stock solution that was 40 mM leucine in 20 mM potassium acetate buffer was prepared, and dilutions of this solution were made with 20 mM potassium acetate buffer to give solutions that were 10-30 mM leucine. These solutions were used to obtain a leucine calibration curve for the optical experiments with leucine-enkephalin in which the protocol for the ninhydrin method was modified (as described later in this chapter).

For the mass spectrometric experiments, 16 mM leucine was prepared in the 20 mM potassium acetate buffer containing 5.4 mM alanine. Dilutions of this solution were made with the 20 mM potassium acetate buffer/5.4 mM alanine to obtain solutions that were 4-12 mM leucine. Stock solutions (mass spectrometric) for glycine and phenylalanine were prepared in the same manner each at a concentration of 10.0 mM. Dilutions of these solutions were made with the 20 mM potassium acetate buffer/5.4 mM alanine to obtain solutions that were 1.25-5 mM in the amino acid.

Carboxypeptidase-Y (CPY) Solution: A stock solution containing 44.4 units CPY/mL was prepared. One unit of activity is defined as the amount of enzyme that hydrolyzes 1 μmole of the substrate N-CBZ-Phe-Leu per minute at a pH of 6.75 at 25°C [2]. The enzyme was obtained from Sigma as the lyophilized powder containing approximately 20% protein. The manufacturer-reported sample specification for the enzyme was 100 units/mg of protein. Thus, a 10 mg sample of "enzyme" (containing 2 mg of protein) was dissolved in 4.5 mL of water to give a solution of 44.4 units of CPY/mL. This stock solution was stored in the refrigerator at 4°C for at least 24 hours prior to use to restore its activity [2]. Dilutions of this stock solution were made prior to each experiment as described in the Protocol section. Full activity of the enzyme was confirmed using the method of Hayashi [10]: The ninhydrin method was used to determine the extent of hydrolysis of N-CBZ-Phe-Leu (0.5 mM) by CPY (1μg/mL) after a 10 minute reaction time. "Approximately 30% hydrolysis occurs" under these conditions [10]. It was found that 29% hydrolysis of N-CBZ-Phe-Leu occurred using the assay for peptidase activity as described by Hayashi [10].

2.5 Protocol

2.5.1 Mass Spectrometry

The following procedure was used when doing mass spectrometry. Refer to Figure 2.4 for the following discussion. A 500 μL gastight syringe (Hamilton; TLL 81220) was connected to the source end of capillary #9 and was used (with a syringe pump) to back-flush the tee (#7) and capillaries (#9 and #6) with 40 mM potassium hydroxide for approximately 30 minutes. (Syringes A and B were not connected to the tee assembly at this time.) Approximately 100 μL was flushed through the tee during this time, or roughly 140 times the total volume of the tee and capillaries. Subsequently, the tee was rinsed by back-flushing in the same manner with water for about 30 minutes. Next, syringes A and B were attached to the unions (#4) and the syringe pumps were used to flush the tee for at least 30 minutes with the "blank" sample. The flow rate of each syringe pump during this process was 3 μL/hr. (During data acquisition, the flow rate of each syringe pump was 2.7 μL/hr.) This procedure up to this point serves to clean the tee and capillaries [47-49] as well as precondition the capillaries with a sample similar to that about to be run. (However, it contaminates the reaction syringe with TG and PTSA; see below.) The 10 μL reaction reservoir (#11 in Figure 2.5) was cleaned by flushing with 20 μL of 40 mM KOH solution followed by 20 μL of water. It was then preconditioned by flushing with about 20 μL of a solution containing 5.4 mM alanine in 20 mM potassium acetate buffer.

EHMS was always performed first with the blank sample in both syringes (using the configuration in Figure 2.4) to obtain stable emission. This served as a "check" to make sure everything was working properly (i.e., no leaks, electrical or

physical). Once the present configuration of the tee assembly was decided upon (see Appendix C), no problems such as electrical leakage or sample leakage were encountered. The blank sample was run for at least 30-45 minutes. During this time, spectra were usually not recorded. Next, syringe A (see Figure 2.5) was rinsed and filled with a solution containing 5.4 mM alanine in 20 mM potassium acetate buffer, and syringe B was filled with the 60/40 (v/v) water/TG solution described in Section 2.4. The 10 μL reaction reservoir was attached (see Figure 2.5), and EHMS was performed for at least another 30-45 minutes. Finally, the potassium acetate buffer solution was replaced with either an amino acid standard solution or a hydrolysis mixture. The 10 μL reaction reservoir was only used with hydrolysis experiments (not for calibration curves).

To obtain mass spectrometric calibration curves, data was collected for 30 minutes with each standard (in syringe A; matrix in syringe B). The 30 minute time interval was chosen to assure steady state emission. The data was smoothed (10 scans at a time) using the sliding Savitzky-Golay smoothing routine as described previously. Ratios (analyte: internal standard) from the smoothed data were then calculated and averaged in order to obtain an average and standard deviation (error bar) for the calibration curve.

For the hydrolysis of N-CBZ-Val-Leu, the stock solution containing 44.4 units CPY/mL was diluted 1:11 with water to produce a solution that was 4.04 units CPY/mL. A 200 μL volume of this solution was pipetted (using an SMI Airpettor pipet) into a cylindrical glass vial (approximately 3 mL) containing 800 μL of a solution that was 6.33 mM N-CBZ-Val-Leu and 6.7 mM alanine in 25 mM potassium acetate buffer. (Neither the enzyme nor the substrate solutions was thermostatted at 25°C prior to use. Room temperature was typically 22-24°C.)

Assuming a neglible volume of mixing, this produced 1 mL of a solution that was 5.06 mM N-CBZ-Val-Leu and 5.4 mM alanine in 20 mM potassium acetate buffer with 0.8 unit CPY/mL. This sample was mixed for 5 seconds after the addition of the CPY using a Thermolyne Model 37600 vortex mixer. Syringe A was filled with this reaction mixture (after being rinsed with it at least 3 times), the 10 μL reaction reservoir was flushed with 20 μL of the reaction mixture, syringe A was refilled with the reaction mixture, and EHMS was used to monitor the hydrolysis for 2.5 hours. Hydrolyses were also monitored mass spectrometrically with this substrate at a concentration of 4.45 mM (concentration after addition of CPY). Hydrolyses were performed in triplicate and initial rates were averaged.

For the hydrolysis of N-CBZ-Phe-Gly, 100 μL of the 44.4 units CPY/mL stock solution was diluted to 8.8 mL with water to produce a solution that was 0.505 unit CPY/mL. A 200 μL volume of this solution was mixed with 800 μL of a solution containing N-CBZ-Phe-Gly and 6.7 mM alanine in 25 mM potassium acetate buffer. (Both the enzyme and the substrate solutions were thermostatted at 25^{o}C prior to use.) Substrate concentrations after addition of CPY were 7.95, 5.93, 3.98, 3.23, and 2.39 mM in solutions containing 5.4 mM alanine, 20 mM potassium acetate, and 0.1 unit CPY/mL. After mixing, rinsing the reaction reservoir, and filling the syringe (as above), each hydrolysis was monitored for 2 hours. Hydrolyses were performed in triplicate and initial rates were averaged.

For the hydrolysis of leucine-enkephalin, 100 μL of the 44.4 units CPY/mL stock solution was diluted to 400 μL with water to produce a solution that was 11.1 units CPY/mL. A 200 μL volume of this solution was mixed with 800 μL of a solution that was 19.0 mM leucine-enkephalin and 6.7 mM alanine in 25 mM potassium acetate buffer. (Both the enzyme and the substrate solutions were

thermostatted at 25°C prior to use.) Assuming a neglible volume of mixing, this produced 1 mL of a solution that was 15.2 mM leucine-enkephalin and 5.4 mM alanine in 20 mM potassium acetate buffer with 2.2 units CPY/mL. Hydrolyses were performed in triplicate and were monitored continuously for 24 hours. The syringes had to be refilled twice during the hydrolysis. The reaction mixture used to refill the syringes was thermostatted. A hydrolysis was also monitored at a final substrate concentration of 6.1 mM. This hydrolysis was not replicated and was monitored for 3 hours.

2.5.2 Optical Spectroscopy

The following procedure for the formation of the ninhydrin complex is based on that described by Moore and Stein [5]. A 1 mL volume of an analyte or standard solution was placed in a test tube (15 cm long, 15 mm diameter), which was then incubated in the constant temperature bath (at 25°C) for at least 10 minutes. A 1 mL volume of ninhydrin solution (Section 2.4) was then added, and the test tube was immediately immersed in a boiling water bath to allow formation of the ninhydrin complex. The test tube was kept in the boiling water bath for 15 minutes. Maximum color development occurs under these conditions [5]. Afterwards, the test tube was placed in an ice water bath for at least 10 minutes. The solution was then further diluted with 5 mL of a 50/50 (v/v) 2-propanol/water mixture. The solution was then placed back into the 25°C constant temperature bath for at least 10 minutes. A 1 mL aliquot of the solution was finally diluted with 9 mL of the 2-propanol/water mixture. The absorbance of this solution was then measured at 570 nm using the HP8452 UV/Vis

spectrophotometer described previously. Water was used as the blank.

Calibration curve data was obtained in triplicate for both leucine and glycine. The whole procedure of forming the complex was replicated. The average absorbance from each of five standard solutions (1-5 mM leucine or glycine in 20 mM potassium acetate buffer) was plotted versus concentration to provide calibration curves for leucine and glycine.

For the hydrolysis of N-CBZ-Val-Leu, 800 μL of 5.51 mM N-CBZ-Val-Leu in 25 mM acetate buffer were placed in each of four test tubes. These were put in the constant temperature bath (25°C) for at least 10 minutes. The stock solution of 44.4 units CPY/mL was diluted 1:11 with water to produce a solution that was 4.04 units CPY/mL. (This solution was not thermostatted at 25°C prior to use.) Before starting the hydrolysis, each test tube was removed momentarily from the temperature bath to allow addition of 200 μL of 4.04 units CPY/mL and mixing. The total volume in the test tube was 1 mL, assuming neglible volume of mixing. The final substrate and enzyme concentrations were 4.41 mM and 0.8 unit CPY/mL, respectively. Except for the omission of alanine (used as an internal standard in the EHMS experiments), these solutions duplicated those loaded into syringe A in Section 2.5.1 above. Alanine was omitted to reduce the background absorbance. The solutions were mixed for 5 seconds and then placed back into the temperature bath. After 15 minutes, the reaction in the first test tube was quenched by the addition of 1 mL of ninhydrin solution, followed by immersion in a boiling water bath. The remainder of the ninhydrin experiment, as described above, was performed.

The same procedure was used with the remaining three test tubes, with the reaction time increasing from 15 minutes to 60 minutes in 15 minute intervals

(hereafter referred to as the "reaction interval"). From the absorbance data, along with data from the leucine calibration curve, a plot of the concentration of leucine released versus time was obtained. Hydrolyses of N-CBZ-Val-Leu were repeated at a final peptide concentration (i.e., after the addition of CPY) of 2.60 mM. However, it was found that more than 30% of the substrate had been hydrolyzed within 60 minutes. Therefore, the linear approximation used in determining the initial velocity (as described in Chapter 1) is not valid. A shorter reaction interval of 10 minutes and a total reaction time of 40 minutes was used so that the initial velocity of the reaction could be determined (using 4 time/absorbance data pairs) before 30% of the peptide was hydrolyzed. Hydrolyses were performed in triplicate and initial rates were averaged.

Hydrolyses of N-CBZ-Phe-Gly were performed using a final CPY concentration of 0.1 unit CPY/mL and final substrate concentrations of 8.01, 5.92, 3.98, 3.21, and 2.40 mM. Again, this duplicated the corresponding MS solutions, except for the omission of alanine. The enzyme and substrate solutions were thermostatted at 25^{o}C prior to use. The reaction was monitored from 20 minutes to 80 minutes using a reaction interval of 20 minutes. Hydrolyses were performed in triplicate and initial rates from each replicate were averaged.

Hydrolyses of leucine-enkephalin were performed using a final CPY concentration of 2.2 units CPY/mL and a substrate concentration of 15.2 mM in 20 mM acetate buffer. Because of the larger concentration of substrate used and the larger background anticipated (due to the fact that the amino terminus of leucine-enkephalin was not protected with CBZ, and therefore could react with ninhydrin), the procedure for the quenching and subsequent dilutions of the reaction mixture was modified slightly. A single 3 mL reaction mixture was used

for each replicate. At specified time intervals, a 200 μL aliquot of the thermostatted reaction mixture was withdrawn and mixed with 200 μL of ninhydrin solution in a test tube, which was then immersed in boiling water for 15 minutes. The test tube was then placed in an ice water bath as described previously. A 9 mL volume of 50/50 (v/v) 2-propanol/water was added, and the solution in the test tube was mixed and thermostatted. From this solution, a 700 μL aliquot was added to 9 mL of the 2-propanol/water mixture, and the solution in this test tube was mixed. The absorbance of this solution was then determined. This modified procedure was necessary so that the resulting absorbances would not be greater than one absorbance unit. The reaction was monitored after 2 hours, 3 hours, and then every three hours for 24 hours (i.e. at 2, 3, 6, 9, . . ., 24 hours). Hydrolyses were performed in triplicate.

Because dilutions of the leucine-enkephalin reaction mixture were different than those of the protected dipeptide reaction mixtures in the formation of the ninhydrin complex (resulting in different final concentrations of water and concomitants, including buffer), a second leucine calibration curve was obtained for use with the data from the modified technique described in the previous paragraph. A 40 mM leucine solution in 20 mM potassium acetate buffer was prepared. Dilutions of this stock solution were made with acetate buffer to obtain solutions that were 10-30 mM leucine. Aliquots (200 μL) of these solutions were subjected to the same procedure as was used during the monitoring of the hydrolyses of the leucine-enkephalin, described in the previous paragraph.

Chapter 3

Results and Discussion

3.1 "Blank" Sample

To assess performance of the revised source, a "blank" sample comprised of 5.4 mM alanine, 50 mM para-toluenesulfonic acid (PTSA), and 10 mM potassium acetate in 80/20 (v/v) water/thioglycerol (TG) was run (using two syringes). This blank was chosen to represent (as closely as possible) the sample matrix. The "blank" sample was found to give stable emission during the electrohydrodynamic (EH) process. Subsequently, EH was always performed on the blank sample as a preliminary step in using the tee assembly. Although a phosphate buffer would be better suited for use at pH 6, the involatility of phosphate salts can lead to problems of clogging and unstable emission; use of the acetate buffer provided more stable operation with only slight compromise in buffer capacity. Such a compromise is not without precedent; Hayashi and coworkers [9] have used an acetate buffer in the pH range of 3.5-6.0 in hydrolyses of protected dipeptides monitored optically using the ninhydrin method.

Figure 3.1 A is an EH mass spectrum of the blank sample. Some of the more prominent peaks include protonated PTSA solvated with TG (suggesting that ion pairing is taking place), protonated TG, and potassium adducts of TG. Table 3.1 lists the identification of the major peaks in the mass spectrum. It is interesting to note the peak attributed to a protonated TG dimer at the m/z value of 215 probably resulting from the formation of a disulfide bond with elimination of H_2, possibly due to TG oxidation. Each thioglycerol molecule contains one sulfhydryl group, -SH. Easily oxidized, two -SH groups can be converted to disulfide

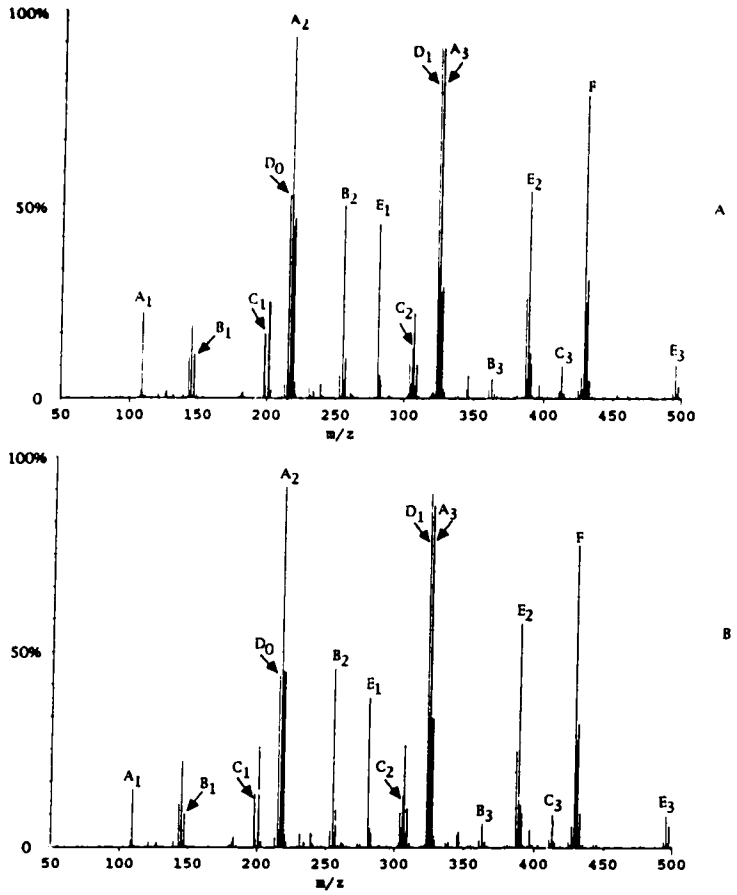


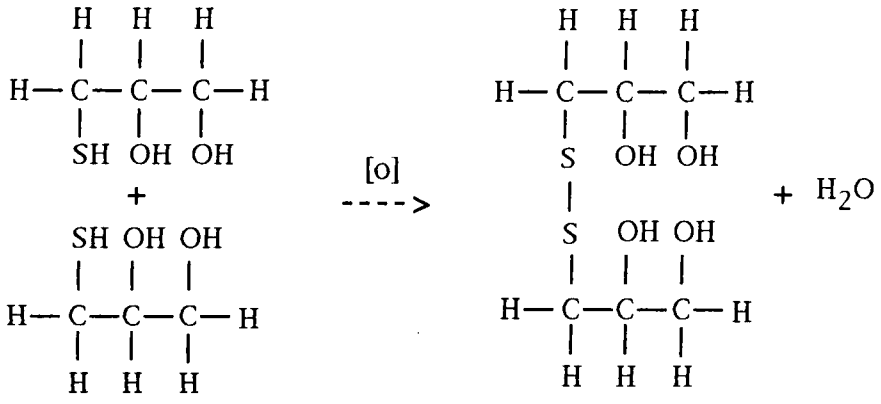
Figure 3.1. "Blank" sample (A) and "separated" sample (B) EH mass spectrum. See text for details. Each spectrum is an average of 60 scans. Series are labelled as follows: A_n = [H + nTG]$^+$; B_n = [K + nTG]$^+$; C_n = [H + Ala + nTG]$^+$; D_n = [H + TG dimer + nTG]$^+$; E_n = [H + PTSA + nTG]$^+$; F = [H + 2TG dimers]$^+$; where TG = thioglycerol, Ala = alanine, TG dimer = disulfide-linked oxidation product, and PTSA = para-toluenesulfonic acid. See Table 3.1 for identification of other prominent ions. "Blank" sample consisted of 50 mM PTSA, 5.4 mM Ala, and 10 mM potassium acetate in 80/20 (v/v) water/TG in both syringes. "Separated" sample consisted of 100 mM PTSA and 5.4 mM Ala in 60/40 (v/v) water/TG in one syringe and 5.4 mM Ala and 20 mM potassium acetate in the other syringe.

Table 3.1. Assignment of prominent ions in the EH mass spectrum of a "blank" comprised of 50 mM PTSA, 5.4 mM alanine, and 10 mM potassium acetate in 80/20 (v/v) water/TG.

m/z	Assignment[a]
109	$[H + TG]^+$
145	$[H + 3TG]^+$ ----> $[H + 2TG]^+$ (metastable ion)
147	$[K + TG]^+$
198	$[H + alanine + TG]^+$
201	$[H + TG + G]^+$ impurity
215	$[H + TG\ dimer]^+$
217	$[H + 2TG]^+$
255	$[K + 2TG]^+$
281	$[H + PTSA + TG]^+$
306	$[H + alanine + 2TG]^+$
307	$[H + TG\ dimer + G]^+$ impurity
309	$[H + 2TG + G]^+$ impurity
323	$[H + TG + TG\ dimer]^+$
325	$[H + 3TG]^+$
363	$[K + 3TG]^+$
389	$[H + PTSA + 2TG]^+$
429	$[H + 2TG\ dimers]^+$
497	$[H + PTSA + 3TG]^+$

[a] TG = thioglycerol; G = glycerol; PTSA = para-toluenesulfonic acid; TG dimer = disulfide-linked oxidation product (Scheme 3.1)

links, -S-S- [50] (see Scheme 3.1). The peaks at the m/z values of 323 and 429



Scheme 3.1

correspond to a protonated TG dimer solvated by one TG molecule and a proton-bound dimer of dimers, respectively. Murawski noted the appearance of these peaks as well when doing EHMS of ammonium iodide in TG [51]. Murawski also noted the presence of peaks due to apparent glycerol contamination, like those at m/z 201, 307, and 309, in Figure 3.1. The purity of the TG used in these studies was 98%. Assuming that 1% of the TG is actually glycerol, this corresponds to a glycerol concentration of 27 mM.

As a further test, a "separated" sample (buffer and MS matrix in separate syringes) was run (see Figure 3.1 B). The mass spectrum is very similar to the "blank" mass spectrum, suggesting that there is indeed mixing occurring in the tee.

3.2 Treatment of Data

Although performance with the blank was judged satisfactory, S/N (signal to noise ratio) was low (i.e. relative intensities varied from scan-to-scan), typical of

desorption ionization mass spectrometry. Signal averaging was therefore necessary in order to lessen the effect of random variations (distinct from the time variations of interest) in the relative intensities of the analyte and internal standard peaks. However, care must be taken when averaging because the analyte concentrations are time dependent. For this reason, it is desirable that as few scans be averaged as necessary so as not to lose important information relating to the rate of hydrolysis.

In order to determine the number of scans to average, the improvement in S/N from increasing the number of scans averaged was assessed for repeated scans of a time-invariant system. For this purpose, EHMS was performed using the tee assembly with 8 mM leucine and 5.4 mM alanine in 20 mM potassium acetate buffer in one syringe and 5.4 mM alanine and 100 mM PTSA in 60/40 (v/v) water/TG in the other. Sixty scans were acquired. The single ion chromatograms (SICs) from the most abundant ions due to leucine (at m/z = 348) and alanine (at m/z = 306) are shown in Figure 3.2. In order to treat the data equally, it would be necessary to consider all possible combinations for the number of scans averaged from the sixty acquired. However, this approach gives rise to extremely large numbers of calculations. For example, if all possible combinations of 15 scans were averaged from the sixty acquired, there would be 10^{13} averages. This is too many to consider; therefore, only a subset of the first 15 of the sixty scans was considered for the determination of the number of scans to average.

The data were treated in the following manner: N corresponds to the number of scans averaged. For N = 2, all possible combinations of 2 of the 15 scans were averaged. Because within-scan noise was comparable to that between

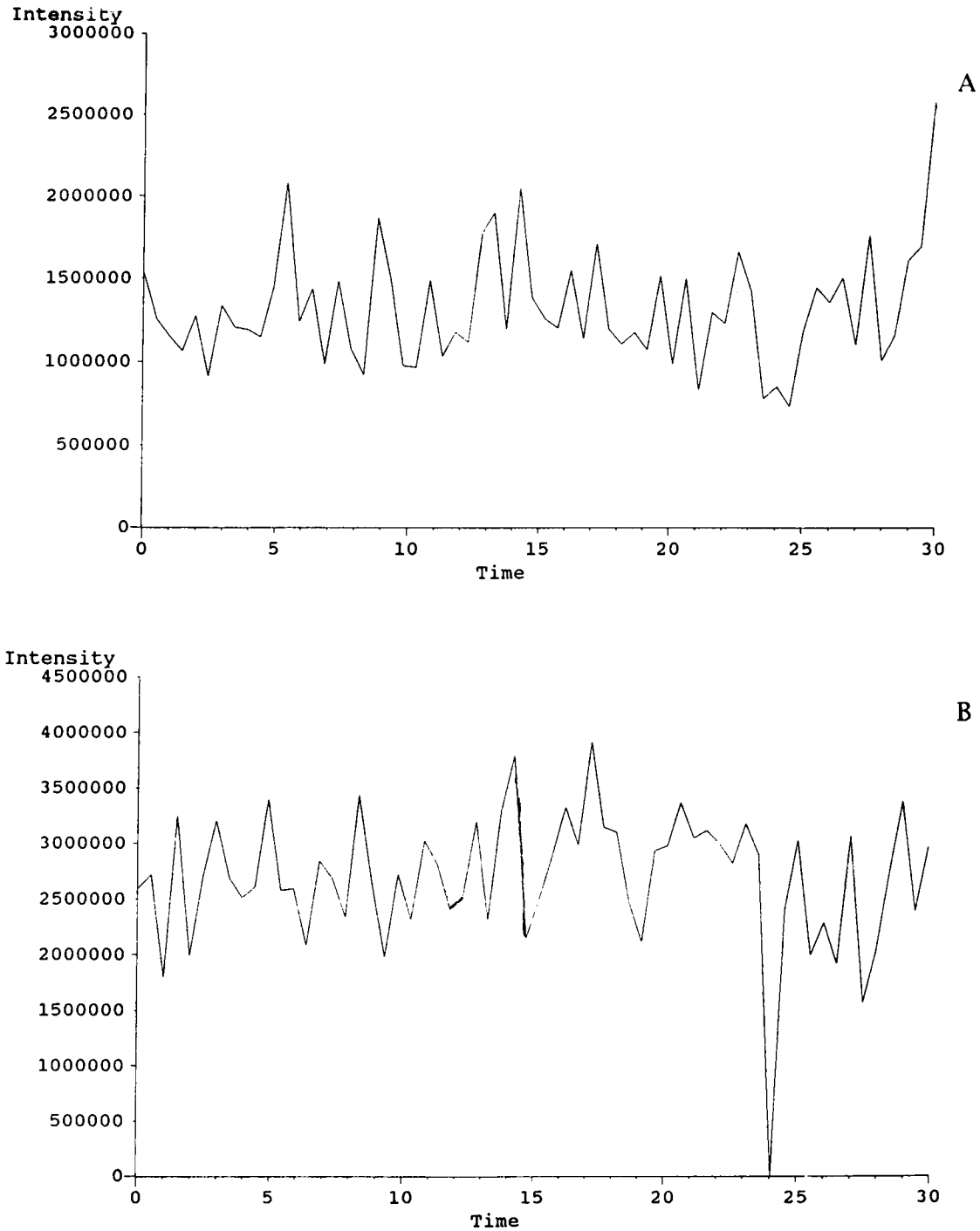


Figure 3.2. Single ion chromatograms of (A) leucine at m/z = 348 ([H + Leu + 2TG]$^+$) and (B) alanine at m/z = 306 ([H + Ala + 2TG]$^+$) resulting from 60 scans; where TG = thioglycerol, Leu = leucine, and Ala = alanine.

scans, the leucine and alanine intensities were averaged separately, before a ratio was taken. There were 105 possible combinations of 2. Each of the 105 averages of the intensities from the leucine data was divided by the corresponding average from the alanine data. An average and standard deviation was calculated for these 105 ratios. The percent relative standard deviation (% RSD) was then calculated using this average and standard deviation. The procedure was then repeated for N = 3, (455 possible combinations), etc. Figure 3.3 is a plot of % RSD versus N. As evident in Figure 3.3, the % RSD decreases as N gets larger, but most of the decrease occurs at smaller N values (i.e. % RSD decreases about 6.5% from N = 2 to 4 and only decreases about 1.5% from N = 8 to 10).

After considering both the data in Figure 3.3 and the fact that it is desirable to average as few scans as possible for these studies, it was determined to average 10 scans at a time when treating data from hydrolyses. The magnet scanned 10 times in approximately 5 minutes. The magnet was not scanned faster to get more averaging in less time because of magnet hysteresis. Because the concentration of substrate and/or enzyme can be adjusted to change the rate of the reaction (i.e. less enzyme can be used to slow the reaction), it was determined that reaction conditions could be adjusted if needed in order that the signal averaging could provide an acceptable S/N ratio.

3.3 Results with N-CBZ-Val-Leu

With a knowledge of suitable scan conditions at hand, it was next necessary to select a suitable substrate. As a first test, N-CBZ-Val-Leu (see Structure 3.1) was chosen because it was readily available and is soluble at millimolar concentra-

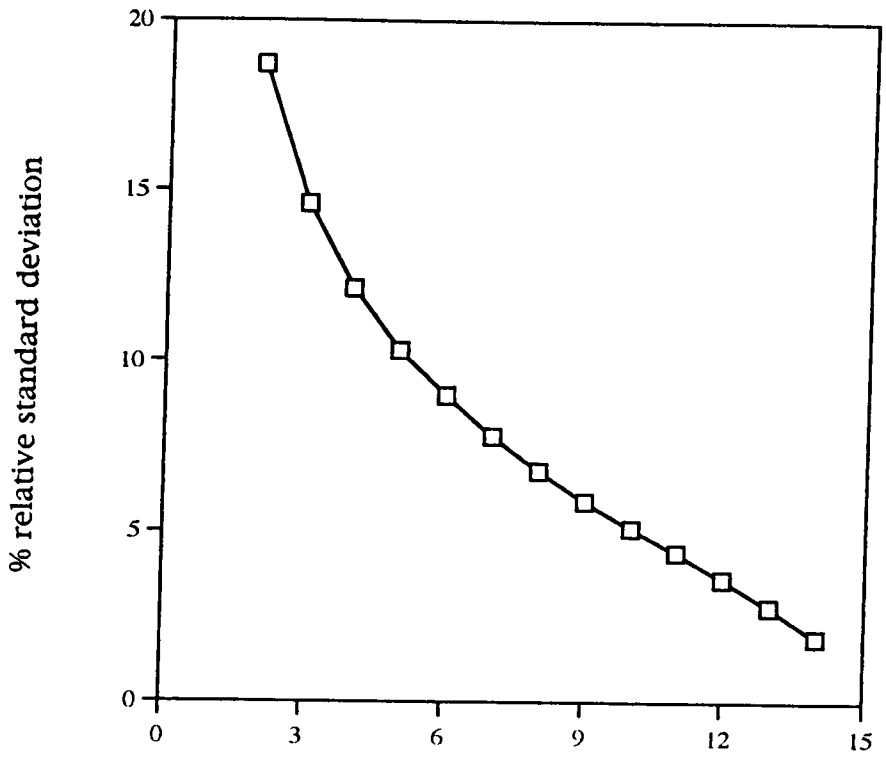
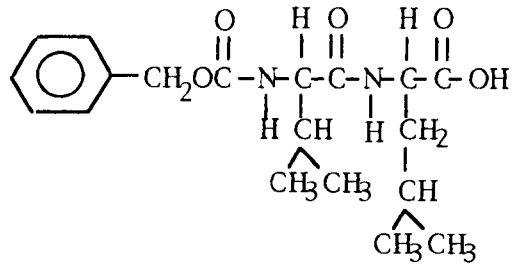


Figure 3.3. Plot of percent relative standard deviation (%RSD) versus N. See text for details.



Structure 3.1

tions. The solubility of this substrate was found to be about 8 mM. Since leucine is released as a result of CPY-catalyzed hydrolysis of this protected dipeptide, a preliminary experiment involved acquisition of a leucine calibration curve. Figure 3.4 shows graphically the results obtained from a mass spectrometric leucine calibration curve. The procedure used to obtain this calibration curve was described in Chapter 2. From this calibration curve, the limit of detection (LOD) for leucine is estimated to be 0.2 mM (using the defintion of three times the standard deviation of the blank divided by the slope of the calibration curve). Table 3.2 includes the slope, y-intercept, and correlation coefficient from the mass spectrometric leucine calibration curve (and others; see below). The rather large blank value resulted from an offset in the "zero" setting of the amplifier on the MS console. This same offset was maintained for all mass spectrometric experiments (calibration curves and hydrolyses) to ensure a blank value greater than zero (thus meeting the minimum threshold required by the data software). The leucine calibration curve is linear up to at least 16 mM leucine. From the range of possible concentrations available, 5 mM was chosen arbitrarily as the substrate concentration for the first hydrolysis.

Figure 3.5 is a plot of leucine concentration released versus time from one of three hydrolyses of 5.06 mM N-CBZ-Val-Leu with 0.8 unit CPY/mL. The first 10-12 minutes are not plotted on this graph because, after the start of the

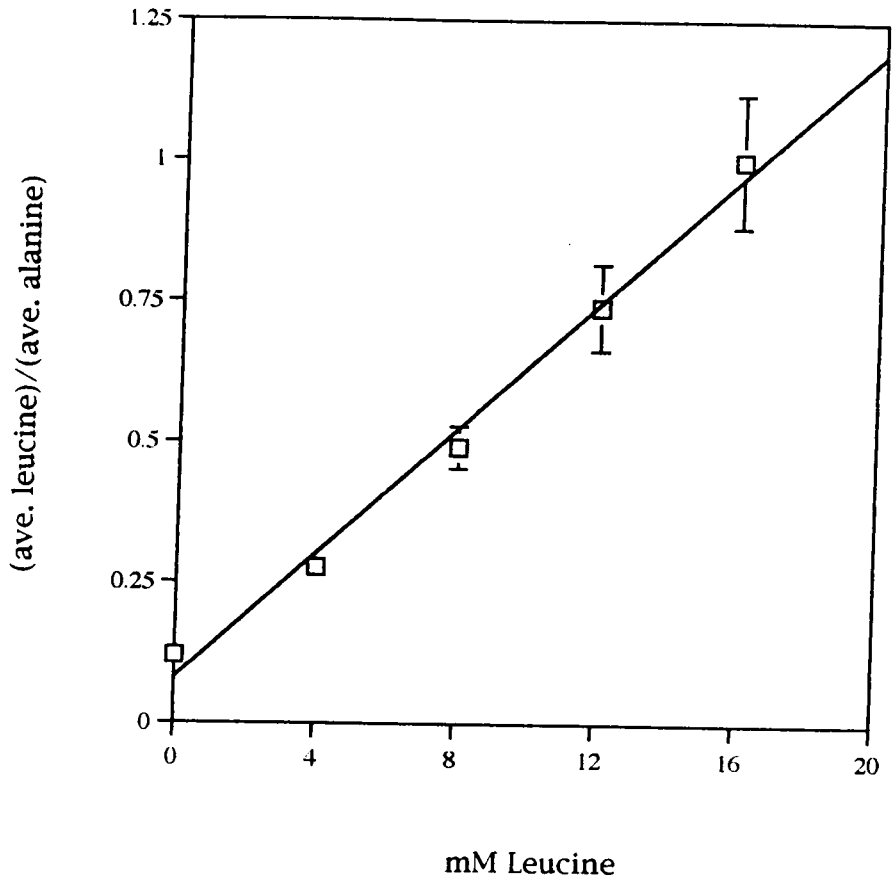


Figure 3.4. Mass spectrometric leucine calibration curve. Error bars represent the standard deviation of the mean. See text for details. Syringe A contained the indicated amount of leucine and 5.4 mM alanine in 20 mM potassium acetate buffer. Syringe B contained 100 mM para-toluenesulfonic acid and 5.4 mM alanine in 60/40 (v/v) water/thioglycerol. See Table 3.2 for slope, y-intercept, and correlation coefficient.

Table 3.2. EHMS amino acid calibration curve data. All uncertainties represent the 95% confidence limits.

amino acid	slope[a]	y-intercept	correlation coefficient
leucine	0.056 (±0.003)	0.080 (±0.029)	0.996
glycine	0.062 (±0.001)	0.075 (±0.007)	0.999
phenylalanine	0.042 (±0.002)	0.078 (±0.010)	0.997

[a] expressed in mM^{-1}

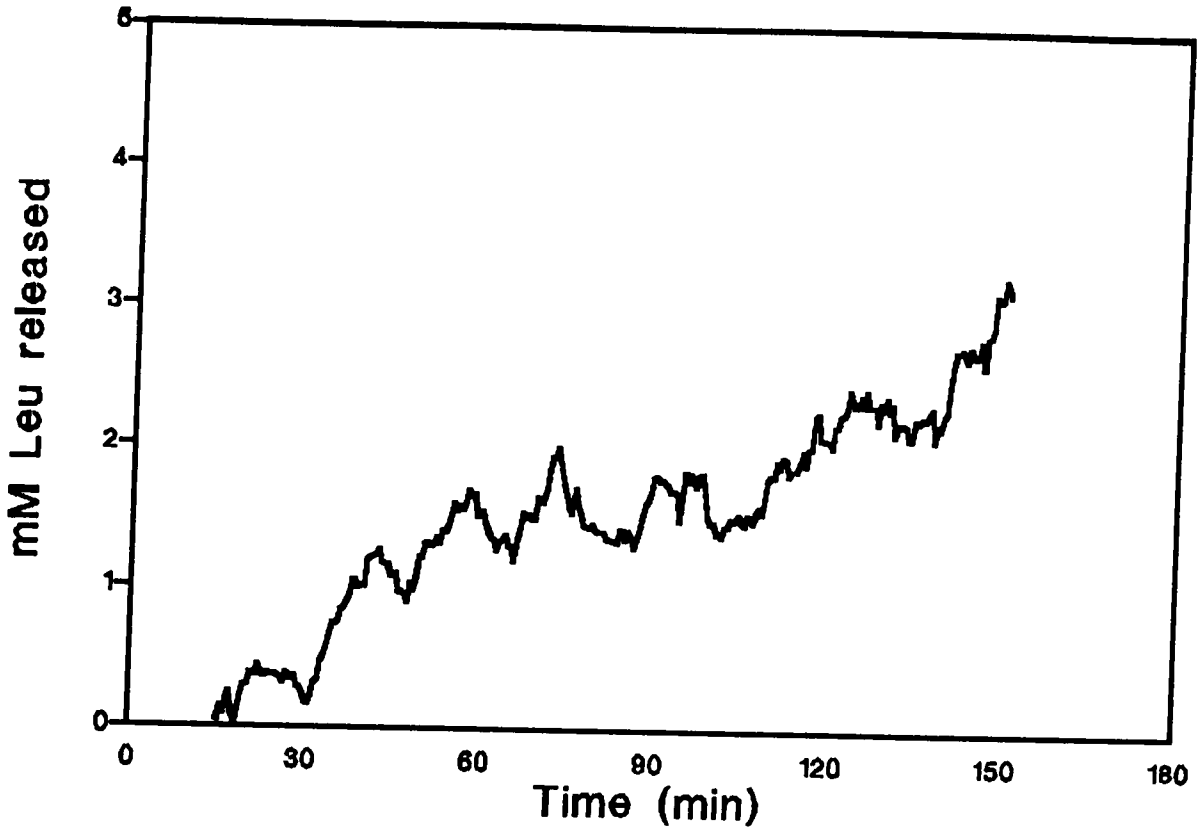


Figure 3.5. Leucine concentration from a hydrolysis of 5.06 mM N-CBZ-Val-Leu with 0.8 unit CPY/mL monitored by EHMS (10-point Savitzky-Golay smoothed). Syringe A contained substrate, enzyme, and 5.4 mM alanine in 20 mM potassium acetate buffer. Syringe B contained 100 mM para-toluenesulfonic acid and 5.4 mM alanine in 60/40 (v/v) water/thioglycerol.

reaction, this much time was required to rinse the syringe and "reaction reservior" (see Chapter 2) with the reaction mixture and to obtain emission.

The slope obtained from a linear least squares fit to the data in Figure 3.5 represents the average rate of release of leucine during the hydrolysis. However, in order to use a linear approximation to determine the initial velocity, the hydrolysis should be no more than about 30% complete (see Chapter 1). The data in Figure 3.5 reveals that the reaction is approximately 30% complete at 60 minutes (assuming release of 5.03 mM leucine when the reaction is 100% complete). Therefore, the slope was determined using data up to 60 minutes into the hydrolysis (approximately 90 scans). Two additional hydrolyses were performed at a substrate concentration of 5.06 mM and three were performed at 4.45 mM. The top portion of Table 3.3 summarizes the data from the hydrolyses of N-CBZ-Val-Leu monitored mass spectrometrically.

The third column in the top of Table 3.3 contains the average rate at each concentration as well as the 95% confidence limits calculated from pooled standard deviations (reflecting the uncertainty in the individual rate measurements; see Appendix A). The rate of 0.024 $\pm$0.003 mM leucine/min determined at 4.45 mM lies outside the uncertainties of the remaining two rates in the subset, indicative of a systematic error. There are at least two possibilities for the source of the systematic error. One possible source of error is from preconditioning of the tee assembly and capillaries as described in Chapter 2. Flushing both capillaries with the "blank" solution (containing thioglycerol) could have contaminated both the syringe that later contained the reaction mixture as well as the capillary that connected the syringe to the tee. Although the syringe was rinsed several times with the buffer (see Chapter 2) before it was filled with a

Table 3.3. Data from hydrolyses of N-CBZ-Val-Leu. All uncertainties represent the 95% confidence limits. All rates are expressed in units of mM leucine/min.

Concentration[a]	Rate[b]	Ave. Rate[c]	Ave. Rate[d]	Turnover Number[e]
Mass Spectrometric Data				
5.06	0.027 (±0.002) 0.020 (±0.004) 0.026 (±0.004)	0.024 (±0.003)	0.024 (±0.009)	3.0 (±1.1)
4.45	0.016 (±0.002) 0.024 (±0.003) 0.016 (±0.004)	0.019 (±0.003)	0.019 (±0.011)	3.3 (±1.4)

Concentration[a]	Ave. Rate[f]	Turnover Number[e]
Optical Data		
4.41	0.021 (±0.002)	2.6 (±0.2)
2.60	0.022 (±0.002)	2.8 (±0.2)

[a] expressed in mmol/L
[b] rates from replicate analyses with 95% confidence limits; N = 90 scans; t = 1.99
[c] average rates with 95% confidence limits calculated from pooled standard deviations; N = 90; t = 1.99
[d] average rates with 95% confidence limits calculated from simple standard deviations; N = 3; t = 4.30
[e] expressed in sec^{-1}
[f] average rates with 95% confidence limits; N = 12; t = 2.23

hydrolysis mixture, a variable TG residue could have remained. A second possible source of variation was imperfect temperature regulation. There was approximately 15 cm of capillary from the "capillary coil" (submerged in the constant temperature bath) that ran from the bath to the union (see Figure 2.5 in Chapter 2) followed by 15 cm from the union to the tee. The aqueous reaction mixture was at an unregulated temperature during the time it was in this section of capillary (approximately 8 minutes). Room temperatures (measured with a wall thermometer) fluctuated from day-to-day between 22-24^{o}C. This is probably insignificant due to the fact that the sample only spends about 8 minutes at an unregulated temperature. Furthermore, CPY studies by Lewis and Schuster [52] reveal that the rates of hydrolysis of the substrate N-CBZ-Phe-Ala by CPY during incubation at temperatures of 4 and 25^{o}C differ only by about 2%. Therefore, it is expected that the rate would be affected little by the 8 minute time interval during which the temperature varied by one to three degrees. The syringe and connecting capillary before the temperature bath are also not thermostatted, but sample in these regions never reaches the emitter at a flow rate of 2.7 μL/hr. This assumes neglible diffusion and mixing prior to the tee. Thus, while there are at least two possible sources of systematic error, contamination by TG is the more probable.

Because of systematic error, the precision of the rate determination within each replicate measurement at 4.45 mM was better than the between-run precision of the rates. The pooled 95% confidence limit does not reflect the between-run variation and therefore underestimates the true uncertainty in the average rate. It is more appropriate in this case to average the three rates from the triplicate analyses and calculate a 95% confidence limit based on a simple

standard deviation of this average (see column 4 of the top portion of Table 3.3). As shown in the Table 3.3, the rates at 5.06 and 4.45 mM agree within the 95% confidence limit, even with the over-optimistic precision estimate. Michaelis-Menten kinetics predict that the rate at 4.45 mM would be slower than that at 5.06 mM unless the enzyme is saturated. Since the systematic errors appeared to affect only one of six rate determinations, it was determined to continue and assess the reliability of the average rates in relation to an established method.

In order to assess the validity of the EHMS reaction rate determinations, optical experiments were undertaken using the widely accepted ninhydrin method [5]. Figure 3.6 is the leucine calibration curve obtained by the ninhydrin method. Table 3.4 includes the slope, y-intercept, and correlation coefficient for the leucine calibration curve (and a glycine calibration curve; see below). This curve in Figure 3.6 was used to obtain Figure 3.7, a plot of mM of leucine released versus time from separate hydrolyses of 4.41 mM N-CBZ-Val-Leu with 0.8 unit CPY/mL quenched at various times. Notice that Figure 3.7 has a negative y-intercept. The enzyme had not been thermostatted at 25^{o}C prior to its addition to the substrate solution (see Chapter 2). It is possible that the protocol could have caused an induction period giving rise to the negative y-intercept in Figure 3.7; however, this appears not to have affected the slope (see below). Figure 3.7 contains 12 data points (3 at each chosen time) because there were 12 separate reactions; three were stopped at 15 minutes, three at 30 minutes, etc. (see Protocol section in Chapter 2). The data in Figure 3.7 confirm that the hydrolysis is less than 30% complete at 60 minutes, based on an expectation of 4.41 mM leucine released when the hydrolysis is 100% complete. Therefore, the data up to the 60 minute reaction time was used for the initial velocity determination. A

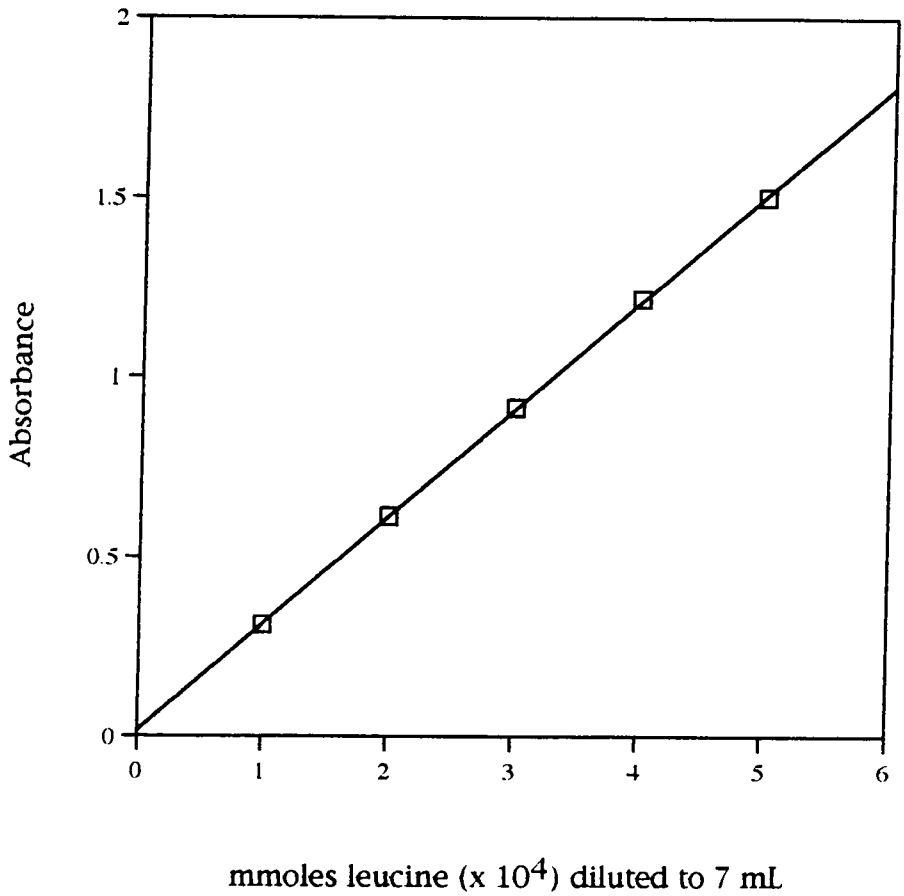


Figure 3.6. Leucine calibration curve obtained by the ninhydrin method. Error bars are shown but are smaller than the points. See Table 3.4 for slope, y-intercept, and correlation coefficient.

Table 3.4. Optical calibration curve data. All uncertainties represent the 95% confidence limits.

amino acid	slope[a]	y-intercept	correlation coefficient
leucine	2.09 ($\pm$0.05) x 10^4	0.019 ($\pm$0.026)	0.999
glycine	2.06 ($\pm$0.05) x 10^4	0.022 ($\pm$0.019)	0.999

[a] expressed in $M^{-1} \times cm^{-1}$

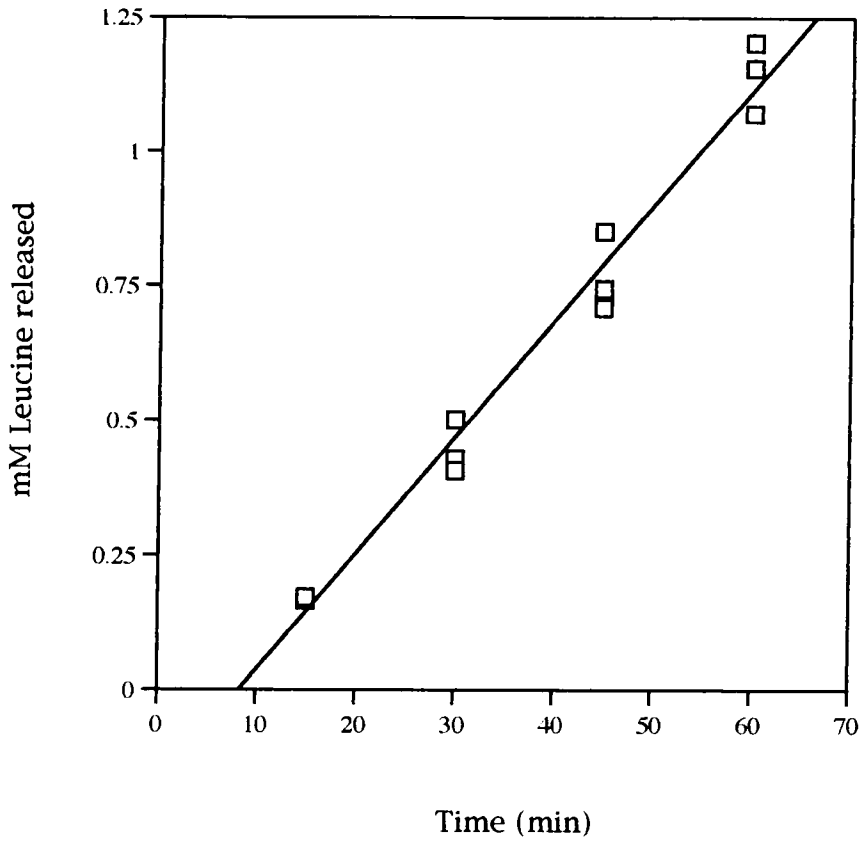


Figure 3.7. Hydrolyses of 4.41 mM N-CBZ-Val-Leu with 0.8 unit CPY/mL in 20 mM potassium acetate buffer monitored using the ninhydrin method.

least squares fit was performed on this data, and the resulting slope was used as a measure of the average rate of release of leucine during the hydrolysis. Three hydrolyses with this substrate at a concentration of 2.60 mM were also monitored by the ninhydrin method. The average rate at each concentration as well as the 95% confidence limits are shown in the bottom portion of Table 3.3.

From the data in Table 3.3, it is clear that the rates determined by mass spectrometry and the ninhydrin method agree within the experimental error (despite the apparent systematic error(s) in the EHMS data), both using a given method at different concentrations and using either method at a given concentration. It is apparent that the rate remains constant in the concentration range from 2.60 to 5.06 mM N-CBZ-Val-Leu. The maximal rate (V_{max}) has evidently been reached, indicating that the enzyme is saturated with substrate in this concentration range (see Figure 1.2 in Chapter 1). Michaelis-Menten kinetics predicts that K_m cannot be determined under these conditions. However, the turnover number can be determined from the maximal velocity using Equation (4) in Chapter 1. The numbers obtained are included in Table 3.3.

Hayashi and coworkers found a K_m value of 0.1 mM for N-CBZ-Val-Leu under reaction conditions (25°C and pH of 6) [1] similar to those employed here. Cleland has recommended that a suitable substrate concentration range for the evaluation of K_m and V_{max} is from 0.2 K_m to 5 K_m which will produce initial velocities from 0.16 V_{max} to 0.83 V_{max} (see Equation (5) in Chapter 1) [19]. For N-CBZ-Val-Leu, this corresponds to 0.02-0.5 mM, barely overlapping the 0.2 mM leucine limit of detection. It could therefore have been predicted that this substrate would be a poor choice for assessment of K_m using EHMS. To proceed with the kinetics studies, it was necessary to identify another substrate that

possessed a larger K_m and whose solubility would not be a limiting factor.

3.4 Results with N-CBZ-Phe-Gly

N-CBZ-Phe-Gly was tried next because it is soluble at concentrations up to 8 mM and has a reported K_m value of 4 mM under the slightly different reaction conditions of 25°C and pH of 6.5 [9] (a pH of 6.0 was used in this study). As a preliminary experiment, a glycine mass spectrometric calibration curve was obtained (Figure 3.8). The procedure used to obtain this curve was described in Chapter 2. Table 3.2 reveals the slope, y-intercept, and correlation coefficient from this curve.

Figure 3.9 is a plot of glycine concentration released versus time from one of three hydrolyses of 7.95 mM N-CBZ-Phe-Gly with 0.1 unit CPY/mL monitored by EHMS. The data were treated in the same way as that for N-CBZ-Val-Leu described above, except that initial velocities were determined from the start of the reaction to 80 minutes into the hydrolysis (130 scans), because the reaction is no more than approximately 30% complete under these conditions (see Figure 3.9). Two additional hydrolyses were performed at a substrate concentration of 7.95 mM, and three were performed at substrate concentrations of 2.39, 3.23, 3.98, and 5.93 mM.

Table 3.5 contains data analogous to that for N-CBZ-Val-Leu (in Table 3.3). Systematic errors (the differences between the rates from replicates at a given concentration; see column 2 of Table 3.5) are larger than for the corresponding N-CBZ-Val-Leu data, even though the precision of the rate determination within each replicate measurement is generally better than in Table 3.3. The within-run

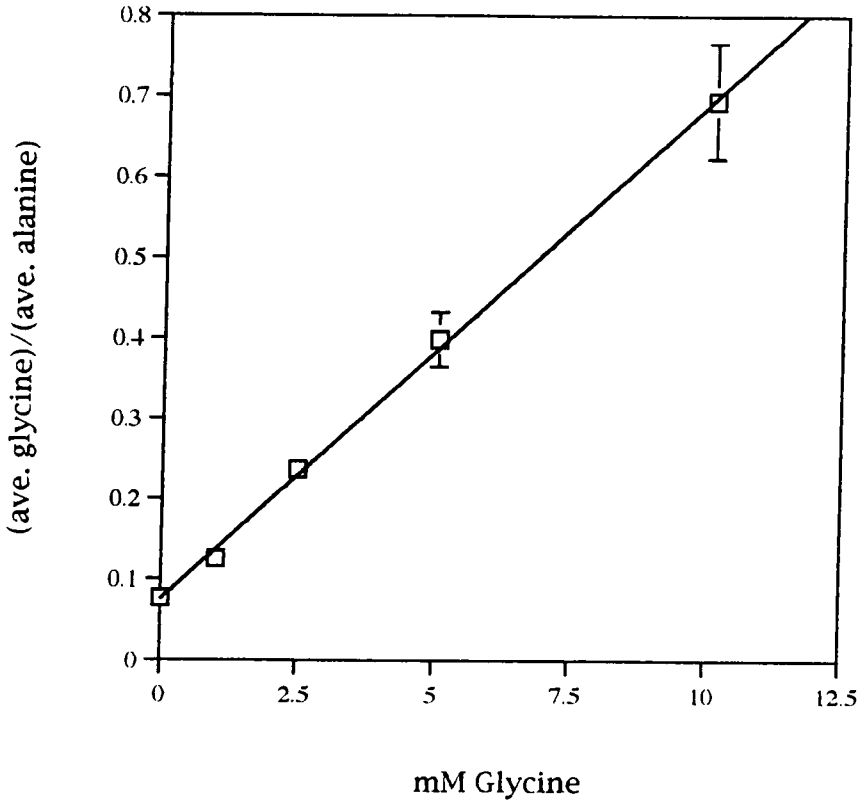


Figure 3.8. Mass spectrometric glycine calibration curve. Error bars represent the standard deviation of the mean. See text for details. Syringe A contained the indicated amount of glycine and 5.4 mM alanine in 20 mM potassium acetate buffer. Syringe B contained 100 mM para-toluenesulfonic acid and 5.4 mM alanine in 60/40 (v/v) water/thioglycerol. See Table 3.2 for slope, y-intercept, and correlation coefficient.

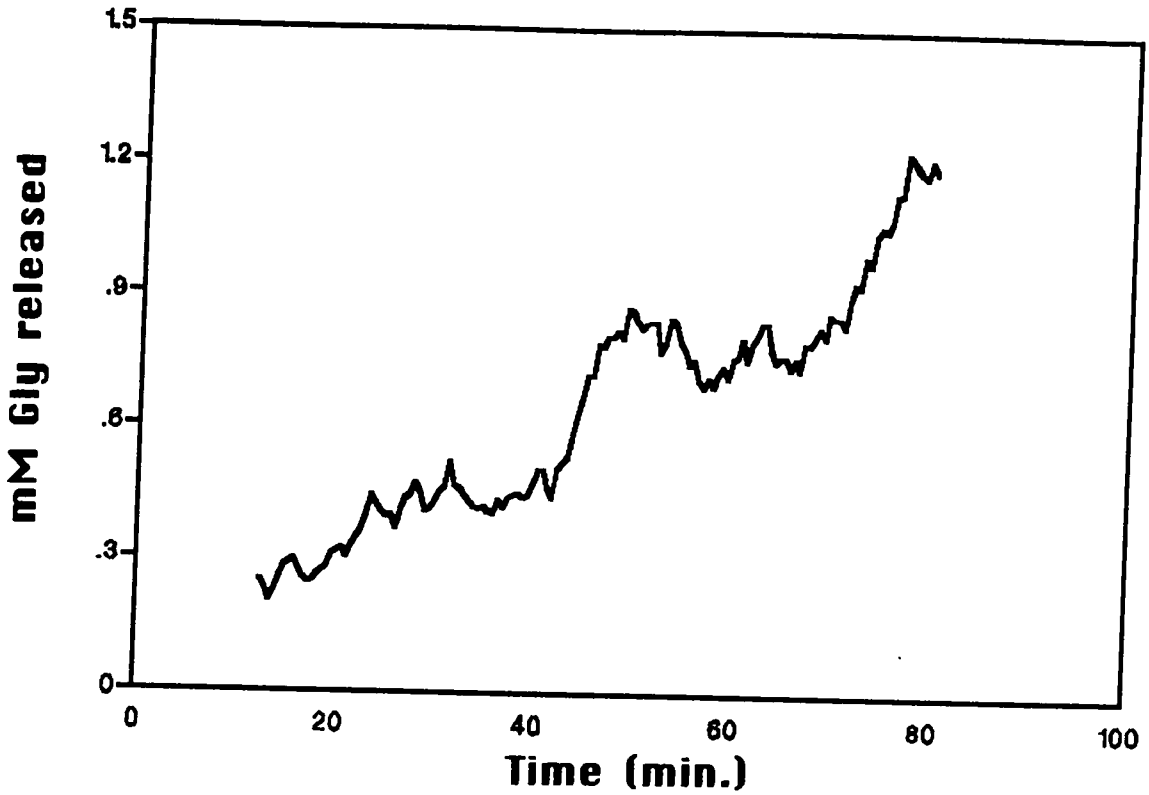


Figure 3.9. Glycine concentration from a hydrolysis of 7.95 mM N-CBZ-Phe-Gly with 0.1 unit CPY/mL monitored by EHMS (10-point Savitzky-Golay smoothed). Syringe A contained substrate, enzyme, and 5.4 mM alanine in 20 mM potassium acetate buffer. Syringe B contained 100 mM para-toluenesulfonic acid and 5.4 mM alanine in 60/40 (v/v) water/thioglycerol.

Table 3.5. Data from hydrolyses of N-CBZ-Phe-Gly. Uncertainties represent the 95% confidence limits. All rates are expressed in units of mM glycine/min.

Concentration[a]	Rate[b]	Ave. Rate[c]	Ave. Rate[d]
Mass Spectrometric Data			
2.39	0.005 (±0.001)		
	0.011 (±0.001)	0.0084 (±0.0008)	0.0084 (±0.0072)
	0.009 (±0.001)		
3.23	0.013 (±0.002)		
	0.009 (±0.002)	0.013 (±0.001)	0.013 (±0.009)
	0.017 (±0.001)		
3.98	0.010 (±0.001)		
	0.014 (±0.003)	0.014 (±0.002)	0.014 (±0.008)
	0.017 (±0.001)		
5.93	0.016 (±0.001)		
	0.021 (±0.002)	0.016 (±0.002)	0.016 (±0.012)
	0.011 (±0.002)		
7.95	0.014 (±0.001)		
	0.013 (±0.001)	0.013 (±0.001)	0.013 (±0.004)
	0.011 (±0.001)		

Concentration[a]	Ave. Rate[e]
Optical Data	
2.40	0.0104 (±0.0014)
3.21	0.0124 (±0.0018)
3.98	0.0134 (±0.0023)
5.92	0.0149 (±0.0009)
8.01	0.0160 (±0.0021)

[a] expressed in mmol/L
[b] rates from replicate analyses with 95% confidence limits; N = 130; t = 1.98
[c] average rates with 95% confidence limits calculated from pooled standard deviations; N = 130; t = 1.98
[d] average rates with 95% confidence limits calculated from simple standard deviations; N = 3; t = 4.30
[e] average rates with 95% confidence limits; N = 12; t = 2.23

precision is improved in the N-CBZ-Phe-Gly data relative to the N-CBZ-Val-Leu data, but the between-run precision deteriorated. It must be noted that, in contrast to the experiments with N-CBZ-Val-Leu, both the substrate and enzyme solutions were thermostatted prior to the initiation of the hydrolysis. This improved technique in the EHMS method may have helped the precision despite the uncontrolled parameter(s) of TG contamination and/or temperature in the tee assembly mentioned in Section 3.3. The reaction is slower and monitoring time longer for the substrate N-CBZ-Phe-Gly. This could improve precision in the EHMS data. The same experimental procedure used for monitoring the CPY-catalyzed hydrolyses of N-CBZ-Val-Leu by EHMS (as described in Chapter 2) was used with the substrate N-CBZ-Phe-Gly (except for thermostatting the enzyme and substrate solutions as mentioned above) because the existence of the systematic error(s) had not yet been realized.

As a result of these errors, it can be seen from column 4 in Table 3.5 that all of the mass spectrometrically determined rates agree within experimental error. Nevertheless, the trend is that predicted by Michaelis-Menten kinetics, except that the rates from the triplicate analyses at 7.95 mM are lower than two of the three at 5.93 mM. This may represent saturation of the enzyme. This behavior is not uncommon. In fact, while the Michaelis-Menten equation is obeyed at lower substrate concentrations, the velocity often *decreases* at high concentrations [6]. This can be due at least in part to a "crowding" of the enzyme by substrate molecules. Many enzymes have several active sites that combine with a particular part of the substrate molecule. One substrate molecule interacts with all of these sites in an "effective" enzyme-substrate complex [6]. An "ineffective" complex can form if some of the binding groups on the enzyme are combined with one

substrate molecule and the other groups combine with a different substrate molecule. The chances of an ineffective enzyme-substrate complex increase at higher substrate concentrations. Since the precision of the mass spectrometric experiments is poor, it is difficult to assess whether enzyme saturation is taking place at 7.95 mM. It is evident from Table 3.5 that the three rates from the triplicate analyses (in column 2) at 5.93 and 7.95 mM are higher than those at 2.39 mM in agreement with Michaelis-Menten kinetics (see Figure 1.2 in Chapter 1); therefore, at least some *qualitative* information can be obtained from the EHMS data.

At this point, optical experiments were undertaken for comparison with the mass spectrometric results. Because glycine is liberated from N-CBZ-Phe-Gly during CPY-catalyzed hydrolysis, an optical glycine calibration curve (see Figure 3.10) was acquired for use in the optical experiments (as described in Chapter 2). Table 3.4 includes the slope, y-intercept, and correlation coefficient from this curve. Note the similarity of the slope with that from the leucine calibration curve obtained previously (see Figure 3.6), suggesting that ninhydrin forms a complex with glycine to the same degree as it does with leucine and that the spectroscopy of the two complexes is similar (λ_{max} = 570 nm and $\varepsilon = 2 \times 10^4$ for both). Figure 3.10 was used to obtain Figure 3.11, a plot of glycine released versus time from hydrolyses of 8.01 mM N-CBZ-Phe-Gly with 0.1 unit CPY/mL. The data in Figure 3.11 confirms that the hydrolysis is less than 30% complete in 80 minutes. Therefore, the data up to the 80 minute reaction time was used for the initial velocity determination. A least squares fit was performed on this data, and the resulting slope was used as a measure of the average rate of release of glycine during the hydrolysis. Unlike Figure 3.7, Figure 3.11 does not have a negative

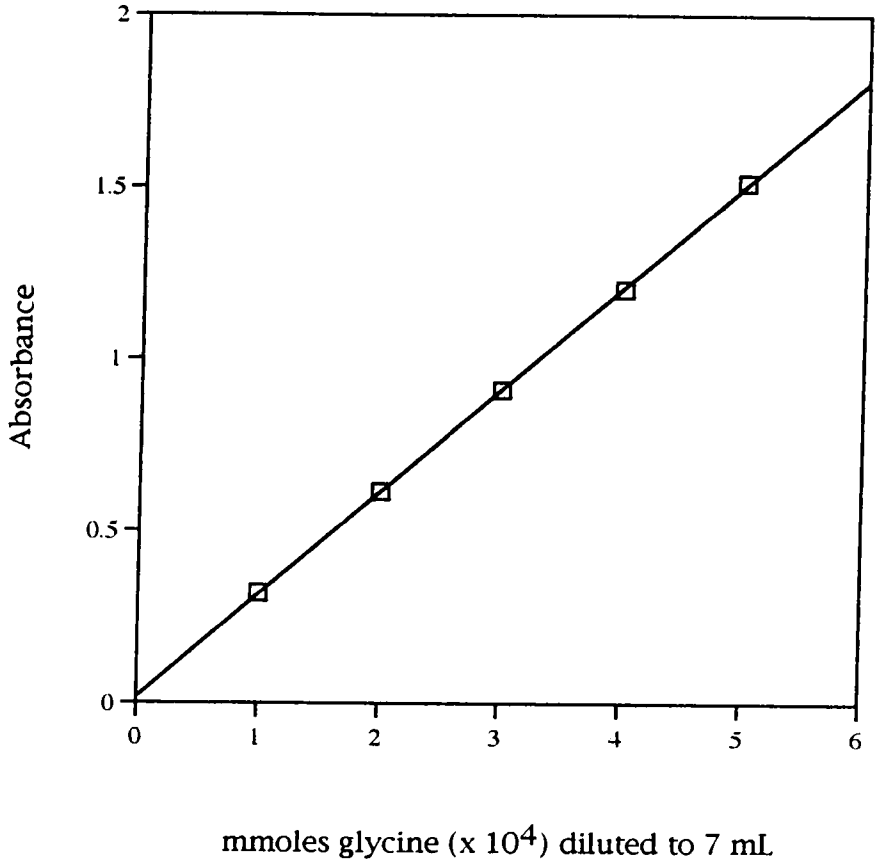


Figure 3.10. Glycine calibration curve obtained by the ninhydrin method. Error bars are shown but are smaller than the points. See Table 3.4 for slope, y-intercept, and correlation coefficient.

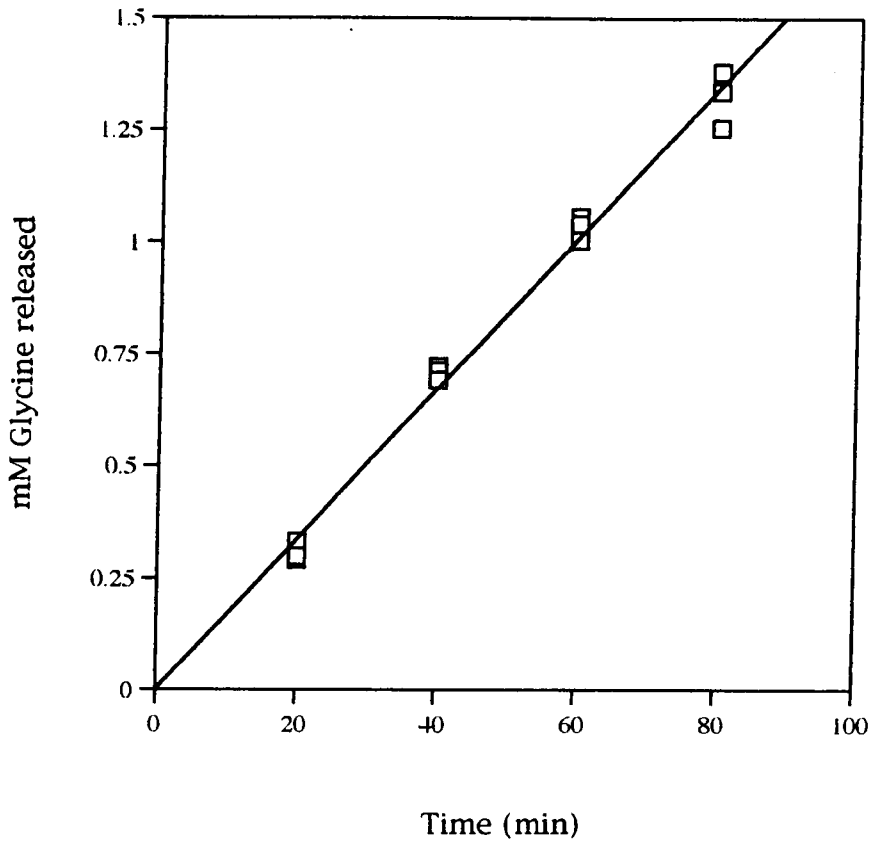


Figure 3.11. Hydrolyses of 8.01 mM N-CBZ-Phe-Gly with 0.1 unit CPY/mL in 20 mM potassium acetate buffer monitored using the ninhydrin method.

y-intercept. This is could be due to the fact that, for the hydrolyses of N-CBZ-Phe-Gly, the enzyme solution was thermostatted at 25^{o}C prior to being added to the substrate solution (see Chapter 2). Hydrolyses of N-CBZ-Phe-Gly were also monitored optically at concentrations of 2.40, 3.21, 3.98, and 5.92 mM. The data from the hydrolyses of N-CBZ-Phe-Gly monitored optically are presented in the bottom portion of Table 3.5. Although confidence intervals overlap, the average rates differ by more than the uncertainty in each. Thus it can be concluded from the optical data that the initial rate increases as the substrate concentration increases (as Michaelis-Menten kinetics predicts).

When the rate varies at different substrate concentrations, a Hanes plot (see Figure 3.12) is useful for the determination of K_m and V_{max} (see Chapter 1). For EHMS data, each rate from the replicate measurements in the Hanes plot is shown in order to illustrate and emphasize the rather large deviations present (Figure 3.12 A). Figure 3.12 B shows a Hanes plot of the optical data. The values for K_m, V_{max}, and the turnover number determined from both Hanes plots are presented in Table 3.6. Because the enzyme is not saturated, V_{max}/K_m represents the first order rate constant for the reaction E + S ---> E + P [17] (see Chapter 1). This value is also shown in Table 3.6.

The K_m value from the optical data (2.16 $\pm$0.44; see Table 3.6) agrees with the value from the EHMS data (1.52 $\pm$2.56) within the 95% confidence limit. It is also apparent that the Hanes plot of the optical data is more nearly linear than that of the mass spectrometric data. The large scatter in the Hanes plot of the EHMS data (Figure 3.12 A) gives rise to large standard deviations of both the slope and the y-intercept for the least squares line. Thus, K_m determined by EHMS has a rather large 95% confidence limit because it is found by dividing the

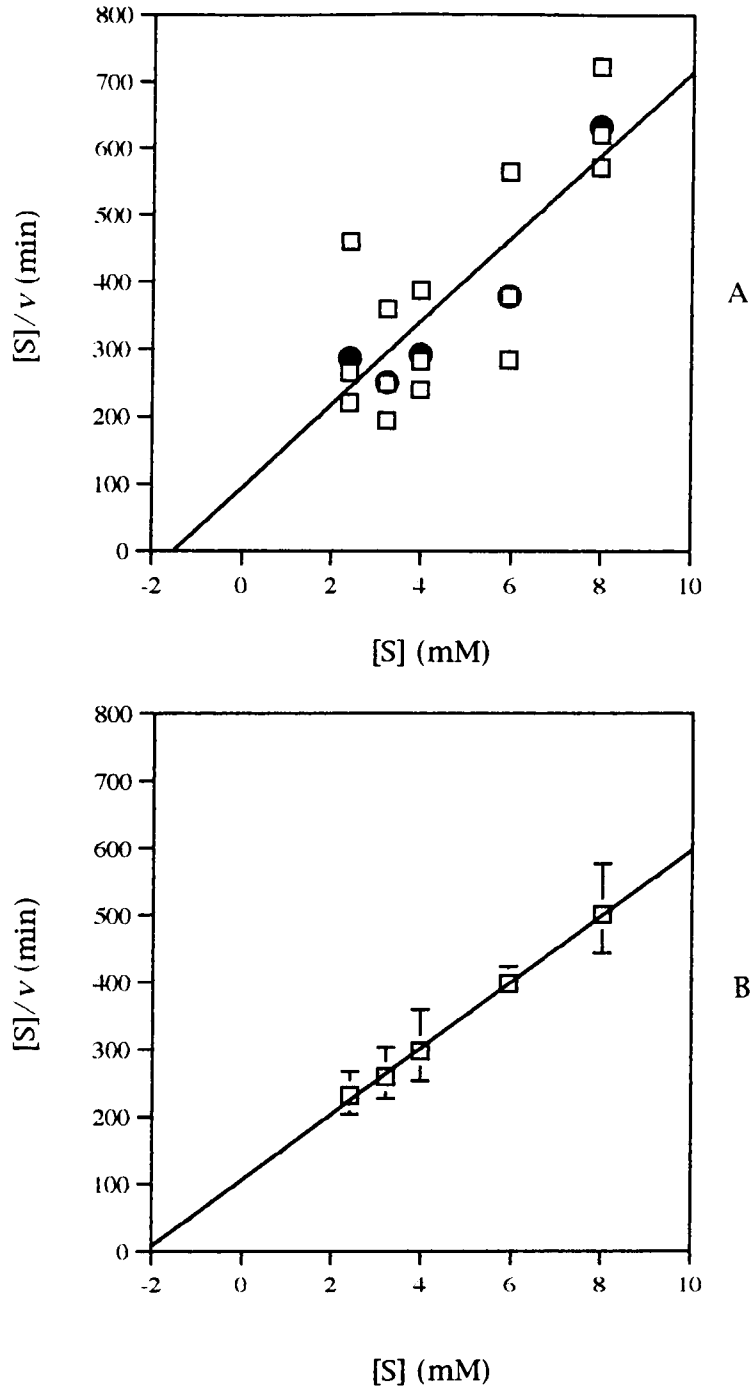


Figure 3.12. Hanes plots from hydrolyses of N-CBZ-Phe-Gly monitored by (A) EHMS and (B) the ninhydrin method. In (A), clear boxes represent individual replicates. Dark circles represent averages. In (B), error bars represent the 95% confidence limits.

Table 3.6. Kinetic parameters for N-CBZ-Phe-Gly. All uncertainties represent the 95% confidence limits.

Method	K_m[a]	V_{max}[b]	Turnover number[c]	k[d]
EHMS	1.52 (±2.56)	0.016 (±0.008)	16 (±8)	0.011 (±0.018)
ninhydrin	2.16 (±0.44)	0.020 (±0.002)	20 (±2)	0.0093 (±0.0021)

[a] Michaelis constant expressed in mmol/L

[b] maximal velocity expressed in units of mM glycine released/min

[c] expressed in sec^{-1}

[d] first order rate constant (V_{max}/K_m) for the reaction E + S ---> E + P expressed in units of min^{-1}

y-intercept by the slope (see Chapter 1). V_{max}, the turnover number, and the first order rate constant also have large 95% confidence limits. However, the values for K_m, V_{max}, the turnover number, and the rate constant determined by EHMS do agree within experimental error with those determined by the ninhydrin method.

Despite poor precision, these results indicate that EHMS should provide useful qualitative and semi-quantitative information. In a multistep reaction, such as CPY hydrolysis of a larger peptide, mass spectrometry should give information about the rate of release of each amino acid (i.e. a rate constant). Mass spectrometry may also be capable of identifying reaction intermediates. The data from a multistep reaction monitored by the ninhydrin method must be deconvoluted to provide information such as rate constants. Even though the precision in the mass spectrometric experiments is poorer than that of the optical experiments, the fact that more data is obtained by continuous monitoring of the reaction by EHMS may compensate and provide better precision than that obtained by deconvoluting the optical data in order to extract the rate constants. This issue was addressed in the final studies undertaken.

3.5 Results with Leucine-Enkephalin

Leucine-enkephalin is a neuroactive peptide that has been used in previous EHMS studies [39] (see Section 1.3). Bel and Cook [39] used EHMS to monitor a CPY-catalyzed hydrolysis of leucine-enkephalin. Because of this precedent, this pentapeptide (Tyr-Gly-Gly-Phe-Leu; Tyr = tyrosine, Gly = glycine, Phe = phenylalanine, Leu = leucine) was used as the substrate for a multistep reaction

with CPY. It has a molecular weight of 555.6 g/mol; therefore, it (and all CPY-catalyzed reaction products and intermediates) does not exceed the mass limit (1000 daltons) of the MS-9 mass spectrometer. The first step in the hydrolysis is the cleavage of leucine from the pentapeptide. According to Hayashi [1], this first step is favorable because catalysis by CPY is most effective when the penultimate and/or terminal amino acids have aromatic or aliphatic side chains. The cleavage of the phenylalanine residue from the tetrapeptide is expected to be slow (compared to that of the leucine) because "when glycine is placed in the penultimate position, the release of the terminal amino acid is very slow" [1]. The second step in the hydrolysis will give the tripeptide (Tyr-Gly-Gly) and phenylalanine. There are glycine residues in both the penultimate and terminal positions of this tripeptide. Hayashi has reported that "cleavage of tripeptides is difficult" with CPY regardless of the identity of the amino acids in the tripeptide [1]. He further found that, under reaction conditions similar to those used in this study, the substrate N-CBZ-Gly-Gly was not hydrolyzed by CPY after a 10 minute reaction time [9]. Kuhn and coworkers observed that, after a reaction time of 2 hours, N-CBZ-Gly-Gly was hydrolyzed only negligibly and "kinetics constants were not measurable" using the ninhydrin method [8]. Therefore, it is expected that the release of glycine will be small at best and may not be observed.

To extract rate constants for each step, the competing reactions for formation and consumption of intermediates must be considered. The hydrolysis of leucine-enkephalin was modelled assuming that the first step in the hydrolysis is simple first order (true provided the enzyme is not saturated). The first step involves release of leucine to give a tetrapeptide:

$$\text{Tyr-Gly-Gly-Phe-Leu} \text{ ----> } \text{Tyr-Gly-Gly-Phe} + \text{Leu} \quad (1)$$

The following first order rate equation represents this reaction:

$$dA(t)/dt = -k_1A(t) \quad (2)$$

where A(t) is the concentration of the pentapeptide at time t and k_1 is the first order rate constant. Solving this equation for A(t) gives

$$A(t) = A_oe^{-k_1t} \quad (3)$$

where A_o is the initial concentration of the pentapeptide. The second step involves the release of phenylalanine from the tetrapeptide to give a tripeptide:

$$\text{Tyr-Gly-Gly-Phe} \text{ ----> } \text{Tyr-Gly-Gly} + \text{Phe} \quad (4)$$

The following equation represents the rate of change in the concentration of the tetrapeptide:

$$dB(t)/dt = k_1A(t) - k_2B(t) \quad (5)$$

where B(t) is the concentration of the tetrapeptide at time t and k_2 is the rate constant for the hydrolysis of the tetrapeptide. This differential equation can be rewritten to separate the variables:

$$dB(t)/dt + k_2B(t) = k_1A(t) \quad (6)$$

Multiplying both sides of the equation by e^{k_2t}, substituting from Equation (3), and rewriting gives the following equation:

$$dB(t)/dt * e^{k_2t} + k_2B(t)e^{k_2t} = k_1A_oe^{(k_2-k_1)t} \quad (7)$$

$$d(B(t)e^{k_2t})/dt = k_1A_oe^{(k_2-k_1)t} \quad (8)$$

$$d(B(t)e^{k_2t}) = k_1A_oe^{(k_2-k_1)t}\,dt \quad (9)$$

By imposing the initial condition that B(t) equals zero at time equals zero, both sides of Equation (9) can be integrated and B(t) can be determined:

$$B(t)e^{k_2t} = [k_1A_o/(k_2-k_1)] * [e^{k_2t}e^{-k_1t} - 1] \quad (10)$$

$$B(t) = [k_1A_o/(k_2-k_1)] * [e^{-k_1t}-e^{-k_2t}] \quad (11)$$

The rate of change of the concentration of leucine is given by the following equation:

$$dP_1(t)/dt = k_1A(t) \quad (12)$$

$P_1(t)$ is the concentration of leucine, which can be obtained by solving Equation

(12):

$$P_1(t) = A_o[1-e^{-k_1 t}] \quad (13)$$

The rate equation for the release of phenylalanine is

$$dP_2(t)/dt = k_2 B(t) \quad (14)$$

$P_2(t)$ is the concentration of phenylalanine. Substituting for B(t) from Equation (11) and solving for $P_2(t)$ gives

$$P_2(t) = Le^{-k_2 t} - [(k_2 Le^{-k_1 t})/k_1] + (k_2 L/k_1) - L \quad (15)$$

where

$$L = k_1 A_o/(k_2-k_1) \quad (16)$$

The concentration of tripeptide, C(t), is equal to the concentration of phenylalanine released:

$$C(t) = P_2(t) \quad (17)$$

It was desired to monitor the hydrolysis of leucine-enkephalin by EHMS and by the ninhydrin method. In principle, EHMS is capable of determining

separately P_1, P_2, A, B, and C. k_1 can be determined by using Equation (13) and P_1. k_2 can be determined through the use of P_2, k_1, and Equations (15) and (16). By contrast, *only* $P_1 + P_2 + A + B + C$ ($= P_1 + P_2 + A_o$) can be determined by the optical method. k_1 and k_2 can be obtained from the optical method only as simultaneous fitting parameters for the sum of Equations (3), (11), (13), (15), and (17).

EHMS was used to monitor hydrolyses of 15.2 mM leucine-enkephalin in 20 mM potassium acetate buffer with 2.2 units CPY/mL. The substrate and enzyme concentrations were chosen to provide reasonable detectability and rates, based on Bel's earlier results [39]. Each hydrolysis was monitored continuously for 24 hours. Figure 3.13 is a mass spectrum acquired approximately 22 hours into the hydrolysis. Some of the seemingly intense isotope clusters are due to the fact that the ion intensities for some of the major ions are offscale. Such peaks were not related to the substrate, intermediates, or products, and therefore were not of concern. (See Table 3.1 for identification of unlabelled prominent peaks.) As expected (but in contrast to Bel's results; see discussion below), only the release of leucine and phenylalanine were observed.

Unfortunately, the intact pentapeptide, the intermediate tetrapeptide, and the resulting tripeptide were not detected. This would be a disadvantage if an unknown were being sequenced. It is preferable when sequencing an unknown using exopeptidases to monitor the substrate and intermediates rather than the released amino acids because of the possibility of repetitive amino acids in the peptide. The magnet was scanned from 600-50 daltons (Chapter 2); therefore, solvated pentapeptide peaks would not have been detected. The protonated pentapeptide was not observed. Nevertheless, information about the rate of

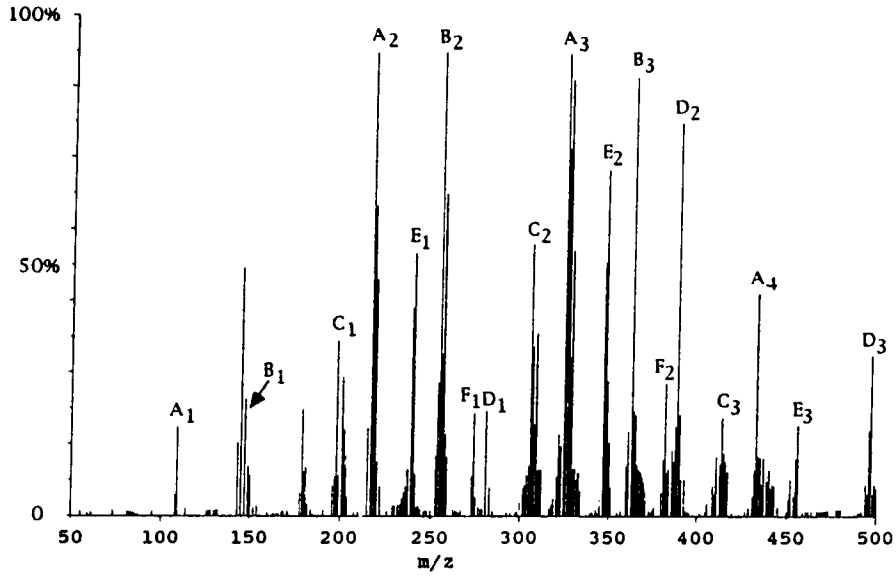


Figure 3.13. EH mass spectrum taken approximately 1300 minutes into a hydrolysis of 15.2 mM leucine-enkephalin with 2.2 units CPY/mL in 20 mM potassium acetate buffer. Series are labelled as follows: A_n = $[H + nTG]^+$; B_n = $[K + nTG]^+$; C_n = $[H + Ala + nTG]^+$; D_n = $[H + PTSA + nTG]^+$; E_n = $[H + Leu + nTG]^+$; F_n = $[H + Phe + nTG]^+$; where TG = thioglycerol, Ala = alanine, PTSA = para-toluenesulfonic acid, Leu = leucine, and Phe = phenylalanine. See Table 3.1 for identification of other prominent ions.

release of the first two amino acids can be obtained.

The inability to detect the peptides is probably not a consequence of the ionization method employed, but rather the choice of organic cosolvent and instrumentation used. As noted previously, Bel and Cook [39] monitored the CPY-catalyzed hydrolysis of 16 mM leucine-enkephalin with 2.2 units CPY/mL in 40/60 (v/v) water/thiodiethanol (TDE) with 500 mM NaI as supporting electrolyte (a matrix that would not give stable emission with the current source). The same mass spectrometer was used as in the present study; however, Bel used a different source which required premixing of the aqueous reaction solution with the TDE in a single syringe located inside the mass spectrometer source housing. The intact pentapeptide, as well as the intermediate tetra- and tripeptide, were detected in addition to the liberated amino acids. However, instability of the emission was problematic, prompting the present work to try to improve on his system.

Stable emission could not be established using Bel's solution composition with the present tee assembly (same sample premixed in both syringes). In fact, all attempts at using TDE as a cosolvent were unsuccessful. Therefore, another cosolvent had to be chosen.

Cook and Murawski [38] used a 30/70 (v/v) water/glycerol matrix to monitor a CPY-catalyzed hydrolysis of the substrate N-CBZ-Phe-Leu, but were unable to detect the intact protected dipeptide. For this reason, Bel did not use glycerol in his hydrolysis experiment [39]. Thioglycerol (TG) was never used as a cosolvent by Murawski or Bel because it totally inactivates CPY [38,39]. However, thioglycerol can be used to monitor a CPY-catalyzed hydrolysis with the present tee assembly because the reaction mixture and the TG are mixed "post column" just prior to entering the mass spectrometer. TG is more acidic than glycerol and

therefore is sometimes prefered as a FAB matrix for peptides such as angiotensin because it is better able to protonate analytes [53]. The use of TG resulted in stable emission for EHMS with the new source, and it was therefore adopted in these studies.

Figure 3.14 shows the concentration of leucine and phenylalanine released over time during the hydrolysis of leucine-enkephalin. (Phenylalanine concentration was determined through use of a phenylalanine calibration curve (see Figure 3.15 and Table 3.2).) The ions monitored were m/z = 348 ($[H + Leu + 2TG]^+$), 274 ($[H + Phe + TG]^+$), and 306 ($[H + Ala + 2TG]^+$) because these were typically the most intense ions (but not the only analyte ions; see Figure 3.13) for each amino acid. The two 20 minute gaps in the data at about 550 minutes and 1100 minutes are a result of the syringes having to be refilled. The bulk solution used to refill was thermostatted. From the appearance of the data in Figure 3.14, it is evident that the leucine is cleaved at a faster rate than the phenylalanine. The concentration of leucine rises during the first 200-300 minutes and then levels off afterwards. At approximately 100-150 minutes, the phenylalanine starts to be detectable and it continues to increase in concentration throughout the remainder of the experiment. The data in Figure 3.14 becomes more and more noisy as the reaction proceeds. There does not appear to be as much noise during the initial release of the leucine as there is after the concentration of leucine levels off or during the release of the phenylalanine.

One possibility for explaining the noise is that precipitation was occurring as the reaction progressed. At the start of the reaction, leucine-enkephalin is the only material of limited solubility present. As the reaction progresses, the tetrapeptide and leucine are present. At approximately 300 minutes, all of the

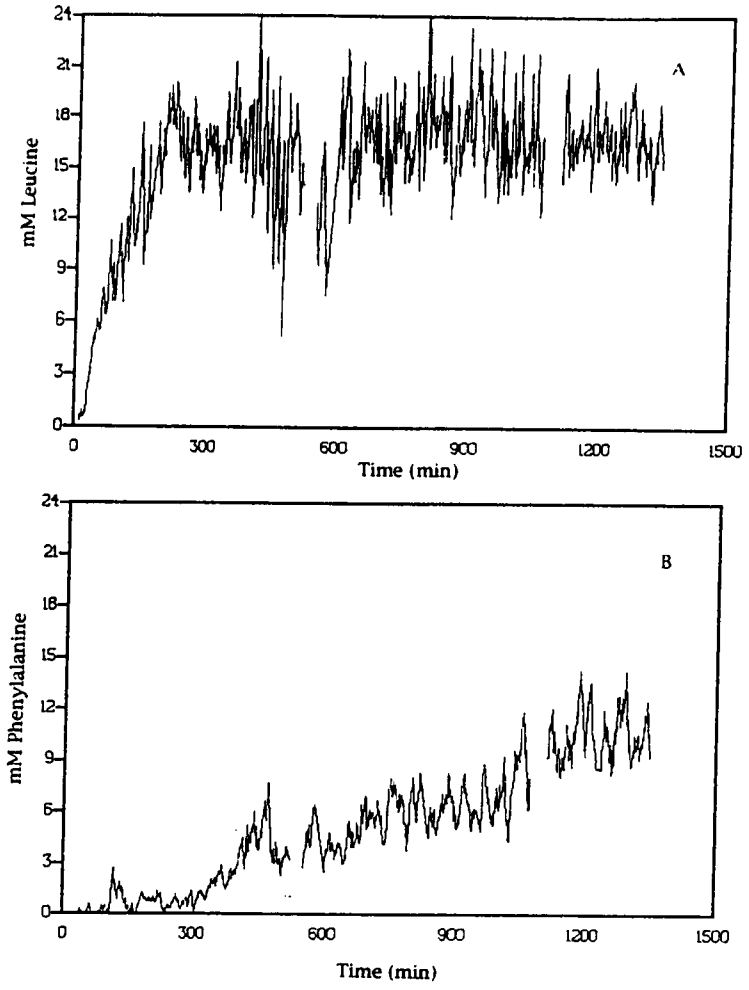


Figure 3.14. Concentration of (A) leucine and (B) phenalanine released during a hydrolysis of 15.2 mM leucine-enkephalin with 2.2 units CPY/mL in 20 mM potassium acetate buffer monitored by EHMS (10-point Savitzky-Golay smoothed). Interruptions at approximately 550 minutes and 1100 minutes are due to both syringes being refilled.

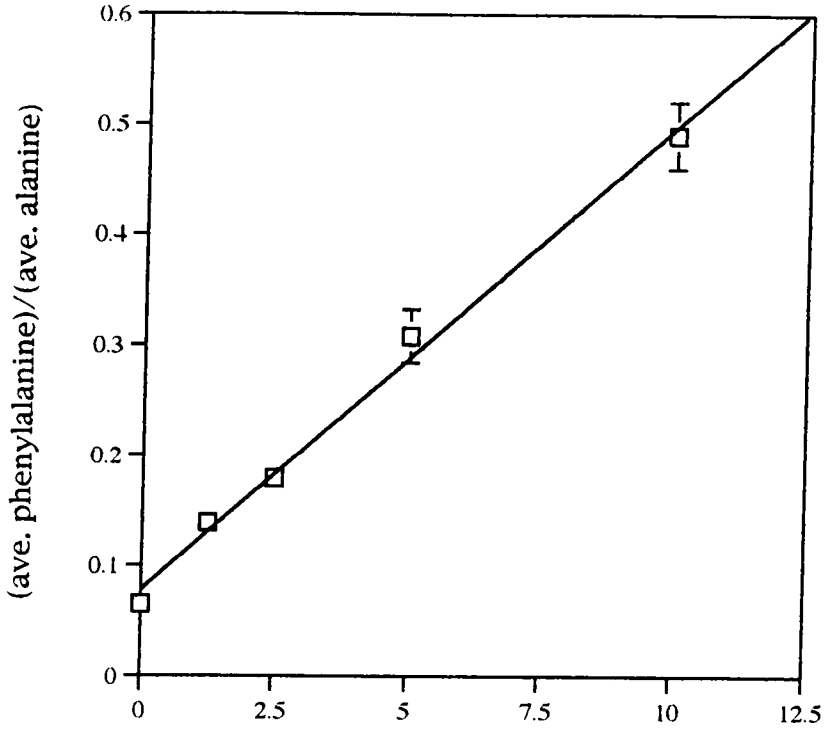


Figure 3.15. Mass spectrometric phenylalanine calibration curve. Error bars represent the standard deviation of the mean. See text for details. Syringe A contained the indicated amount of phenylalanine and 5.4 mM alanine in 20 mM potassium acetate buffer. Syringe B contained 100 mM para-toluenesulfonic acid and 5.4 mM alanine in 60/40 (v/v) water/thioglycerol. See Table 3.2 for slope, y-intercept, and correlation coefficient.

leucine has been liberated (15.2 mM). After about 1400 minutes, approximately 60% (or 9 mM) of the phenylalanine has been liberated from the tetrapeptide. At this point, present in the solution is 15.2 mM leucine, approximately 6 mM Tyr-Gly-Gly-Phe, 9 mM phenylalanine, and 9 mM Tyr-Gly-Gly in the aqueous reaction mixture. In comparing the top portion of Figure 3.14 to the data from the hydrolysis of N-CBZ-Val-Leu (Figure 3.5) or N-CBZ-Phe-Gly (Figure 3.9) it is obvious that Figure 3.14 is noisier. A comparison of the absolute ion intensities reveals that intensities from the leucine-enkephalin hydrolyses were approximately 2 to 3 times larger than those from the protected dipeptides. Different substrates are being hydrolyzed in each case, and the concentration of the substrate (and therefore that of the amino acids released) is higher for the leucine-enkephalin experiment, increasing the possibility of precipitation. After each of the three 24 hour hydrolyses of leucine-enkephalin was monitored by EHMS, the Pt capillary emitter and fused silica capillary were inspected using a microscope; however, there was no visual evidence of precipitation observed. The solubilities of all peptides and amino acids involved were determined. The acetate salt of leucine-enkephalin was used in these experiments. It was readily soluble at 15.2 mM in both the acetate buffer and the MS matrix. Leucine and phenylalanine are soluble at >90 mM and >60 mM, respectively, in buffer and MS matrix. Tyr-Gly-Gly is soluble at > 40 mM in each matrix. (Tyr-Gly-Gly-Phe was not available to be tested.) Therefore, precipitation was probably not occurring. The appearance of Figure 3.14 could also be a result of a "shot noise limited response." As the signal gets larger, the noise gets larger. It can be seen from Figures 3.4 and 3.8 (leucine and glycine calibration curves) that this appears to be the trend.

It is also apparent in Figure 3.14 that the final leucine concentration is greater than 15.2 mM. The alanine internal standard was prepared separately in both the MS matrix solution and in the aqueous reaction solution. If a weighing error was made which resulted in alanine being present at a concentration below 5.4 mM, it would give the appearance that more than 15.2 mM leucine would be released at the end of the hydrolysis. Another possible explanation is that the other species present during the course of the reaction (intermediates and released amino acids) suppressed alanine by competing for available protons.

In spite of the noise, the mass spectrometric data can be analyzed to determine the rate constants for the release of leucine and phenylalanine. The simple first order reaction model derived above can be used, provided that the enzyme is not saturated with substrate (see Chapter 1). To test for saturation, a single hydrolysis was monitored by EHMS at a lower substrate concentration (6.1 mM). The enzyme and buffer concentrations were the same as for the hydrolysis of 15.2 mM substrate. Figure 3.16 shows the concentration of leucine released during the hydrolysis at a substrate concentration of 6.1 mM. This hydrolysis was monitored for three hours, and the release of phenylalanine was not observed during this time. Figure 3.16 shows that as the reaction proceeds, the data becomes more noisy (not unlike in Figure 3.14). The initial velocities of reaction were determined for the three hydrolyses at 15.2 mM and for the one at 6.1 mM in the same manner as for the two protected dipeptides in Sections 3.3 and 3.4, and results are tabulated in Table 3.7. For the rates determined at 15.2 mM, the replicates do not agree within the experimental precision; a systematic error is again evident. Nevertheless, the initial velocity determined at 6.1 mM is substantially lower than the three replicates at 15.2 mM, suggesting that the

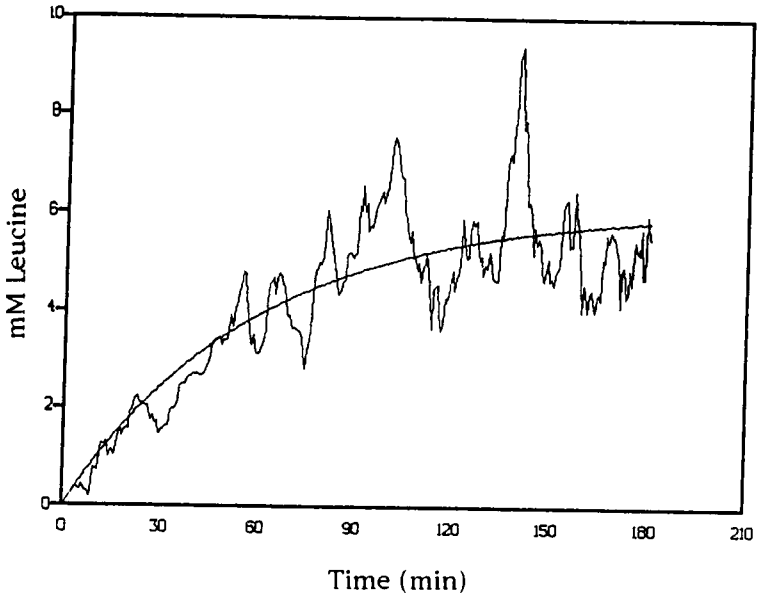


Figure 3.16. Concentration of leucine released during a hydrolysis of 6.1 mM leucine-enkephalin with 2.2 units CPY/mL in 20 mM potassium acetate buffer monitored by EHMS (10-point Savitzky-Golay smoothed). Solid line is predicted concentration of leucine based on rate constant determined from the least squares fit ($k_1 = 1.70 \times 10^{-2}$ min^{-1}).

Table 3.7. Initial velocities determined from hydrolyses of leucine-enkephalin. All uncertainties represent the 95% confidence limits.

Concentration[a]	Rate[b]	Ave. Rate[c]	Ave. Rate[d]
6.1	0.061 (±0.012)	----------	----------
15.2	0.167 (±0.013)		
15.2	0.084 (±0.014)	0.128 (±0.012)	0.128 (±0.103)
15.2	0.134 (±0.007)		

[a] expressed in mmol/L

[b] rates from each replicate analysis with 95% confidence limits; N = 60; t =2.00; for hydrolysis at 6.1 mM, N = 40; t = 2.02

[c] average rates with 95% confidence limits calculated from pooled standard deviations; N = 60; t = 2.00

[d] average rates with 95% confidence limits calculated from simple standard deviations; N = 3; t = 4.30

enzyme is at least not saturated by 6.1 mM substrate. If the rate constants for the release of leucine determined at 6.1 and 15.2 mM agree, it can be concluded that the enzyme is also not saturated at 15.2 mM.

A nonlinear least squares program called NLIN [54] was used on the University of Tennessee VAX computer in order to estimate the rate constants by fitting each set of smoothed data to Equations (13) and (15) (see Appendix B). Each value of k_1 obtained from the leucine data was used in Equation (15) to determine a value for k_2 for that replicate. The program also calculates the 95% confidence limits for each rate constant.

The top of Table 3.8 contains the values for k_1 and k_2 as well as the 95% confidence limits from each of the hydrolyses monitored by EHMS. Also listed are the average values of k_1 and k_2. Because the differences in the individual k_1s (and k_2s) are greater than the 95% confidence limits for the 15.2 mM data, the 95% confidence limits of each replicate were not pooled in order to calculate the 95% confidence limits for the averages. Rather, the three values for each rate constant were averaged, and a standard deviation and confidence limit was calculated for this average. Table 3.8 reveals that the k_1 obtained at 6.1 mM by EHMS agrees within experimental error with the k_1 obtained at 15.2 mM. It can therefore be concluded that the enzyme is not saturated at 15.2 mM.

Figure 3.17 contains plots of mM leucine and phenylalanine released versus time for the first replicate at 15.2 mM as observed in the lab as well as the concentration of each amino acid released versus time as predicted by substituting for k_1, k_2, and A_o into Equations (13), (15), and (16). In Figure 3.17, the "best" values of k_1 and k_2 for this data set were used (i.e. $k_1 = 1.26 \times 10^{-2}$ min^{-1} and $k_2 = 7.88 \times 10^{-4}$ min^{-1}). Because A_o is equal to 15.2 mM, the

Table 3.8. Rate constants for the release of leucine and phenylalanine from leucine-enkephalin. Uncertainties represent the 95% confidence limits.

Method	Conc.[a]	k_1 x 1000[b]	k_2 x 1000[b]	Ave. k_1 x 1000[b]	Ave. k_2 x 1000[b]
EHMS	15.2	12.6 (±0.7)	0.788 (±0.091)		
	15.2	14.5 (±0.9)	0.544 (±0.073)	14 (±4)[c]	0.82 (±0.72)[c]
	15.2	16.1 (±1.0)	1.124 (±0.142)		
	6.1	17.0 (±1.0)	N/A[d]	----------	----------
ninhydrin	15.2	2.49 (±0.28)	0.356 (±0.088)		
	15.2	2.70 (±0.84)	0.264 (±0.197)	2.9 (±1.4)[c]	0.33 (±0.15)[c]
	15.2	3.59 (±0.96)	0.381 (±0.159)		

[a] concentration expressed in mmol/L

[b] expressed in units of min^{-1}

[c] average rate constants with 95% confidence limits calculated from simple standard deviations; N = 3; t = 4.30

[d] release of phenylalanine not observed

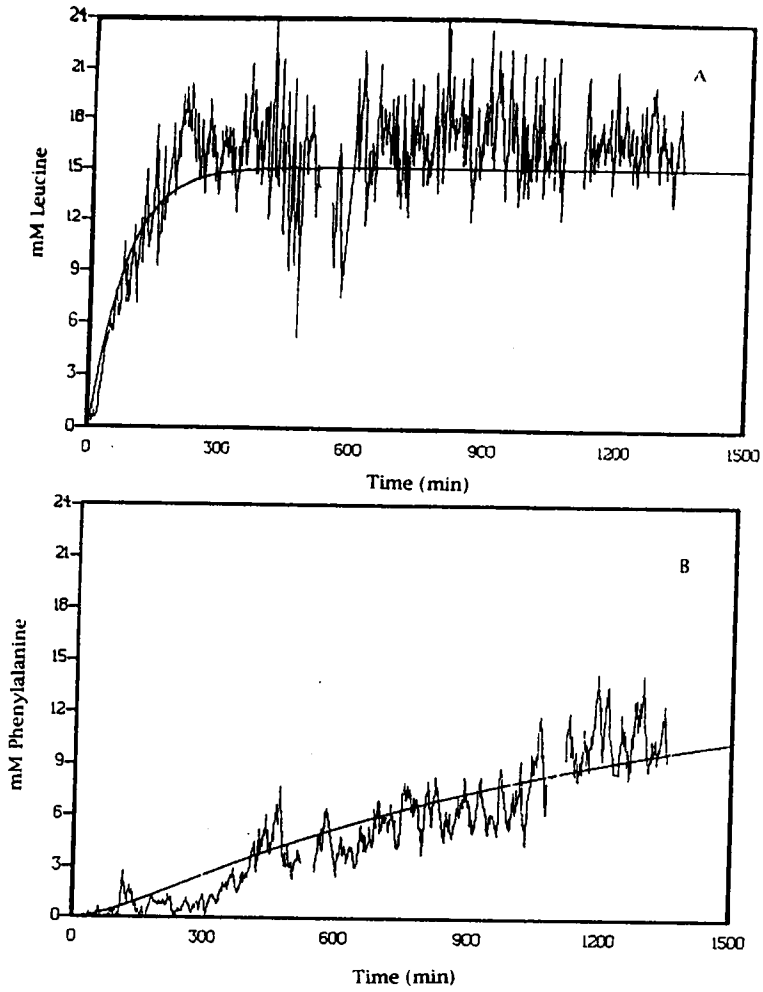


Figure 3.17. Concentration of (A) leucine and (B) phenylalanine released and predicted during the first hydrolysis of 15.2 mM leucine-enkephalin with 2.2 units CPY/mL in 20 mM potassium acetate buffer monitored by EHMS (10-point Savitzky-Golay smoothed). Interruptions at approximately 550 minutes and 1100 minutes are due to both syringes being refilled. Solid line is predicted concentration of (A) leucine and (B) phenylalanine based on rate constants determined from the best least squares fit (k_1 = 1.26 x 10^{-2} min^{-1} and k_2 = 7.88 x 10^{-4} min^{-1}).

predicted maximum leucine concentration will not exceed 15.2 mM. As can be seen from Figure 3.17, the predicted rate constants fit the data well. Figure 3.16 contains a plot of mM leucine released versus time during the hydrolysis of 6.1 mM leucine-enkephalin as well as the concentration of leucine released based on substituting 1.7×10^{-2} min^{-1} for k_1 in Equation (13). Figures 3.16 and 3.17 show that the rate constants predicted appear to represent (or "fit") the data from individual replicates. It was desired to assess whether the average values for k_1 and k_2 fit the individual replicate data sets despite the systematic error present.

Figure 3.18 contains the same mass spectrometric data as in Figure 3.17 (i.e. from the first replicate); however, the nonlinear least squares line shown is from the average k_1 (= 1.4×10^{-2} min^{-1}; see Figure 3.18 A) and k_2 (= 8.2×10^{-4} min^{-1}; see Figure 3.18 B). The average k_1 does not fit the initial part of the curve (i.e. before 300 minutes) in Figure 3.18 A as well as it does in Figure 3.17 A. The average k_1 over-estimates the amount of leucine released. Figure 3.18 B is very similar to Figure 3.17 B because the k_2 value from the first replicate is very close to the average k_2 value (see Table 3.8). Figures 3.19 and 3.20 each contain plots of mM leucine and phenylalanine released versus time from the second and third replicates, respectively, as well as the concentrations predicted based on the average values for k_1 and k_2.

It can be concluded from Figures 3.18, 3.19, and 3.20 that the average k's do not fit most of the data very well. The average k_1 fits the third "replicate" the best (at least for the first 300 minutes; see Figure 3.20 A) while the average k_2 fits the first "replicate" the best (see Figure 3.18 B). There is a significant difference among the "replicates" as evidenced by the release of phenylalanine in Figures 3.19 B and 3.20 B. Figure 3.19 B shows that, after about 1400 minutes,

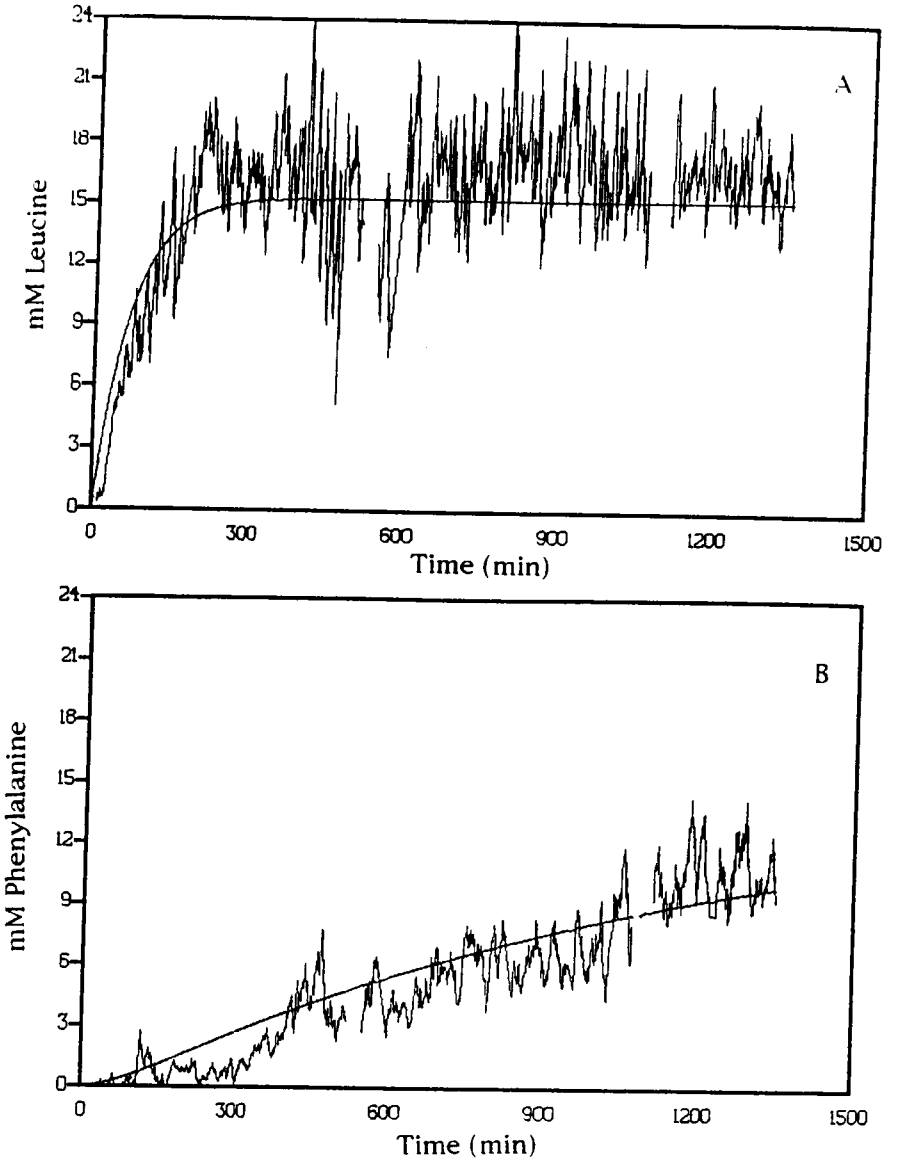


Figure 3.18. Average rate constants with data from first hydrolysis. Concentration of (A) leucine and (B) phenylalanine released and predicted during the first hydrolysis of 15.2 mM leucine-enkephalin with 2.2 units CPY/mL in 20 mM potassium acetate buffer monitored by EHMS (10-point Savitzky-Golay smoothed). Interruptions at approximately 550 minutes and 1100 minutes are due to both syringes being refilled. Solid line is predicted concentration of (A) leucine and (B) phenylalanine based on average rate constants determined from the least squares fit (k_1 = 1.4 x 10^{-2} min^{-1} and k_2 = 8.2 x 10^{-4} min^{-1}).

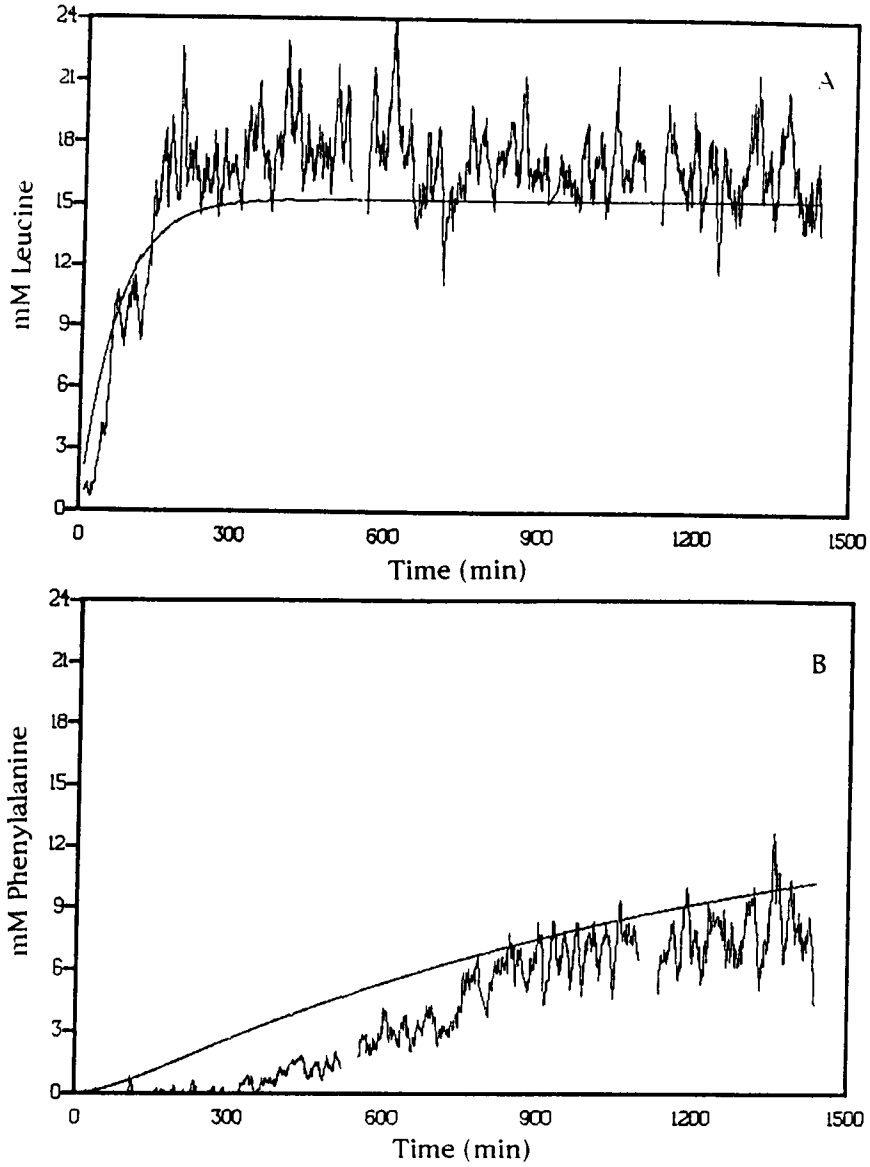


Figure 3.19. Average rate constants with data from second hydrolysis. Concentration of (A) leucine and (B) phenylalanine released and predicted during the second hydrolysis of 15.2 mM leucine-enkephalin with 2.2 units CPY/mL in 20 mM potassium acetate buffer monitored by EHMS (10-point Savitzky-Golay smoothed). Interruptions at approximately 550 minutes and 1100 minutes are due to both syringes being refilled. Solid line is predicted concentration of (A) leucine and (B) phenylalanine based on average rate constants determined from the least squares fit (k_1 = 1.4 x 10^{-2} min^{-1} and k_2 = 8.2 x 10^{-4} min^{-1}).

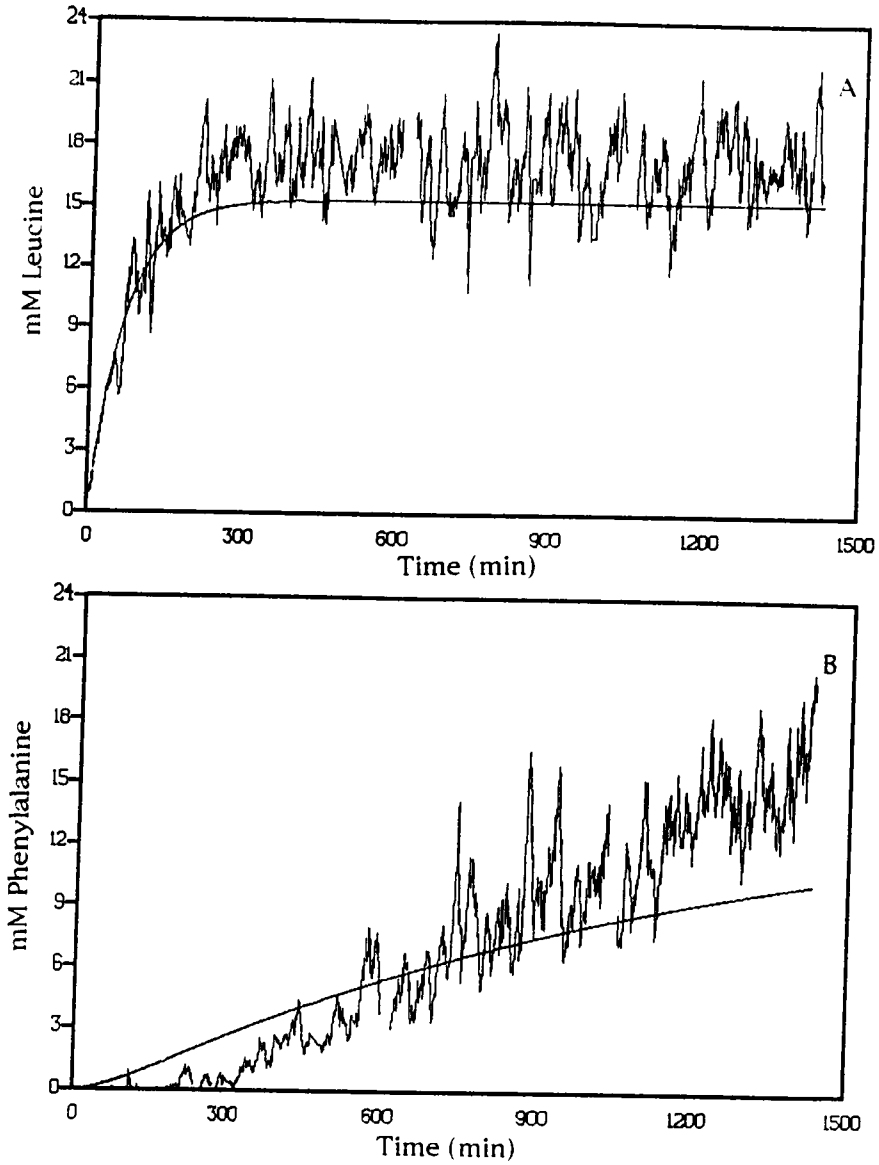


Figure 3.20. Average rate constants with data from third hydrolysis. Concentration of (A) leucine and (B) phenylalanine released and predicted during the third hydrolysis of 15.2 mM leucine-enkephalin with 2.2 units CPY/mL in 20 mM potassium acetate buffer monitored by EHMS (10-point Savitzky-Golay smoothed). Interruptions at approximately 600 minutes and 1050 minutes are due to both syringes being refilled. Solid line is predicted concentration of (A) leucine and (B) phenylalanine based on average rate constants determined from the least squares fit (k_1 = 1.4 x 10^{-2} min^{-1} and k_2 = 8.2 x 10^{-4} min^{-1}).

approximately 6 mM phenylalanine has been released, compared to almost 15 mM in Figure 3.20 B. Therefore, the three hydrolyses at 15.2 mM are not true replicates. Systematic errors, such as contamination by thioglycerol due to preconditioning, may be the cause for the discrepency between the individual runs.

At this point, optical studies were undertaken for comparison with the EHMS results. A second leucine calibration curve was obtained (for reasons decribed in Chapter 2) for use with data from the hydrolyses of leucine-enkephalin monitored by the ninhydrin method (see Figure 3.21). Hydrolyses of leucine-enkephalin were monitored optically at a substrate concentration of 15.2 mM and an enzyme concentration of 2.2 units CPY/mL in 20 mM potassium acetate buffer. The reaction mixture was sampled after two and three hours, and then every three hours afterwards for 24 hours (i.e. at 2, 3, 6, 9, . . ., 24 hours). Figure 3.22 is a plot of the concentration of released amino acid versus time from each of the three hydrolyses monitored optically. Concentrations were estimated using the calibration curve of Figure 3.21 and were corrected for the constant absorbance due to the peptide residues. (Since the tyrosine amino group of the peptides is not blocked with a protecting group (such as the carbobenzoxy group), ninhydrin will form a complex with the peptides in addition to the released amino acids.) The leucine calibration curve in Figure 3.21 was used for the total amine concentration. This assumes that the ninhydrin complex forms to the same extent with all of these amines and that the spectroscopy of all complexes is the same. A comparison of the data in Table 3.4 (optical calibration curves data for leucine and glycine under identical conditions) supports this assumption.

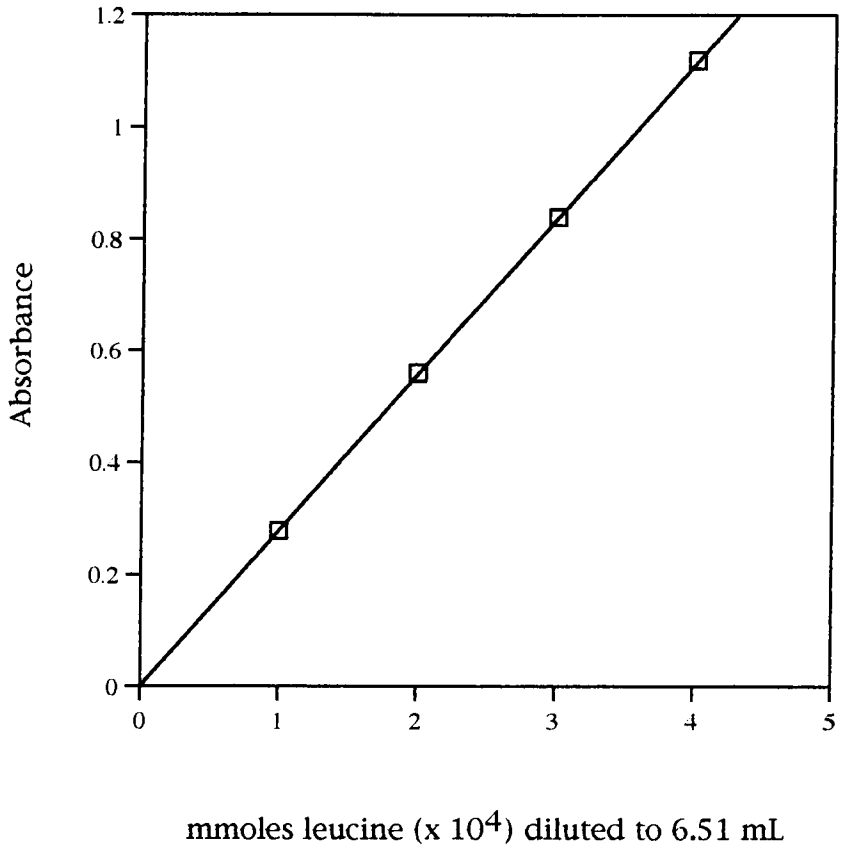


Figure 3.21. Leucine calibration curve obtained by the ninhydrin method as modified for high initial substrate concentration (see Section 2.5.2).

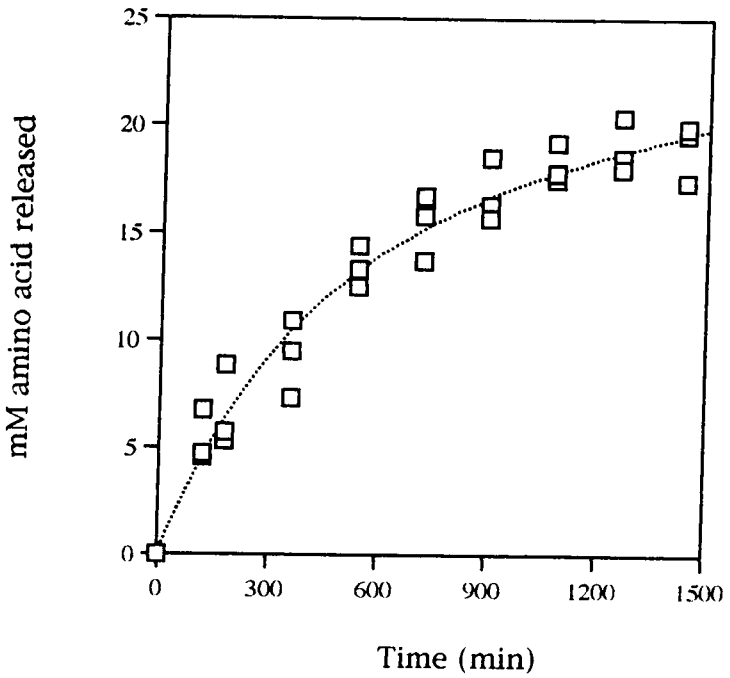


Figure 3.22. Concentration of amino acids released during hydrolyses of 15.2 mM leucine-enkephalin with 2.2 units CPY/mL in 20 mM potassium acetate buffer determined by the ninhydrin method. Dotted line is predicted concentration of amino acids based on $k_1 = 2.9 \times 10^{-3}$ min^{-1} and $k_2 = 3.3 \times 10^{-4}$ min^{-1}.

It must be emphasized that, during the hydrolysis of the pentapeptide, phenylalanine is being released from the tetrapeptide before all of the leucine has been released from the pentapeptide. The absorbance read at any time is the sum of the absorbances of each amino acid released as well as the "background" absorbance from the peptides present. This background absorbance was determined by forming a ninhydrin complex with a blank which consisted of the substrate with no enzyme. The absorbance of the amino acids present was determined by subtracting the background absorbance due to the peptides from the absorbance determined at each time interval. The concentration of amino acids (sum) at time t was determined by dividing the corrected absorbance by the absorption coefficient from the leucine calibration plot (Figure 3.21). The total absorbance never exceeded one absorbance unit. The overall equation fitted by the computer program was:

$$\text{sum} = P_1 + P_2 \tag{18}$$

The bottom of Table 3.8 contains the values of k_1 and k_2 determined by this fit, as well as the 95% confidence limits. Figure 3.22 contains a plot of the concentration of amino acids released versus time as observed in the lab as well as the concentration of amino acids released versus time as estimated by the computer program using the least squares k's.

In analyzing the data from Table 3.8, it is evident that the value of k_1 determined by EHMS is a factor of 5 larger than that determined by the ninhydrin method. The value of k_2 determined by EHMS is a factor of 2.5 larger than that determined by the ninhydrin method; however, confidence limits for the k_2

values overlap. The discrepencies cannot be due to thioglycerol contamination, as this would slow the hydrolysis and cause the rate constants for the EHMS data to be lower than those determined by the ninhydrin method. One possible explanation for the higher rate constants determined by EHMS could be due to the problem resulting from the preparation of the alanine internal standard mentioned previously. Apparent release of a greater amount of leucine than would be expected will lead to a higher rate constant as calculated by the model decribed previously.

In order to determine qualitatively how sensitive each nonlinear least squares fit was to the values of k_1 and k_2, the model predictions based on the average optical k values were plotted with the EHMS data (Figure 3.23) while those from the EHMS data were plotted with the optical data (Figure 3.24). It must be concluded based on Figures 3.23 and 3.24 that the two systems were chemically different and/or one or the other method was not representative. The EHMS experiments could have been chemically different from the optical experiments due to possible thioglycerol contamination or failure to properly regulate the temperature of the reaction mixture in the syringe. The flawed internal standard approach mentioned previously also could have contributed to the discrepancies between the rate contants determined by each method. Because the ninhydrin method is an accepted method, it is possible that EHMS does not accurately provide the true rate constants under the present experimental conditions.

The results of this study prove that enzymatic hydrolyses occurring in aqueous environments can be monitored continuously by EHMS for up to 24 hours through use of the tee assembly. However, it is possible that there are systematic error(s) present, possibly due to "poisoning" of the enzyme by

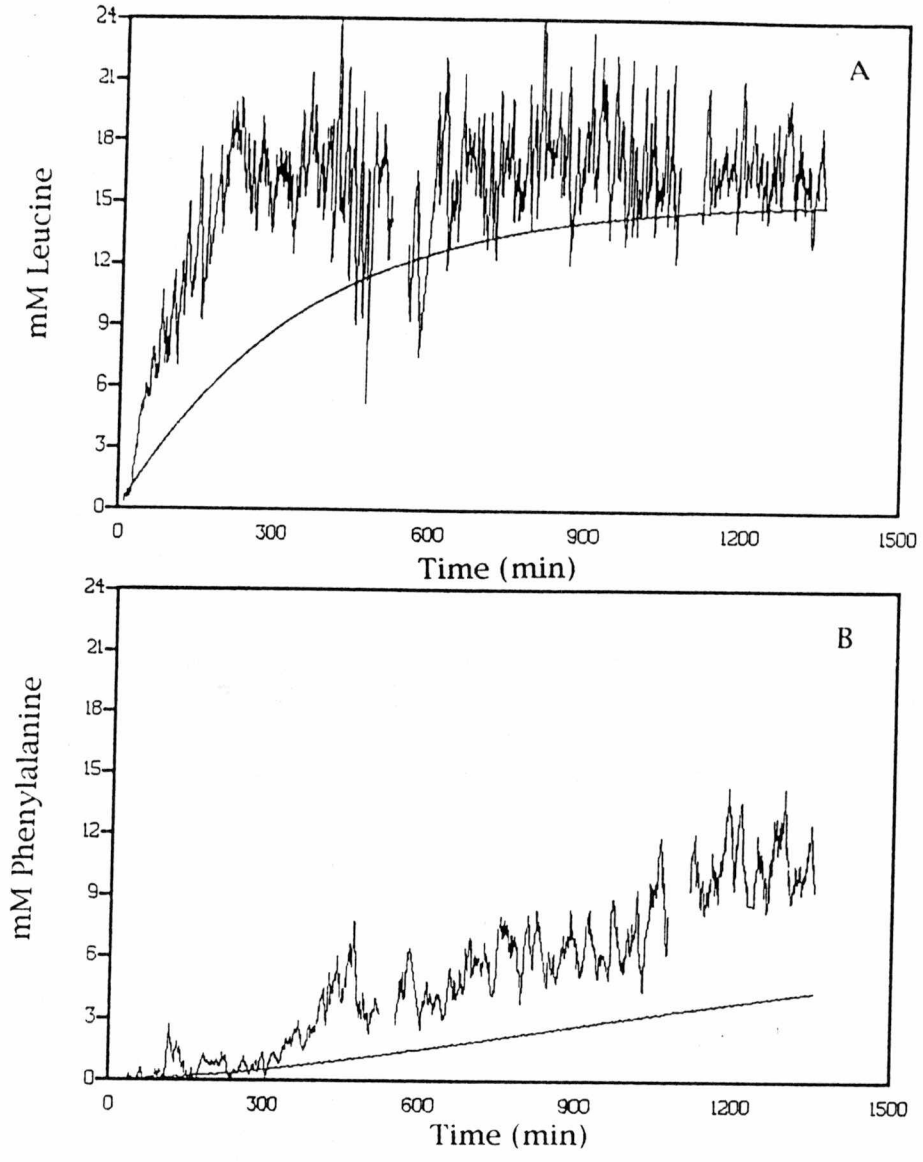


Figure 3.23. Optical rate constants with EHMS data. Concentration of leucine (A) and phenylalanine (B) released during the first hydrolysis of 15.2 m M leucine-enkephalin with 2.2 units CPY/mL in 20 mM potassium acetate buffer monitored by EHMS (10-point Savitzky-Golay smoothed). Interruptions at approximately 550 minutes and 1100 minutes are due to both syringes being refilled. Solid line is predicted concentration of (A) leucine and (B) phenylalanine based on the rate constants determined by the ninhydrin method (k_1 = 2.9 x 10^{-3} min^{-1} and k_2 = 3.3 x 10^{-4} min^{-1}).

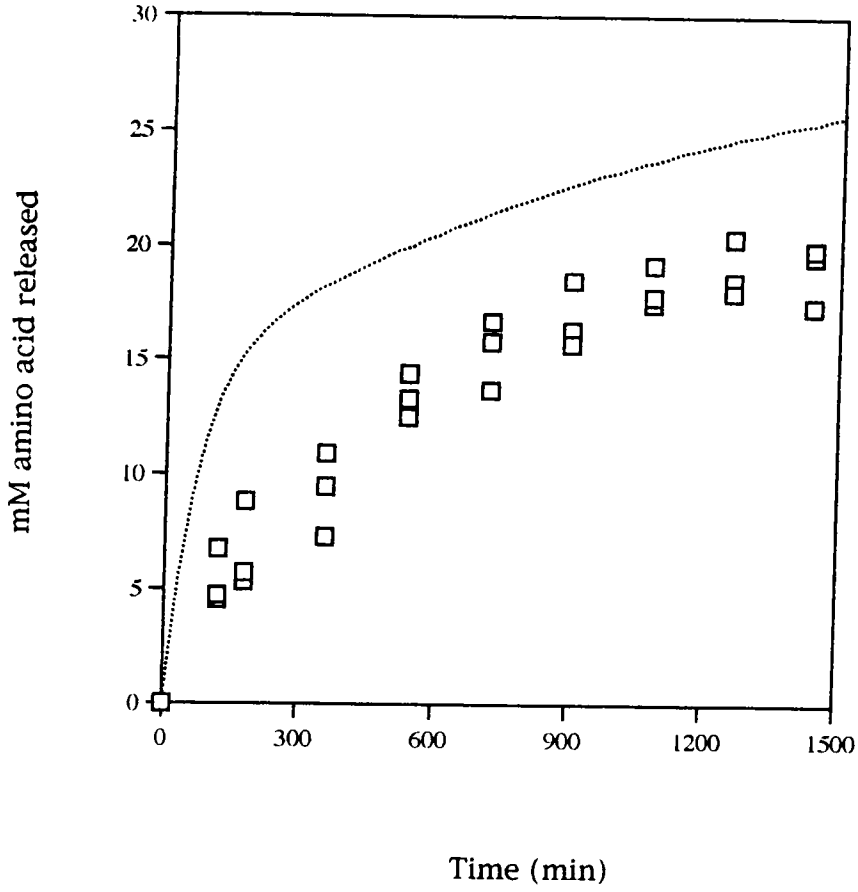


Figure 3.24. EHMS rate constants with optical data. Concentration of amino acids released during hydrolyses of 15.2 mM leucine-enkephalin with 2.2 units CPY/mL in 20 mM potassium acetate buffer determined by the ninhydrin method. Dotted line is predicted concentration of amino acids based the average rate constants determined by EHMS (k_1 = 1.4 x 10^{-2} min^{-1} and k_2 = 8.2 x 10^{-4} min^{-1}).

preconditioning the tee and capillaries with a thioglycerol solution and/or the use of an inappropriate internal standard. Nevertheless, useful qualitative and semi-quantitative information can be obtained.

Chapter 4

Conclusions and Suggestions for Future Work

Enzymatic hydrolyses of peptides taking place in a totally aqueous environment have been monitored for the first time using EHMS. The development of the low dead volume tee assembly has allowed for biochemical reactions to be mixed with an organic cosolvent (needed for the EH ionization process) just prior to entering the mass spectrometer. Reactions have been monitored continuously for up to 24 hours. Two protected dipeptides and one pentapeptide were hydrolyzed by the enzyme carboxypeptidase-Y. Reactions were monitored by both EHMS and the ninhydrin method.

The rates of hydrolyses were determined by EHMS in triplicate. It was discovered that the difference between the rates from replicates (at fixed concentration) differed by a greater amount than the uncertainty in each rate. This is indicative of a systematic error. The preconditioning of the tee and capillaries with the "blank" solution could have contaminated the tee and capillaries with thioglycerol. The syringe could have been contaminated as well. This could have lead to poisoning of the enzyme and run-to-run variation in the rates of reaction. This preconditioning step should be omitted. Another source of error could be the fact that the reaction mixture was not temperature-regulated for about 5% of the time for the hydrolyses of protected dipeptides and 73% of the time for hydrolyses of leucine-enkephalin. The tee assembly should be designed in such a way so as to ensure total regulation of the temperature during hydrolyses. Despite the systematic error(s), this study showed that EHMS can provide useful qualitative data.

For the substrate N-CBZ-Val-Leu, the maximal velocities of the reaction (V_{max}) determined by EHMS and the ninhydrin method agree within the 95% confidence limit. For the substrate N-CBZ-Phe-Gly, the Michaelis constants (K_m) determined by EHMS and the ninhydrin method also agree within the 95% confidence limit; however, precision was very poor.

The hydrolysis of a pentapeptide, leucine-enkephalin, was monitored continuously by EHMS for 24 hours. Rate constants were determined from the EHMS data for the release of the first two amino acids from leucine-enkephalin. These rate constants were also determined using the ninhydrin method by deconvoluting the total amino acid concentration data. The rate constants determined by these methods did not agree within the 95% confidence limit. Although the mass spectrometric data was very noisy, a rather large quantity was collected continuously for 24 hours as compared to timed intervals with the optical method. If the run-to-run variation due to systematic error(s) is overlooked, the within run precisions are comparable.

It was found that the mass spectrometric data from the hydrolyses of leucine-enkephalin was rather noisy. One suggestion for reducing the noise is through the use of ion optics with the EH ion source. In a shot noise limited response, the signal is proportional to the square root of the number of ions impinging upon the detector. Through use of a lens, more ions could be focused through the entrance slit of the MS902 mass spectrometer. Thus, more ions would have a chance to impinge upon the detector. A lens could improve the signal-to-noise ratio.

Another suggestion for improvement is to change the internal standard approach used. It would be much easier to include the internal standard in the

reaction mixture only and not in the MS matrix. Preparation of a single internal standard solution (i.e., weighing the internal standard only once) reduces the number of weighing errors that could be made. In the present studies, alanine was used as an internal standard. In the future, it is suggested that a different internal standard be used in the event that alanine is a constituent amino acid in a peptide sample being hydrolyzed. Good candidates might be non-naturally occurring amino acids or quaternary ammonium salts.

Because there is no fragmentation in EHMS, it is an attractive tool for qualitatively probing enzymatic hydrolyses of peptides. However there are also a few disadvantages of the technique at the present time. Only the released amino acids were detected during the hydrolyses. The substrates and residues were not detected. This can be a disadvantage if trying to obtain sequence information from an unknown and if there are repetitive amino acids in the peptide chain. However, this is probably not a consequence of the ionization method employed, but rather a result of the sampling efficiencies of the analytes in the MS matrix chosen. Therefore, more experiments should be performed to try to optimize the solvent in order to provide better sensitivity to the larger peptides.

Alternatively, the use of *no* organic cosolvent would be desirable. Kriger and coworkers [55] have demonstrated the use of gold-coated fused silica capillaries in electrospray ionization mass spectrometry (ESIMS). At the present time, it is believed that the organic cosolvent is needed with the present EH source for electrical connection from emitter to solution. If the gold-coated capillaries can survive the EH ionization process, no organic cosolvent (and therefore no tee assembly) would be required. Since most large peptides are hydrophobic, they should be sampled more efficiently from water than from a

mixed solvent.

Another undesirable aspect at the present time is the high limit of detection (LOD). It is not known why the LOD is so high, compared to those of other mass spectrometric ionization techniques. Electrospray ionization (ESI) mass spectrometry, for example, is performed routinely with samples in the micromolar concentration range. This could be associated with the choice of organic cosolvent used.

One other disadvantage is the fact that the sample is diluted when analyzed using the tee assembly. However, the amount of dilution can be controlled by the experimenter by changing the composition of the solutions in each syringe and the flow rate from each syringe. In these initial experiments to show the feasibility of the tee assembly, this aspect was not considered. In these studies, the reaction mixture was diluted by a factor of two because each syringe had the same flow rate. The use of the gold-coated capillaries would eliminate sample dilution.

The mass spectrum of the "blank" and "separated" samples revealed the possibility of glycerol contamination (see Section 3.1). Some experiments could be designed to determine if 27 mM glycerol in TG would be detected by EHMS. The TG could be spiked with glycerol, and this sample could be analyzed by EHMS. Alternatively, the TG could be re-distilled. EHMS could be performed on a sample dissolved in this TG to determine whether the peaks at m/z 201, 307, and 309 were still evident in the mass spectrum.

Another problem with the present tee assembly is the possibility of thioglycerol diffusion from the tee to the reaction mixture. The diffusion coefficient for thioglycerol in water could not be found in the literature. The

possibility of thioglycerol contamination from diffusion could be eliminated through use of a check-valve between the tee and the reaction reservior.

The use of the low dead volume tee assembly poses an interesting possibility of a means to study enzymatic hydrolyses of peptides using ESIMS. Samples analyzed by ESI are usually dissolved in a methanol or water/methanol solution with a small amount (1% or less) of an acid (such as acetic or trifluoroacetic acid) added. It seems that it should be feasible to mix an aqueous reaction mixture with a methanol/acid solution using the tee assembly and to subsequently analyze the progress of the reaction using ESI.

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APPENDICES

Appendix A

Statistics Equations Used in Calculations

Appendix A

Statistics Equations Used in Calculations

The purpose of this appendix is to provide the equations used in the statistical analyses of the data obtained in this work. In order to perform a linear least squares fit for the determination of the initial rate of release of amino acids from substrates, the following formulas were utilized [56]:

$$S_{xx} = \Sigma x_i^2 - [(\Sigma x_i)^2/N] \quad (1)$$

$$S_{yy} = \Sigma y_i^2 - [(\Sigma y_i)^2/N] \quad (2)$$

$$S_{xy} = \Sigma x_i y_i - [\Sigma x_i \Sigma y_i/N] \quad (3)$$

where x_i and y_i are the individual pairs of data for x and y and N is the number of pairs of data being fitted to a straight line. S_{xx} and S_{yy} are the sum of squares of the deviations from the mean for the individual values of x and y. The average values for the variables x (avx) and y (avy) are

$$avx = \Sigma x_i/N \quad (4)$$

$$avy = \Sigma y_i/N \quad (5)$$

The slope (m) and y-intercept (b) of the least squares line are defined as follows:

$$m = S_{xy}/S_{xx} \tag{6}$$

$$b = avy - m(avx) \tag{7}$$

The standard deviation about the regression (s_r) is defined as

$$s_r^2 = [S_{yy} - (m^2 * S_{xx})]/(N - 2) \tag{8}$$

Note that two degrees of freedom (one from the calculation of the slope and one for the calculation of the y-intercept) are lost in the calculation of s_r. The standard deviation of the slope (σ_m) and y-intercept (σ_b) can be calculated from the following equations [57]:

$$\sigma_m^2 = N (s_r^2) / \Delta \tag{9}$$

$$\sigma_b^2 = s_r^2 (\Sigma x_i^2) / \Delta \tag{10}$$

where $\Delta = N \Sigma x_i^2 - (\Sigma x_i)^2$.

In order to calculate the pooled standard deviation (s_p) for the slopes from the EHMS data, the following formula was used [43]:

$$s_p^2 = (v_1\sigma_{m1}^2 + v_2\sigma_{m2}^2 + v_3\sigma_{m3}^2)/ (v_1 + v_2 + v_3) \tag{11}$$

where v1, v2, and v3 are the number of degrees of freedom of each of the

replicates and σ_{m1}, σ_{m2}, and σ_{m3} are the standard deviations of the slope from each of the replicates. Because each replicate contained the same number of degrees of freedom, $\nu 1 = \nu 2 = \nu 3$, equation (11) reduces to

$$s_p^2 = (\sigma_{m1}^2 + \sigma_{m2}^2 + \sigma_{m3}^2) / 3 \qquad (12)$$

To calculate the 95% confidence limit using the pooled standard deviation, the appropriate t value was multiplied by s_p.

Appendix B

Nonlinear Least Squares Program

NLIN

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Nonlinear Least Squares Program

NLIN

The program NLIN (NonLINear regression) [54] was used on the University of Tennessee VAX computer to perform a nonlinear least squares fit to the EHMS and optical data in order to estimate the rate constants k_1 and k_2 for the hydrolysis of leucine-enkephalin. The typical run time for the program was approximately one minute.

To successfully execute this program, a regression expression must be written (i.e., a model; e.g., the sum of Equations 3.13 and 3.15), parameter names (e.g., k_1 and k_2) as well as starting values must be declared, and partial derivatives of the model with respect to each parameter must be supplied (e.g., partial derivative of the model with respect to k_1). The Gauss-Newton iterative method was utilized [54]. The program basically finds the values of the parameters which minimize the residual sum of squares. The best fit is obtained when there is no change in the residual sum of squares.

As an example, the following statements were written in order to determine the rate constants k_1 and k_2 for the optical data. Temp1 to temp7 are variables. TOTAL is the model (sum of equations 3.13 and 3.15). der.k1 and der.k2 are the partial derivatives of TOTAL with respect to k_1 and k_2, respectively. All other lines are briefly described in brackets below.

```
options ls=80; [allows output to be viewed on screen]
data a; infile 'indata.dat' dsd; [opens the data file "indata.dat"]
```

```
input t aa; [reads time and amino acid concentration data]
proc nlin; [starts the program NLIN]
 parms k1=0.001     [declares starting value for k1]
          k2=0.0001; [declares starting value for k2]
 A0 = 15.2   [initial leucine-enkephalin concentration]
 temp1 = exp(-k2*t)
 temp2 = A0 * k1 /[(k2 - k1) * (k2 - k1)]
 temp3 = [A0 * k2 - A0 * (k2 - k1)]/[(k2 - k1) * (k2 - k1)]
 temp4 = exp(-k1*t)
 temp5 = A0 * k2/[(k2 - k1) * (k2 - k1)]
 temp6 = [(k2 - k1) * A0 + A0 * k1]/[(k2 - k1) * (k2 - k1)]
 temp7 = A0/(k2 - k1)
 model TOTAL = A0 - A0 * temp4 + k1 * temp7 * temp1 - k2 * temp7 * temp4 +
                    k2 * temp7 - k1 * temp7
 der.k1 = A0 * t * temp4 + temp6 * temp1 + k2 * t * temp7 * temp4 - temp5 *
           temp4 + temp5 - temp6
 der.k2 = -k1 * t * temp7 * temp1 - temp2 * temp1 + temp3 * temp4 - temp3 +
           temp2
```

Appendix C

Helpful Hints for Successful Operation of the Tee Assembly

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Helpful Hints for Successful Operation of the Tee Assembly

The purpose of this appendix is to serve as a possible "trouble-shooting" guide of sorts for the operation of the low dead volume tee. Helpful hints will also be provided. Throughout the next few paragraphs, some of the obstacles that had to be overcome before the tee worked properly will also be revealed.

The end of the 25 μm i.d. fused silica capillary that extends into the mass spectrometer must have a square cut in order for stable emission to be achieved. Inspection of the capillary under a microscope will aid in determining if the end of the capillary is acceptable. It is recommended that the end of the capillary be inspected before and after each use. It will probably be necessary to make a new cut after several hours of use. This can be accomplished by using an Alltech ceramic "Allscribe" capillary cutter (or similar capillary cutting tool). It must also be stressed that, in the event that stable emission cannot be established with a solution that should provide stable emission, the cut at the end of the capillary is by far the easiest item to check and remedy.

The PEEK tubing that is used in all unions and the tee is essential. In early experiments, teflon ferrules were used to make connections between Swagelok fittings and capillary tubing. This was found to give rise to irreproducible results, due at least in part to the large dead volumes that arise when 1/16" fittings are used with tubing smaller than 1/16" o.d. Use of the PEEK tubing sleeves greatly reduced this dead volume (from 12 μL to 0.28 μL for the "low dead volume" tee) and improved reproducibility. Stainless steel ferrules were used in

all fittings to clamp down on the PEEK tubing which, in turn, held the fused silica capillary in place. The fittings should be tightened just enough to hold the capillaries in place. Overtightening can lead to deformation of the ferrules, PEEK, and/or the actual fittings themselves resulting in leaks and irreproducibility.

The flow resistance of the fused silica capillaries made it possible to pressurize the syringes to the point that they began to leak, either at the teflon seal (where the removable needles joined the syringe luer), or around the teflon plunger tip. Because the i.d. of the syringe barrel was so small (0.73 mm), it was sometimes difficult by visual inspection to determine if leakage was in fact occurring at the plunger tips. This problem was remedied through checking for leakage by filling the syringes and flushing the tee with a concentrated solution of methylene blue in water. It was much easier to determine if there was leakage at the plunger tips by this method. In one instance, there was also leakage at the luer-to-barrel seal on one of the syringes. An attempt was made to fix this problem by applying glue to this seal, but this served only as a temporary solution. This syringe barrel was soon replaced. It must be stressed that leakage anywhere "along the line" in the tee assembly resulted in an inability to establish emission (stable or otherwise).

One additional problem which led to unstable emission and irreproducibility was electrical leakage through the syringes to the syringe pumps. Because of the 8 kV electrical contact at the emitter, the solution, and therefore the tee, unions, needles, and syringes were all electrically "floating." This problem was remedied as described in Chapter 2 of this thesis.

In early experiments, a different geometry was used with the low dead volume tee assembly. This is represented in the top of Figure C.1. In this

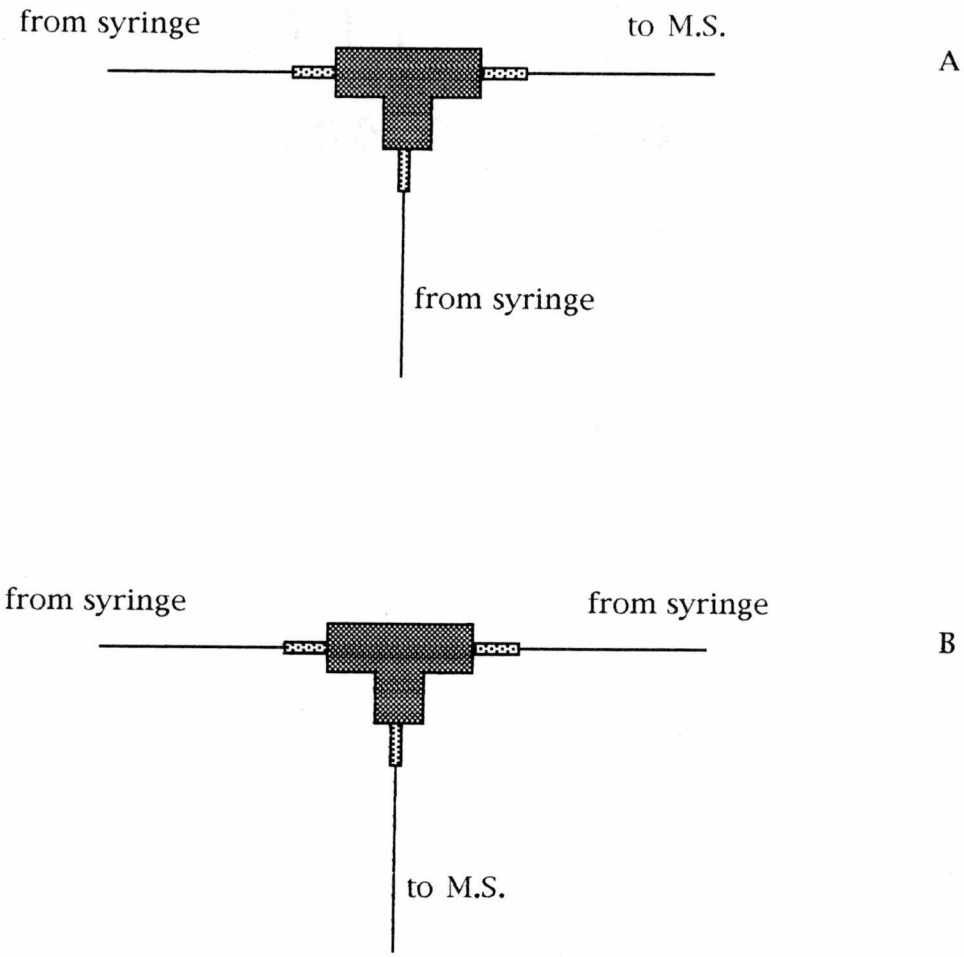

Figure C.1. Different geometries of the tee assembly showing capillaries from syringes oriented (A) 90 degrees and (B) 180 degrees to each other.

arrangement, the capillaries from the syringes were entering the tee at a 90^{o} angle to each other. It was then thought that better mixing would result if the capillaries were oriented at a 180^{o} angle to each other (see bottom of Figure C.1). No noticeable improvement in the performance of the tee assembly resulted from this new configuration; however, this new configuration was kept and used during all of the studies presented in this thesis.

Vita

Steven Walter Salvato, son of Welton and Barbara Hamon, was born on March 12, 1969 in Pasadena, Texas. He grew up in San Angelo, Texas and attended San Angelo Central High School where he graduated fifth in his class in 1987. In the fall of 1987, he entered Angelo State University, where he received a Carr academic scholarship for four years. Steven graduated *summa cume laude* in August 1991 with a B. S. in chemistry.

In the fall of 1991, Steven entered graduate school at the University of Tennessee to pursue an advanced degree in chemistry. He was a teaching assistant in general chemistry from fall 1991 to spring 1992. From fall 1992 to fall 1994, Steven was a teaching assistant in undergraduate analytical chemistry. Steven received a research assistantship from Dr. Kelsey Cook during the spring semester of 1993.

Steven married Julie Beth Hutt on December 28, 1991. After graduation, Steven and Julie will move to Killeen, Texas where Steven will assume duties as an instructor of chemistry at Central Texas College.

Steven is a member of the American Chemical Society (1992), the American Society for Mass Spectrometry (1993), and the East Tennessee Mass Spectrometry Discussion Group (1992).