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EFFECTS OF THIODIETHANOL ON THE KINETICS OF PEPTIDE HYDROLYSIS BY CARBOXYPEPTIDASE-Y

A Thesis

Presented for the

Master of Science

Degree

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K. Someswari Rao

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ABSTRACT

Optimization of a solvent system that can be used in Electrohydrodynamic Mass Spectrometry (EHMS) and effects of that solvent system on kinetics of an enzyme is the topic of present investigation. Thiodiethanol (TDE) was used as the low volatility organic solvent. Carboxypeptidase-Y was used because of its versatility to cleave most amino acids.

Three carbobenzyloxy-protected dipeptides were hydrolysed, and the absorbance of the released amino acids was monitored with a diode array UV/VIS spectrophotometer. The kinetics parameters such as rate constant (k), Michaelis constant (K_m), and maximum velocity (V_{max}) were determined at 0, 10%, 20%, and 40% TDE in phosphate buffer and 0.5 M sodium chloride. A pentapeptide, Leu-Enkephalin, was also hydrolysed at 0 and 20% TDE in phosphate buffer, under similar conditions, and the rate constants were estimated assuming first order kinetics, using a nonlinear approximation procedure.

The results show that TDE inhibits enzyme hydrolysis. For a given concentration of enzyme, as the amount of TDE in the solvent system increased, rate constant for hydrolysis k, and V_{max} decreased, and K_m increased. These results prove that TDE can be used in EHMS, with a maximum of 20% TDE in buffer.

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1. INTRODUCTION

Peptide sequencing is an important chemical problem. One approach is to release one amino acid at a time and identify the released amino acid. Some amino acids are cleaved too fast, however, to determine the exact structure of a peptide. Kinetics studies are of intrinsic interest in this context. Enzyme kinetics are important not only in sequence determination, they also control life processes.

Hydrolysis of peptides can be done by using acids, bases or enzymes. Enzymes that catalyse hydrolytic cleavage are hydrolases [1]. Enzymes that hydrolyse peptides are peptidases. Endopeptidases are enzymes catalysing hydrolysis of interior bonds in polypeptide chains. Peptidases attacking terminal peptide bonds are exopeptidases. Carboxypeptidases are pancreatic enzymes that hydrolyse the peptide bond closest to the carboxy terminal of a polypeptide chain and liberate the terminal residue as a free amino acid (Fig.1)[1]. There are different kinds of carboxypeptidases. Each carboxypeptidase has different amino acid specificity. One significant aspect of carboxypeptidase Y (CPY) is that it can also release proline, which is an imino acid. CPY is very soluble at low ionic strength and is active under denaturing conditions such as in solution of 6 M urea. Stereospecificity

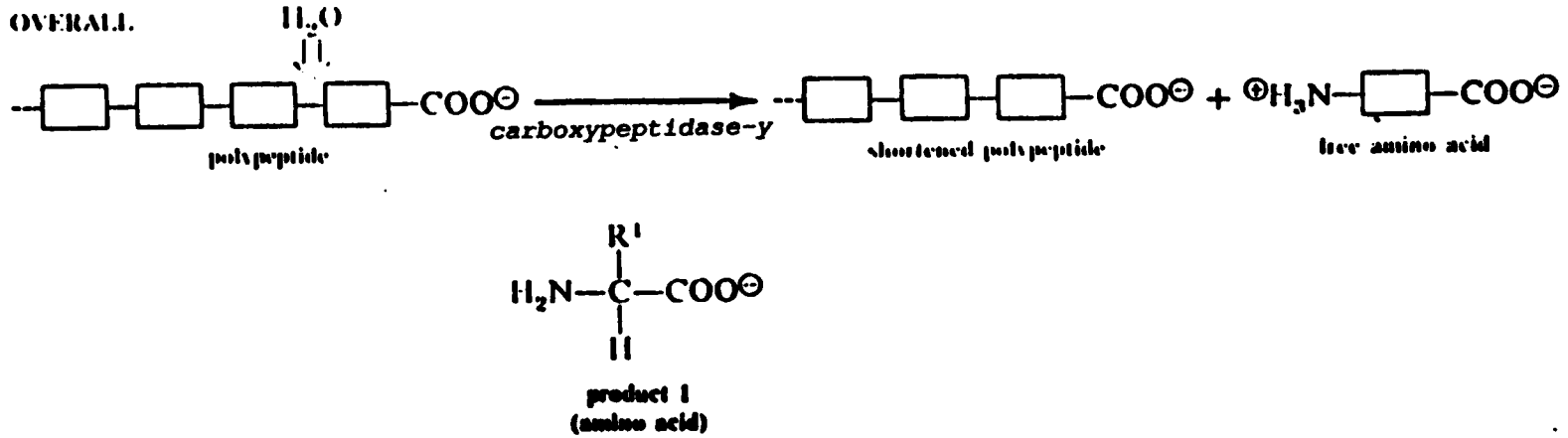

Fig 1. A representation of carboxypeptidase-Y hydrolysing a peptide. [taken from ref.1]

is demonstrated by the failure to hydrolyse peptides containing D-amino acids. These properties render CPY a useful tool in the sequence analysis of proteins[2]. One disadvantage is that the rate at which CPY releases the terminal amino acid is significantly influenced by pH and the structure of the departing as well as the penultimate amino acid [3].

CPY can hydrolyse almost any polypeptide with more than two amino acids. A dipeptide can be cleaved by blocking the amino group with protecting groups such as carbobenzoyloxy (CBZ) or acetyl groups. In one analytical approach involving peptide hydrolysis, the free amino acid released forms a complex with ninhydrin which absorbs at 570 nm. An amino acid with its amino group protected or bound to another amino acid in a peptide cannot form a ninhydrin complex. Thus, when a protected dipeptide is hydrolysed by CPY, the rate of release of the terminal amino acid can be monitored spectrophotometrically [4-10]. During hydrolysis of a polypetide for which more than one amino acid can be released sequentially, after the terminal amino acid is released, CPY proceeds to cleave the next amino acid. The observed rate is a convolution of the individual rates of cleaving amino acids and is referred to as "total rate" [1].

Ninhydrin does not distinguish between individual amino acids. Mass spectrometry, on the other hand, can distinguish among most individual amino acids and can therefore in principle give rates of individual hydrolysis steps. Kinetics

can be monitored with mass spectrometry by periodic or continuous sampling of the hydrolysate. The enzyme, with its high molecular weight, does not interfere with the mass spectra of the smaller peptide analytes and their hydrolysates. Fast Atom Bombardment Mass Spectrometry (FABMS) [11] and Electrohydrodynamic Mass Spectrometry (EHMS) [15,17] are methods that can be used in this kind of kinetics study. A major limitation of these methods is that they need a nonvolatile organic solvent or an organic solvent/water mixture. The most commonly used organic solvents are glycerol, triethanolamine and glycerol/water mixture [11]. Other organic cosolvents (with water) such as thiodiglycol have been used less frequently [17]. With FAB, continuous monitoring times have been limited to roughly 20 minutes although FAB has been used with batch sampling over much longer time frames [11]. Flow FAB extends the time frame for analysis, but with increasing sample requirements. Caprioli has reported continuous operation of flow FAB for 3-4 hrs, consuming roughly 5 µl/min [12-15]. EHMS has been used to monitor the progress of a reaction for as long as 72 hrs [16]. Sample consumption depends on solvent viscosity and/or volatility. Glycerol is consumed at a rate of 5 µl/hr, whereas the corresponding rate for water is approximately 4 µl/min [17]. Spectra are simple, containing solvated quasimolecular ions and little or no fragmentation. These features make EHMS an attractive tool for monitoring enzymatic reactions and for

sequence analysis of peptides. However, sensitivity of this technique is dependent on the solvent matrix as are the kinetics of reaction. The purpose of the present project is to assess the effect of an organic solvent suitable for use in mass spectrometry on enzyme catalyzed hydrolysis using an accepted method: ninhydrin with UV/VIS detection. The data generated can be compared with results obtained from EHMS to see whether MS-determined kinetics accurately reflect system reaction rates. Applications include a series of simple protected dipeptides (where both methods are unambiguous), and an oligopeptide (where only the MS method is unambiguous).

Michaelis-Menten Kinetics

In most enzyme reactions, the rate of product formation is a hyperbolic function of the concentration of substrate. At low substrate levels, doubling the substrate concentration leads to double the rate of the reaction, while at high substrate concentration, the rate becomes independent of substrate concentrations. The key to this behavior was first proposed by V. Henry in 1902 and elaborated by L. Michaelis and M. L. Menten in 1913. The basic concept is that an enzyme-catalyzed reaction involves two steps: 1. the reversible, rapid combination of enzyme (E) and substrate (S) to form a complex (ES); and 2. a slower breakdown of ES to give product (P) and regenerate free enzyme.

$$E+S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \qquad \text{step 1}$$

$$ES \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E+P \qquad \text{step 2}$$

where k_1, k_{-1}, k_2, and k_{-2} are rate constants. The rate of product formation, v is given by

$$v=\frac{k_2[E]_o[S]}{K+[S]} \qquad \text{Eq 1}$$

where K is defined as:

$$K=\frac{k_{-1}+k_2}{k_1} \qquad \text{Eq 2}$$

$[E]_o$ is the total concentration of enzyme, and [S] is the concentration of the substrate. k_2 sometimes is referred to as k_{cat} which is the turnover number of the enzyme. The above equation means that v tends towards a maximum value as [S] increases. The maximum rate will be observed when all the enzyme is in the form of the ES complex. Let the maximum rate = V_{max} ; this is equal to $k_2[E]_o$. Then the above equation can be written as

$$v = \frac{V_{max}[S]}{K + [S]} \qquad \text{Eq 3}$$

When $v = V_{max}/2$, $[S] = K$. K, which thus corresponds to the concentration when the velocity is half maximal, is known as the Michaelis constant, and is normally written as K_m. Eq 3 is generally referred to as the Michaelis-Menten equation (M-M equation). We can calculate k_{cat} as $V_{max}/[E]_o$.

V_{max} is the theoretical limit for the rate of reaction under defined conditions when the substrate concentration is so high that the active site is constantly occupied by substrate. V_{max} is an inherent property of a given enzyme, and depends upon the amount of the enzyme present in a given solution, the pH and the temperature of the system. It is the velocity when the enzyme is saturated with substrate.

K_m and V_{max} can be determined for an enzyme by making measurements under circumstances in which the step 2 is negligible. To measure velocity before the overall reaction is slowed down due to consumption of substrate, "one wants to have a minimum formation of product and a minimum fractional change in substrate concentration during the course of the reaction" [1]. The concentration of the product obtained per unit time will then be proportional to the initial velocity of the reaction. A plot of initial velocity versus substrate concentration (Michaelis-Menten plot or M-M plot (Fig.2)[1]) gives a hyperbolic curve when Michaelis-Menten kinetics are followed [18,19].

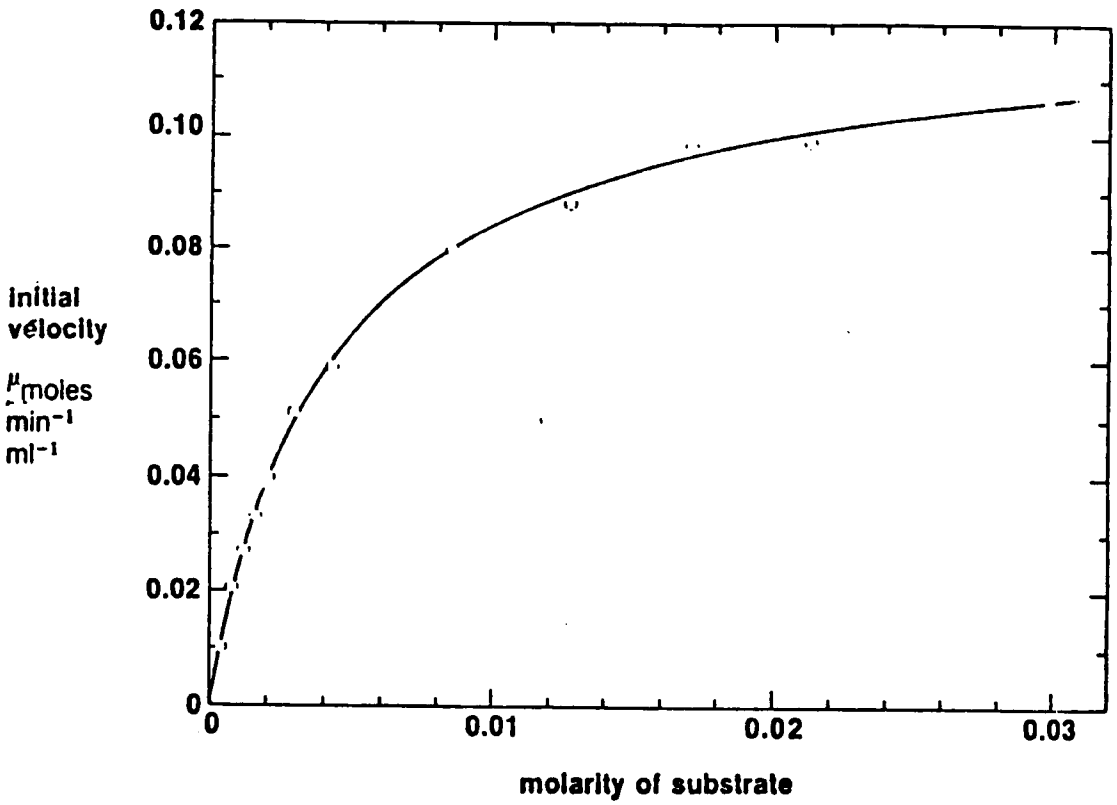

Fig 2. Measured values of the initial velocity of an enzymatic reaction with varying concentrations of substrate. The scatter apparent at the higher substrate concentrations amounts to 2 per cent of the highest velocity, which is quite good for measurement of most enzymes with standard techniques.[taken from ref.1]

The data can be analyzed in several ways. One such analysis is based on inversion and rearrangement of the M-M equation:

$$\frac{1}{v} = \frac{K_m + [S]}{V_{max}[S]} \qquad \text{Eq 4}$$

A plot of 1/v versus 1/[S] should be a straight line (Fig.3)[1]. This linear plot is known as Lineweaver-Burk plot (L-B plot), after the men known for developing this type of analysis. The y-intercept is the reciprocal of V_{max} and the x-intercept is the negative reciprocal of K_m, so both of these constants can be estimated from the plot. Assuming evenly spaced [S], points at high substrate concentrations are close together in this plot, and at low substrate concentrations a gross scatter is seen.

Control of the activities of enzyme molecules frequently involves combination of the enzyme with some other compound, which may block the active site or alter the catalytic activity. Compounds altering rate in this way are called effectors. They may be "activators", which increase the activity, or "inhibitors" that decrease the activity. There are two types of inhibitors. Competitive inhibitors compete with the substrate for an enzyme, either by occupying the catalytic site, or by favoring an enzyme conformation that is unfavorable for substrate binding.

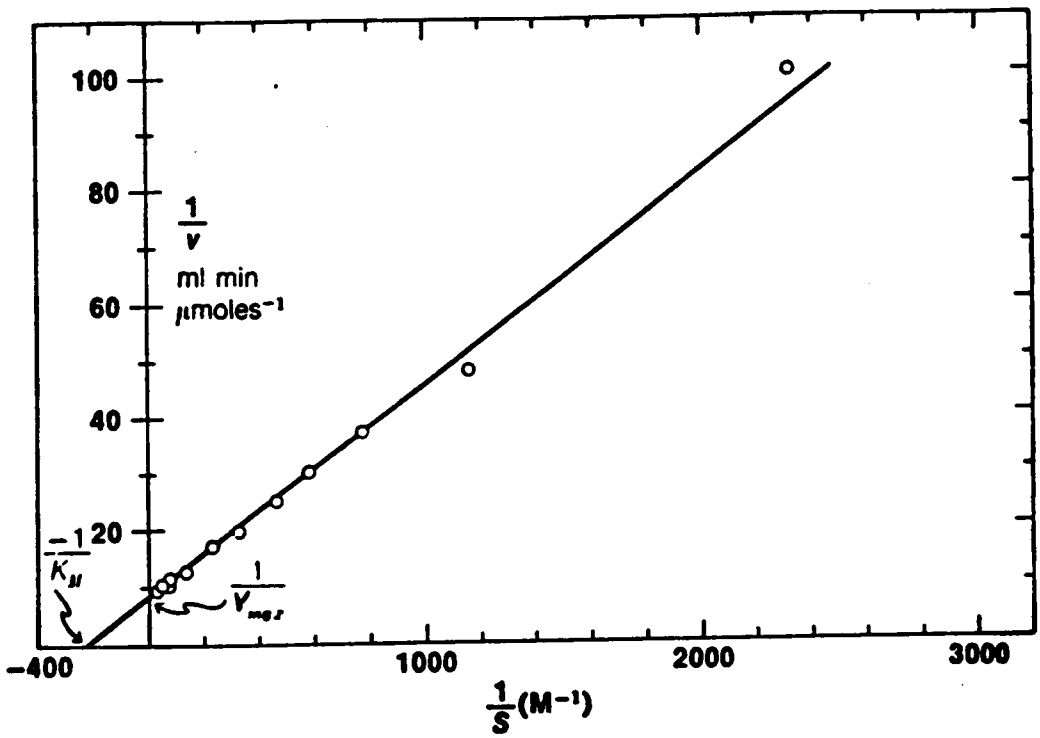

Fig 3. A Lineweaver-Burk plot of the data from Fig 2. Note the gross scatter evident in values obtained at low substrate concentrations, which was not evident in the steeply rising portion of the M-M plot in Fig 2. [taken from ref.1]

Presence of a competitive inhibitor increases the K_m but does not change the V_{max} (Fig.4)[1]. Noncompetitive inhibitors inhibit the reaction by combining with an enzyme in such a way that they are not displaced by the substrate but prevent the enzymatic reaction from occurring. The effect of a noncompetitive inhibitor is a lower value of V_{max}, with K_m unchanged (Fig.5)[1]. Sometimes inhibitors change both K_m and V_{max}, exhibiting mixed behavior. Varying concentrations of inhibitor produce Lineweaver-Burk plots which are straight lines but which do not intersect at the same point, thus changing V_{max} and K_m. In some cases Lineweaver-Burk plots produce curved rather than straight lines, and K_m is calculated from the M-M curve (K_m is [S] at v = half of V_{max}) [19].

The effect of added solvents and salts on the kinetics parameters of enzyme hydrolysis is a subject of interest to biochemists as well as analytical chemists. CPY is a diisopropylphosphorofluoridate (DFP)-sensitive enzyme [20], and is also stoichiometrically and irreversibly inhibited by phenylmethanesulfonyl fluoride (PMSF) [21]. A study of enzymes (unrelated to CPY) done by Klibanov and Zaks [22] showed that the enzyme activity greatly increased by increasing the water content in ethanol. Hayashi [4] has reported that K_m values for CPY increased logarithmically with increasing concentration of ethanol in aqueous substrate solution, suggesting competitive inhibition by ethanol.

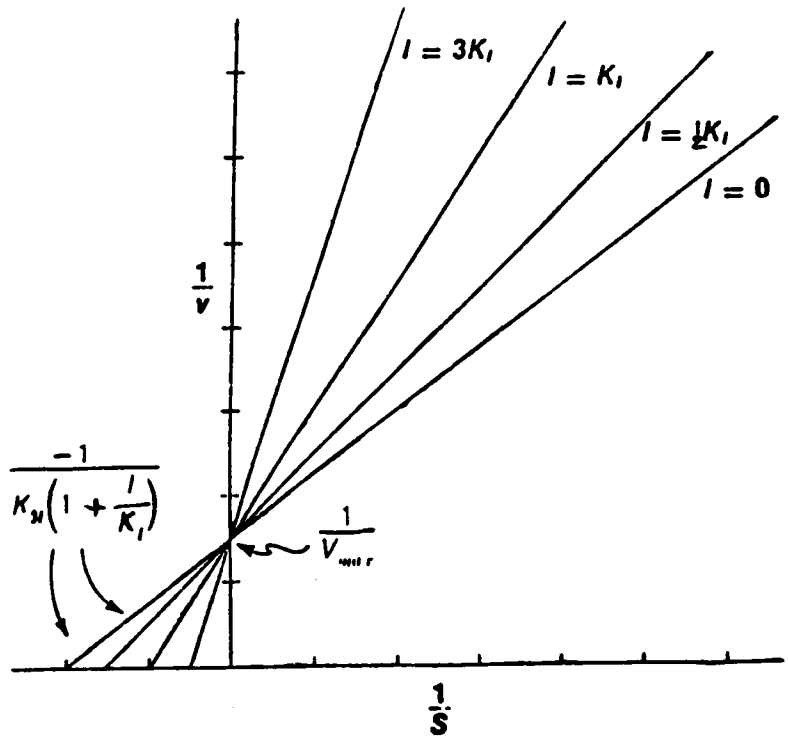

Fig 4. Lineweaver-Burk plots obtained with varying concentration, I, of a purely competitive inhibitor. There is a single intercept on the ordinate (vertical axis) because V_{max} is the same in all cases, but the intercept on the abscissa changes. [taken from ref.1]

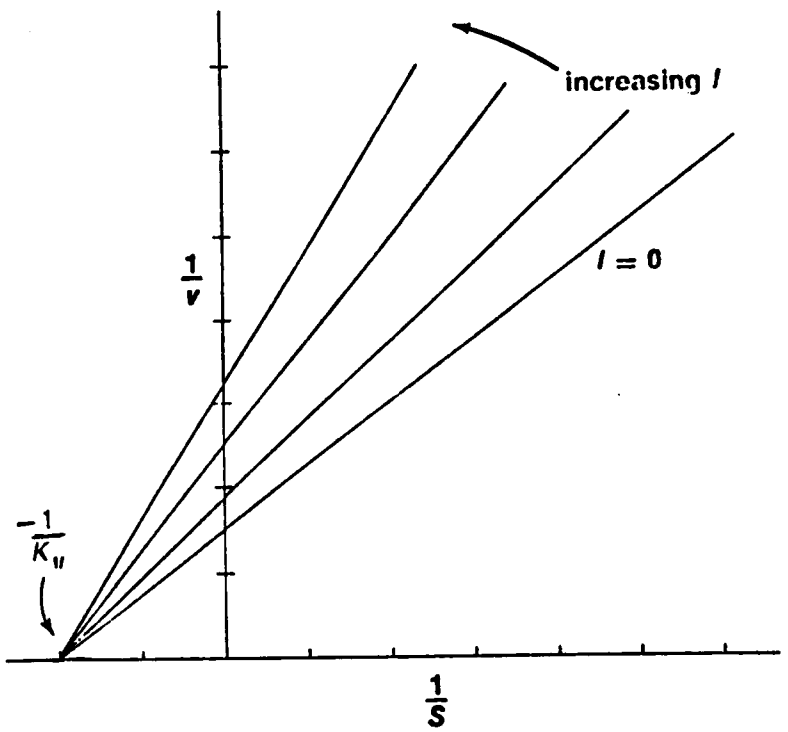

Fig 5. Lineweaver-Burk plots obtained with varying concentrations of a purely non-competitive inhibitor. K_m is constant in all cases, so there is a single intercept on the abscissa. The inhibitor has the same effect as removal of part of the enzyme, so V_{max} and the intercepts on the ordinate change. [taken from ref.1]

Bai and Hayashi [23] have also reported that mercury compounds increased K_m of CPY. Bergmeyer and coworkers [6] have reported that the enzyme is sensitive to metal ions. Cu^{2+}, Ag^{+} or Hg^{2+} result in complete loss of activity, while Cu^{+}, Mg^{2+}, Ca^{2+}, Ba^{2+}, Cr^{3+}, Fe^{2+}, or Ni^{2+} cause a partial loss of activity. EDTA has no effect on enzymatic activity. In general, L-amino acids and NH_2-blocked L-amino acids show the competitive type of inhibition with these metals. Hayashi et al. have reported that potassium chloride has no effect on the kinetics parameters of CPY [4]. In our experiments, we are exploring the effect of thiodiglycol (TDE) on kinetics parameters of CPY, because TDE provides good MS sensitivity to intact substrates without completely deactivating the enzyme, as does thioglycerol. By using different percentages of thiodiglycol in the substrate solution, we assess the maximum amount of this organic solvent that can be used for kinetics experiments using MS. These experiments are also helpful in assessing the type of inhibition (competitive or noncompetitive) thiodiglycol has, if any, on the kinetics of CPY. Sodium chloride was added to the reaction mixture since EH mass spectrometry needs a supporting electrolyte. These experiments also assess the effect of sodium chloride on the activity of CPY.

2. EXPERIMENTAL

Preparation of Solutions

Distilled, filtered water purified in Milli-Q Continental Water Systems Corporation filtration system was used for all aqueous solutions. Chemicals were purchased from Sigma Chemical Company, unless otherwise mentioned. Catalog numbers and lot numbers are listed below (Table 1). All buffers were stored at room temperature. Fresh buffers were made every week, to avoid bacterial contamination. The dipeptides used were N-Carbobenzyloxy (CBZ-) derivatives. The dipeptides used were N-CBZ-Leucine-L-Tyrosine (N-CBZ-Leu-Tyr), N-CBZ-Phenylalanyl-L-Leucine (N-CBZ-Phe-Leu), and N-CBZ-Glutamic acid-L-Tyrosine (N-CBZ-Glu-Tyr). The pentapeptide used was Tyr-Gly-Gly-Phe-Leu (Leu-Enkephalin) (where Gly refers to Glycine). This pentapeptide was sold as the acetate salt to improve solubility. The free base was released, however, as soon as the solid dissolved in buffer, without precipitating out.

Table 1.

Reagents used

Reagent	Product Number	Lot Number
CPY	C-3888	37F-8135
Ninhydrin	N-4876	39F-0692
Hydrindantin	H-2003	79F-0943
Leu-Enkephalin	L-9133	16F-58201
Leucine	L-8000	36F-0765
N-acetyl-L-Tyr	A-2513	119F-0770
N-CBZ-Leu-Tyr	T-1020	48F-08345
N-CBZ-Phe-Leu	C-1141	94F-0545
N-CBZ-Glu-Tyr	C-0257	116F-0324
Thiodiethanol	T-6125	69F-0720
2-Methoxyethanol*	T-6125	5003-KAME
n-Propyl alcohol*	7169	7169 KDTN

* purchased from Mallinckrodt

Phosphate Buffer: To 0.125 M sodium phosphate (monobasic) was added 0.125 M sodium phosphate (dibasic) slowly, while stirring, until a pH of 6.75 was reached. Final concentration of the buffer was 0.125 M. The solution was stored in a volumetric flask at room temperature.

Acetate Buffer: To 60 ml of 2 M sodium acetate trihydrate, glacial acetic acid was added dropwise, with stirring, until a pH of 5.5 was reached. The resulting solution was diluted to 100 ml with water, in a volumetric flask. The final concentration of the buffer was about 1.2 M.

Ninhydrin reagent: 25 ml of methyl cellosolve (2-methoxyethanol) was poured into a three necked round bottomed flask covered with aluminum foil. Dry nitrogen was bubbled through the solvent for 10 minutes. To the flask were added 1 g of ninhydrin and 0.15 g of hydrindantin. The contents were stirred until dissolved. A slow stream of nitrogen was maintained in the solution throughout the experiment, while avoiding exposure to light, since ninhydrin solution is unstable in the presence of light [7]. To the above mixture was added 25 ml of acetate buffer solution, to give a red ninhydrin reagent. This makes a 2% (w/v) solution of ninhydrin. When ninhydrin reagent is exposed to light, the red color fades, indicating that the reagent is decomposed. Due to

its unstable nature, ninhydrin reagent was made fresh daily before each use.

Preparation of Enzyme Stock Solution

For the hydrolysis experiment, 0.1 unit of CPY/ml was used. Manufacturer-reported sample specification for the enzyme was 17 units/mg solid or 105 units/mg of protein, and 16% protein (w/w). One sample of enzyme containing 2 mg of protein was dissolved in 5.5 ml of water. This solution contains 38.18 units of protein per ml. This solution was diluted 1:10 with water to give 3.8 unit/ml solution (0.036 mg protein/ml), and was stored in refrigerator at 4° C. 0.95 unit/ml enzyme solution was prepared by further dilution of this stock solution 1:4 with water at the time of the experiment. The final concentration of the enzyme used was 0.095 units/ml or 0.0009 mg protein/ml, or 0.015 μM. This final dilution was made fresh prior to each experiment.

Leucine solution: A 10 mM leucine solution was prepared by dissolving 0.0131 g of leucine (MW 131.2) in 10 ml phosphate buffer in a volumetric flask. This solution was stored in refrigerator at 4°C. Solutions 0.1 mM to 0.9 mM in leucine were made by dilution of this solution with phosphate buffer.

Tyrosine solution: A 10 mM solution of N-acetyl tyrosine was prepared by dissolving 0.0223 g of N-acetyl tyrosine (MW 223.2) in 10 ml phosphate buffer in a volumetric flask. N-acetyl tyrosine was used, instead of tyrosine, since tyrosine is very sparingly soluble in water. This solution was stored in a refrigerator at 4°C. Solutions 0.1 mM to 0.9 mM in tyrosine were made by dilution of this solution with phosphate buffer.

N-CBZ-Phe-Leu solution: A 10 mM solution of N-CBZ-Phe-Leu was prepared by dissolving 0.1031 g of N-CBZ-Phe-Leu (MW 412.5) in 25 ml of phosphate buffer in a volumetric flask. This solution had to be stirred at least 10 min to make a homogenous solution. This solution was stored in a refrigerator at 4°C. Solutions 1 mM to 9 mM in N-CBZ-Phe-Leu were made by dilution of this solution with phosphate buffer.

N-CBZ-Leu-Tyr solution: A 10 mM solution of N-CBZ-Leu-Tyr was prepared by dissolving 0.1071 g of N-CBZ-Leu-Tyr (MW 428.5) in 25 ml of phosphate buffer in a volumetric flask. The solid was readily soluble in buffer. This solution was stored in a refrigerator at 4°C. Solutions 1 mM to 9 mM in N-CBZ-Leu-Tyr were made by dilution of this solution with phosphate buffer.

N-CBZ-Glu-Tyr solution: A 10 mM solution of N-CBZ-Glu-Tyr was prepared by dissolving 0.1111 g of N-CBZ-Glu-Tyr (MW 444.4) in

25 ml of phosphate buffer in a volumetric flask. The solid was readily soluble in buffer. This solution was stored in a refrigerator at 4°C. Solutions 1 mM to 9 mM in N-CBZ-Glu-Tyr were made by dilution of this solution with phosphate buffer.

Leu-Enkephalin solution: A 0.9 mM solution of Leu-Enkephalin was prepared by dissolving 2.7 mg of Leu-Enkephalin (acetate salt, MW 615.6) in 5 ml of phosphate buffer in a volumetric flask. This compound was purchased as acetate ester for improved solubility. This solution was stored in refrigerator at 4°C. Solutions 1 mM to 9 mM in this analyte were made by dilution of this solution with phosphate buffer.

Instruments Used

An Orion Research Model 701A pH meter was used for pH measurements with a combination glass electrode. The instrument was calibrated with pH 4.01 and pH 6.85 standard solutions available from Sigma Chemical Company. Experimental precision of this instrument was found to be ± 0.001 by using replicates.

An Hewlett Packard diode array UV/VIS spectrophotometer (Model HP8452) was used for absorbance measurements. The measurements were taken at a wavelength of 570 nm, which is the observed λ_{max} for the leucine ninhydrin complex (Fig.6).

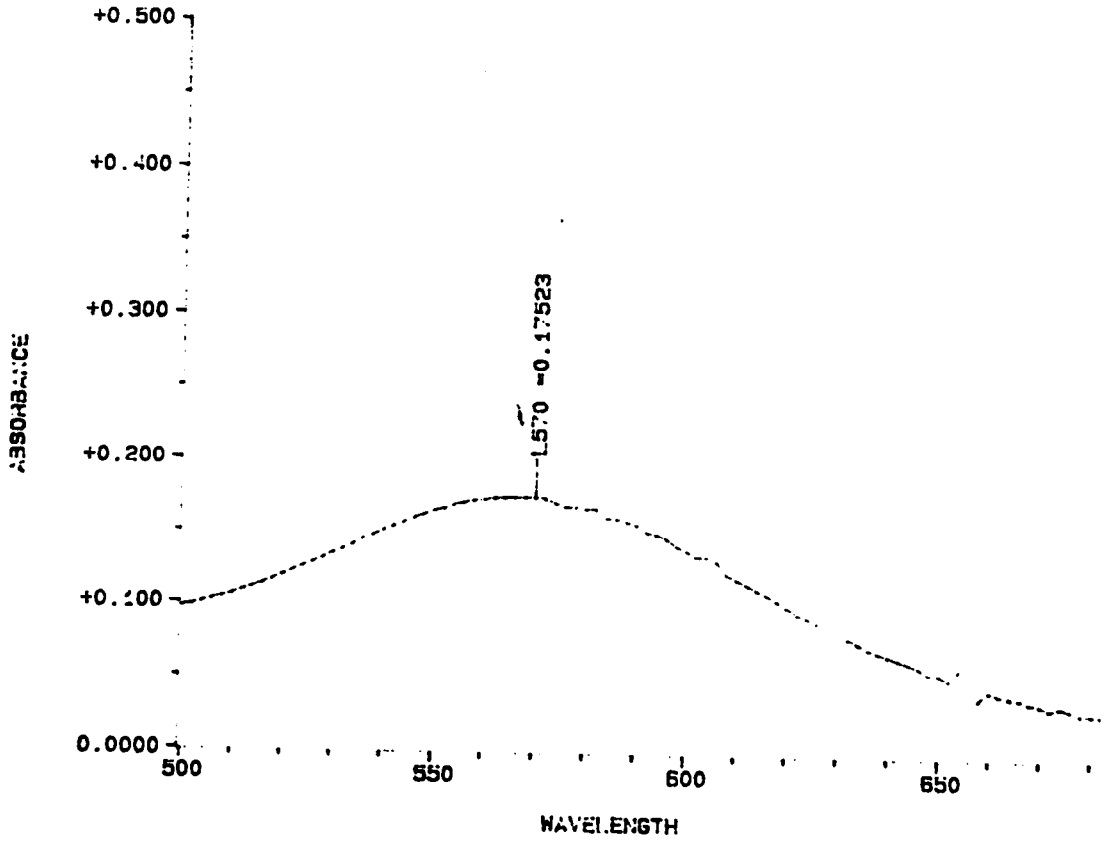

Fig 6. Absorption spectrum of leucine-ninhydrin complex in phosphate buffer, showing absorption maximum at 570 nm. Concentration of leucine: 0.7 mM.

The manufacturer-reported precision agrees with the experimental precision (determined by reading the absorbance of pyrocatechol dye several times) of ± 0.005 for absorbance. The specified precision was ± 2 nm for wavelength.

A vortex mixer (Thermolyne, Model 37600) was used for mixing the solutions. A water bath (MGW LAUDA Co., type MT) was used for maintaining temperature. The precision of this water bath was ± 0.1° C.

3. PROTOCOLS

Amino acid standard solutions : The following were mixed in a 10 ml test tube: 0.1 ml of 5 M sodium chloride solution in water, 0.8 ml of water and 0.1 ml of 0.1 mM to 0.5mM leucine or N-acetyl tyrosine solution in phosphate buffer. Total volume of this solution was 1 ml, assuming negligible volume of mixing. The pH of this solution was 6.5 and the effective concentration of buffer was 0.0125 M. The solution was incubated at room temperature (25°C) in a water bath for 10 minutes.

Calibration solutions using 10% thiodiglycol (TDE) in water were prepared by taking 0.1 ml of 5 M sodium chloride solution in water, 0.7 ml of water, 0.1 ml of TDE and 0.1 ml of 0.1 mM to 0.5 mM leucine or N-acetyl tyrosine solution in phosphate buffer. Solutions using 20% TDE, 40% TDE, 60% TDE and 80% TDE were prepared by adjusting the water and TDE content in the solution. Volume of the substrate solution with TDE was 1 ml.

Formation of ninhydrin complex: 1 ml of freshly prepared ninhydrin solution was added to the amino acid solution and immediately immersed in a boiling water bath to allow formation of the ninhydrin complex. The variation for this step of the reaction was ± 3 seconds. The reaction mixture in

the test tube was kept in a boiling water bath for exactly 15 minutes. A winding stop clock watch was used to keep track of time. To minimize evaporation losses, the test tube was immersed in the water, one inch deep (or until the solution in the test tube was completely immersed in the water). At the end of 15 minutes, the test tube was chilled in an ice bath to stop the complex formation. The amino acid-ninhydrin complex was purple in color. This solution was further diluted with 5 ml of n-propanol-water mixture (50% v/v) and was then allowed to come to room temperature. Total volume of the solution in the test tube was 7 ml. Absorbance of this solution was measured on the HP8452 UV/VIS spectrophotometer, using a 1 cm quartz cuvette. This procedure for complex formation is similar to that described by others [2].

Amino Acid Calibration Curves

The same procedure was repeated with 0.2 mM, 0.3 mM, 0.4 mM and 0.5 mM solutions of leucine or N-acetyl tyrosine in buffer. The absorbance vs concentration of leucine (in initial 1 ml aliquot) was plotted, and coefficients of correlation and slopes for the plots were calculated by regression analysis using the Grapher (R) software. Calibrations for leucine or N-acetyl tyrosine in substrate solutions containing varying amounts of TDE were repeated in a similar manner.

Enzyme Hydrolysis

Hydrolysis experiments for the peptides used were planned in such a way that the absorbance of the complex formed from the released amino acid formed was between 0.1 and 1 absorbance units. The influencing factors here are the concentration of peptide used, the concentration of the CPY used, and the reaction interval (defined in the following section). These factors, however can be changed to suit individual needs; i.e, more enzyme could be used to make the reaction faster, in which case the reaction interval has to be smaller.

Hydrolysis of CBZ-Phe-Leu: The following were mixed in 10 ml test tubes labelled # 1 to # 10: 0.1 ml of 5 M sodium chloride solution in water, 0.7 ml of water and 0.1 ml of 2 mM CBZ-Phe-Leu solution in phosphate buffer. Each solution was mixed on a vortex mixer for 5 seconds. The solutions in these ten test tubes were incubated at room temperature (25°C) for 10 minutes.

To each of the above test tubes, 0.1 ml of 0.95 unit/ml CPY (freshly diluted) was added, 15 seconds apart. Total volume of solution in each test tube was 1 ml, assuming negligible volume of mixing. The hydrolysis in test tube #1 was quenched at 30 seconds by addition of 1 ml of freshly prepared ninhydrin solution, and submersion in boiling water.

The reaction time in test tube #1 was 30 seconds. The remaining part of this experiment was repeated as described in the above paragraph "formation of ninhydrin complex".

The same procedure was repeated with the remaining nine test tubes, with the reaction times increasing from 30 seconds to 270 seconds with each successive tube, with 30 sec intervals (the "reaction interval"). Absorbances for solutions in all 10 test tubes were read successively, to ensure similar conditions. The absorbance of the solution in each test tube was assumed to be proportional to the amount of leucine formed during hydrolysis. Concentration of the amino acid released was calculated by using the calibration curves described above. Absorbances vs time (in seconds) and percentage of peptide hydrolysed vs time (in seconds) were plotted, using the Grapher (R) program.

Substrate solution using 10 % thiodiglycol (TDE) in water was prepared by mixing 0.1 ml of 5 M sodium chloride in water, 0.6 ml of water, 0.1 ml of TDE and 0.1 ml of 2 mM CBZ-Phe-Leu solution in phosphate buffer, adding and mixing each solution in the order mentioned. The solution was then mixed on the vortex mixer for 5 seconds. Solutions using 20% TDE, 40% TDE, 60% TDE and 80% TDE were prepared by adjusting the water and TDE content in the solution. Volume of substrate solution with TDE was 0.9 ml. Ten test tubes of reaction mixture were prepared with each TDE solution. Addition of CPY, quenching of

the reaction, reading of absorbances and plotting were repeated as described above for the case of pure buffer.

The above experiment was used to compare rates of hydrolysis of the dipeptide with and without TDE. The reaction was not followed to completion, but was monitored long enough to see the difference in initial rates.

Hydrolysis of Other Peptides

Hydrolyses of CBZ-Leu-Tyr, CBZ-Glu-Tyr, and Leu-Enkephalin were repeated in a similar manner, with a few differences. The reaction interval was 30 seconds for CBZ-Leu-Tyr but 1 minute for CBZ-Glu-Tyr because the latter peptide reacted more slowly than the other two. For Leu-Enkephalin, the reaction was monitored every 30 minutes for 8 hours. For CBZ-Leu-Tyr, CBZ-Glu-Tyr, and Leu-Enkephalin the rate of reaction was determined with 0%, 10% and 20% TDE.

Calculation of K_m

K_m values for the peptides were calculated from Lineweaver-Burk plots, or M-M plots. Initial velocities were calculated from the slope of absorbance vs time plots, using the first three points in the rate experiment. These three points were chosen such that the amount of the peptide hydrolysed was not more than 20%. In the above experiment,

these three points correspond to three reaction intervals. Table 2 shows the reaction times at which initial slopes were calculated.

Table 2.

Reaction times used in the calculation of the initial slope, for each solvent matrix indicated

Peptide	Matrix	Reaction times
CBZ-Phe-Leu	Buffer	15, 30, 45 Sec
	10 % TDE	1, 1.5, 2 min
	20 % TDE	2, 2.5, 3 min
CBZ-Leu-Tyr	Buffer	1, 2, 3 min
	10 % TDE	2, 4, 6 min
	20 % TDE	10, 15, 20 min
CBZ-Glu-Tyr	Buffer	2, 4, 6 min
	10 % TDE	10, 15, 20 min
	20 % TDE	30, 60, 90 min

4. RESULTS AND DISCUSSION

Calibration data for leucine in buffer and TDE-buffer solutions were obtained by using 0.01 to 0.05 mM solutions of leucine. Leucine-ninhydrin complex was prepared as discussed in the protocol section "formation of ninhydrin complex". An absorption spectrum of the leucine-ninhydrin complex is shown in Fig 6. Absorption spectrum of pure (neat) TDE is shown in Fig 7. This figure shows that TDE does absorb at 570 nm, although the absorbance is very small. The calibration curves for leucine in buffer and TDE are shown in Fig 8. The slopes are almost the same, although the y-intercepts are different. This would appear to be due to the absorbance of TDE in the matrix. However, the extra absorbance for the top curve in Fig 8 (20% TDE) is rouly 0.05 absorbance units, or about as much as the absorbance of pure TDE in Fig 7. The reason for the discrepancy in the absorbance of TDE in the figures 7 and 8 is not clear. The regression data is given in Table 3.

The effect of TDE on the rate of hydrolysis was assessed by hydrolysing CBZ-Phe-Leu using CPY with and without TDE. The procedure was discussed in the protocol section. Absorption spectrum of CBZ-Phe-Leu hydrolysate in buffer and NaCl is shown in Fig 9, indicating the presence of the released amino acid. TDE concentration was varied from 10% to 80%. The curves

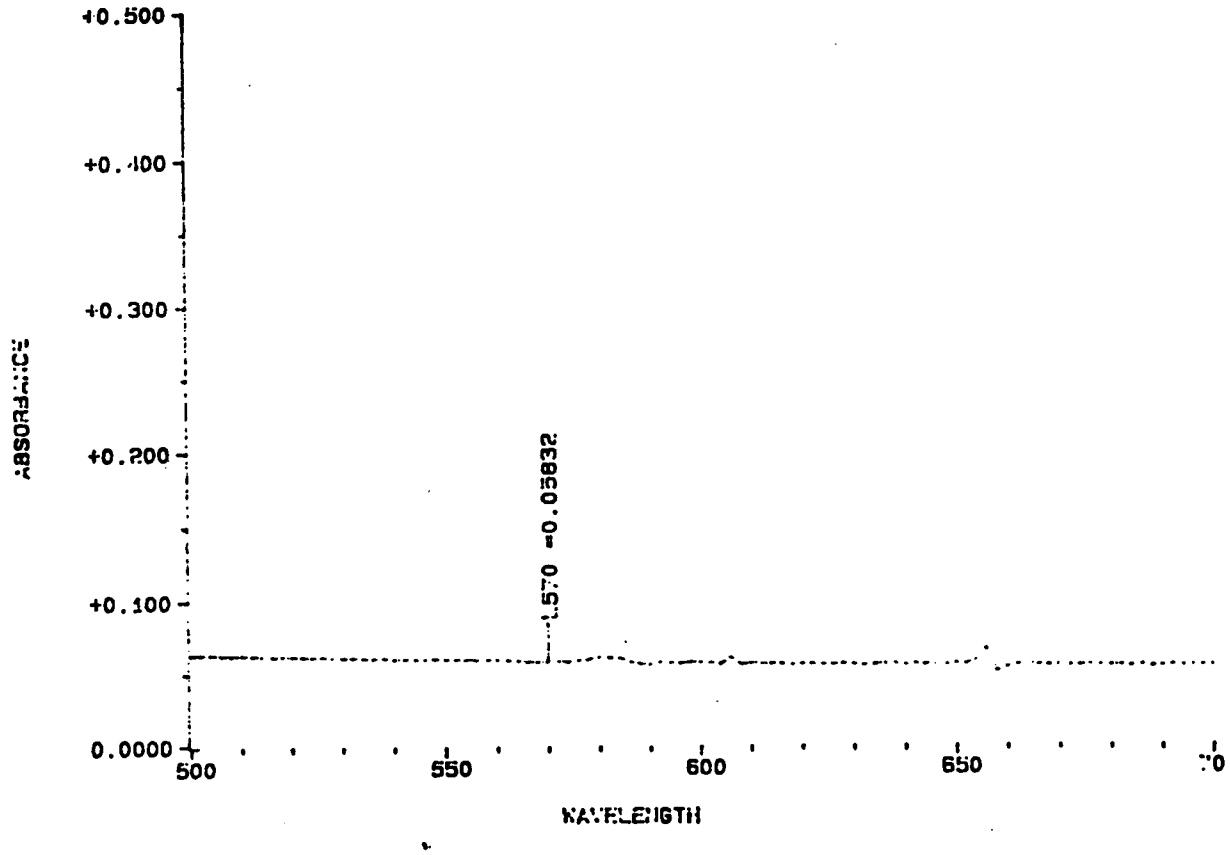

Fig 7. Absorption spectrum of pure TDE, indicating absorbance of the solvent at 570 nm.

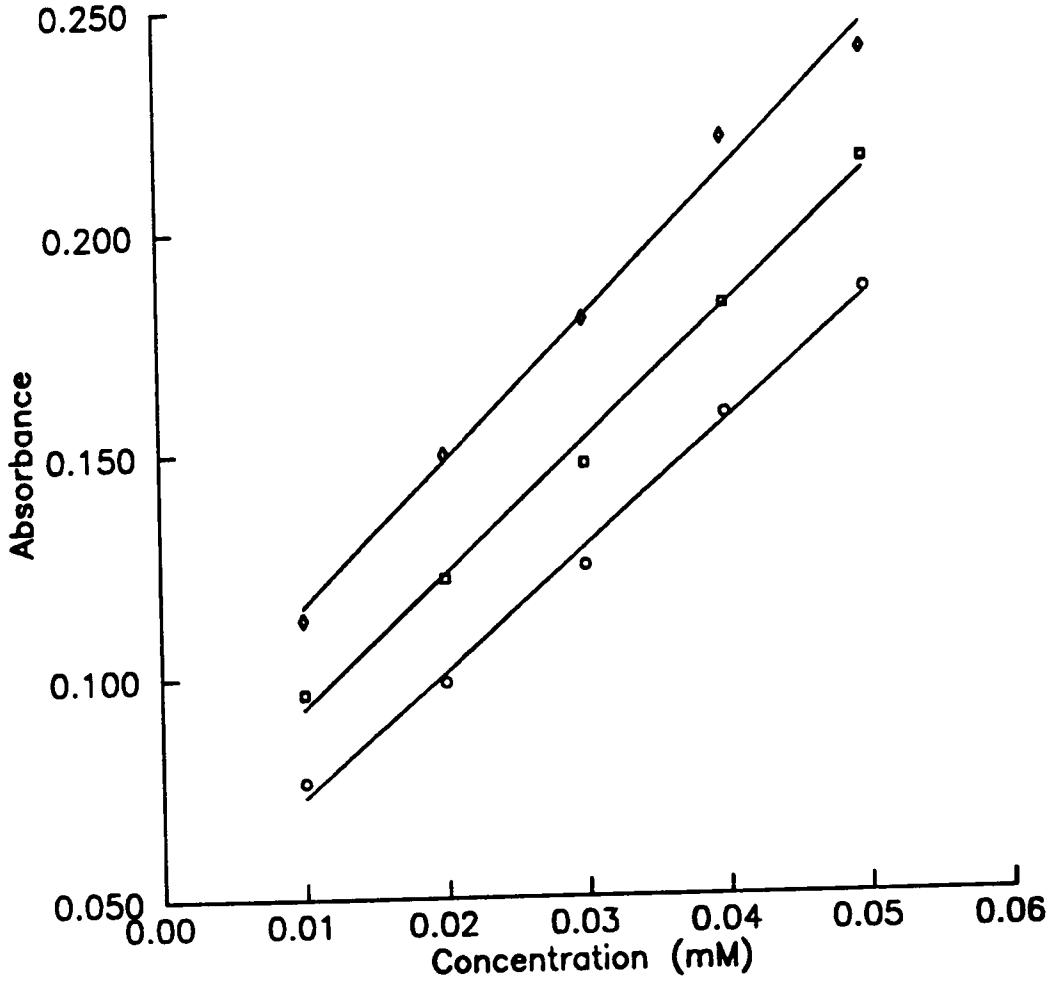

Fig 8. Calibration plots for Leucine in phosphate buffer, 10%TDE, and 20%TDE. O-Buffer, □-10%TDE, ◇-20%TDE

Table 3.

Data for calibration of leucine absorbance at 570 nm.

Matrix	Slope[a]	Y-cept[b]	[c]R^2
Buffer	2.78	0.045	0.995
10% TDE	2.98	0.063	0.994
20% TDE	3.23	0.083	0.991
40% TDE	3.10	0.095	0.993

[a]Slope: mM^{-1} cm^{-1}. Concentration range: 0.01 to 0.05 mM. Results from regression output from Grapher®. [b]Y-cept denotes y-intercept which represents contribution from blank. [c]R^2 is the correlation coefficient, which describes goodness of the fit.

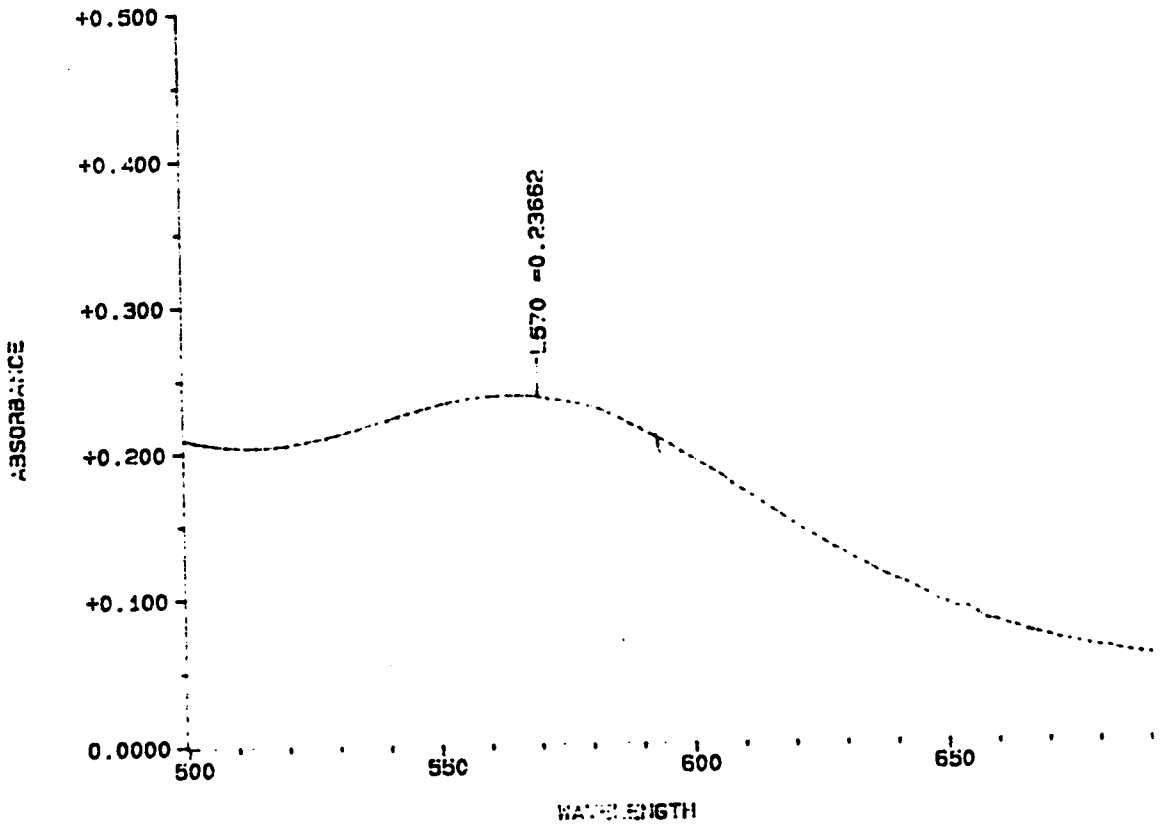

Fig 9. Absorption spectrum of CBZ-Phe-Leu hydrolysate in phosphate buffer and NaCl.

acid. TDE concentration was varied from 10% to 80%. The curves in Fig 10 show the difference in rate of hydrolysis of CBZ-Phe-Leu with and without TDE, with an enzyme concentration of 0.1 unit/ml. At 40% TDE the above reaction was undetectable at the enzyme concentration of 0.1 unit/ml. Rate constants for different concentrations of TDE (Table 4) were calculated using a least squares fit to the pseudo-first-order rate equation:

$$\log(C_t) = -(kt/2.303) + \log(C_o)$$

where C_t is the concentration of the peptide at time t, C_o is the initial concentration of peptide, and k is the pseudo-first-order rate constant. A representative $\log(C_t)$ vs time curve is presented in Fig 11. Comparison of the rate constants proves that TDE has an inhibiting effect on the rate of hydrolysis. Rate curves with and without sodium chloride prove that 0.5 M sodium chloride has no effect on the rate of the reaction (Fig 10).

Sample Michaelis-Menten and Lineweaver-Burk plots for the dipeptides used are shown in Figs 12 and 13. In M-M plots, as the substrate concentrations increased, initial rates also increased, until maximum velocity for the given concentration of enzyme was reached. After the V_{max} is reached, the slope changes showing that the enzyme was saturated. Data for K_m and V_{max} are given in Table 5. Changes in both kinetics parameters K_m and V_{max} with TDE

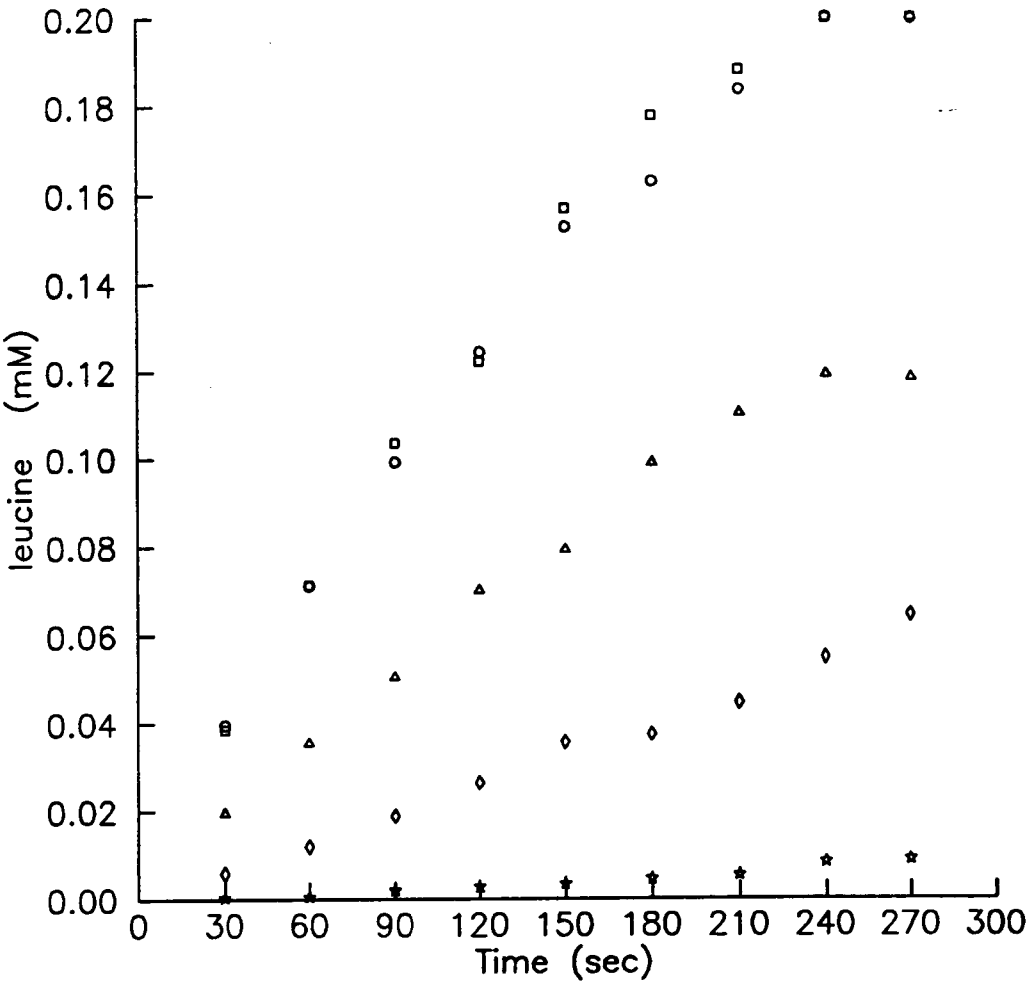

Fig 10. **Absorbance of leucine released during hydrolysis of CBZ-Phe-Leu. ○-Buffer, □-Buffer with NaCl, △-buffer with 10%TDE, ◇-20%TDE, and ★-40%TDE.**

Table 4.

Pseudo-first-order rate constants for hydrolysis of CBZ-Phe-Leu by CPY in indicated solvent

Solvent Matrix	[a]enzyme (unit/ml)	[b]k (min^{-1})	[c]RSD
Buffer	0.1	0.387±0.002	0.010
Buffer & 10% TDE	0.1	0.208±0.001	0.009
Buffer & 20% TDE	0.1	0.063±0.002	0.068
Buffer & 40% TDE	0.1	0.010±0.003	0.300

Amount of substrate used: 0.2 mM; [a]CPY; [b]k (rate constant) values given ± standard error of the mean (Standard deviation/$\sqrt{n}$), n=3. [c]Relative standard deviation : standard deviation / avg

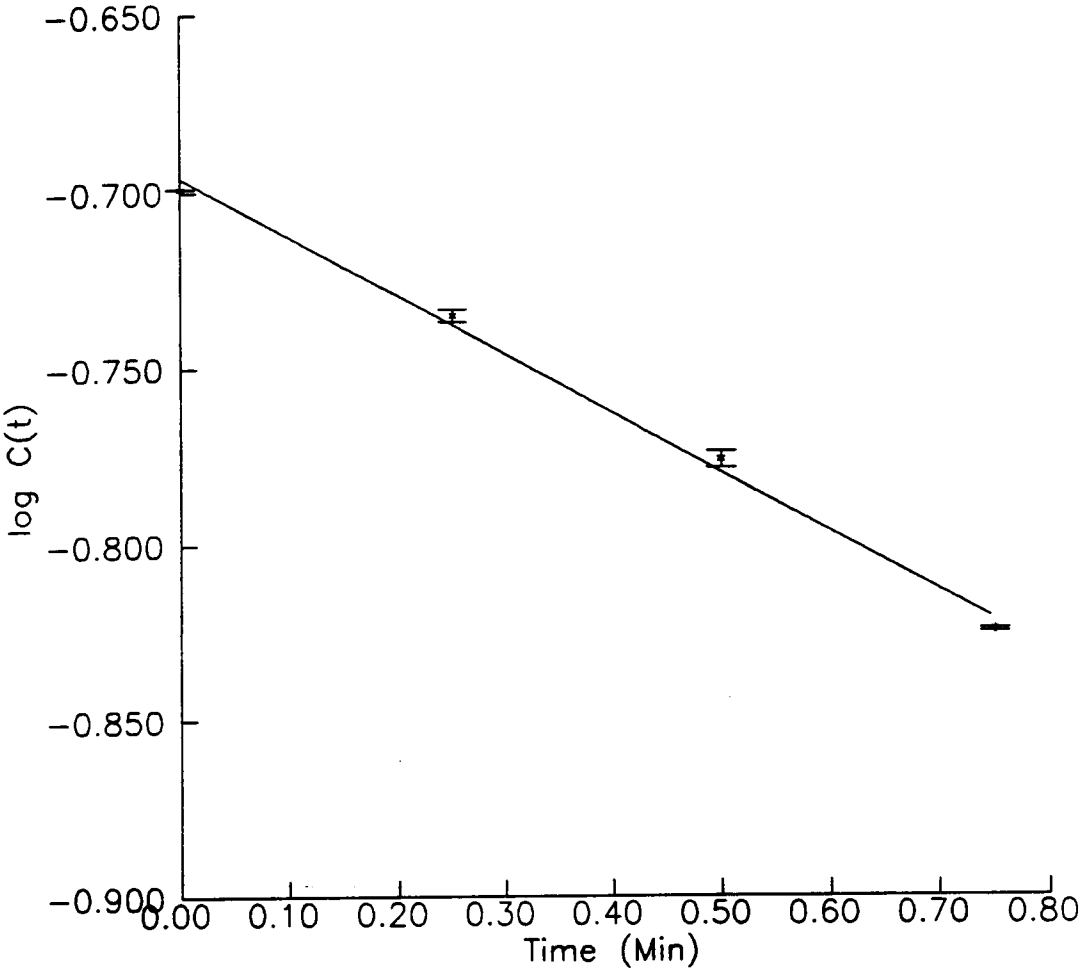

Fig 11. Pseudo-first-order rate plot for CBZ-Phe-Leu in phosphate buffer. Concentration of the peptide : 0.2 mM; Concentration of the enzyme : 0.1 unit/ml. Error bars calculated for standard error of the mean (n = 3)

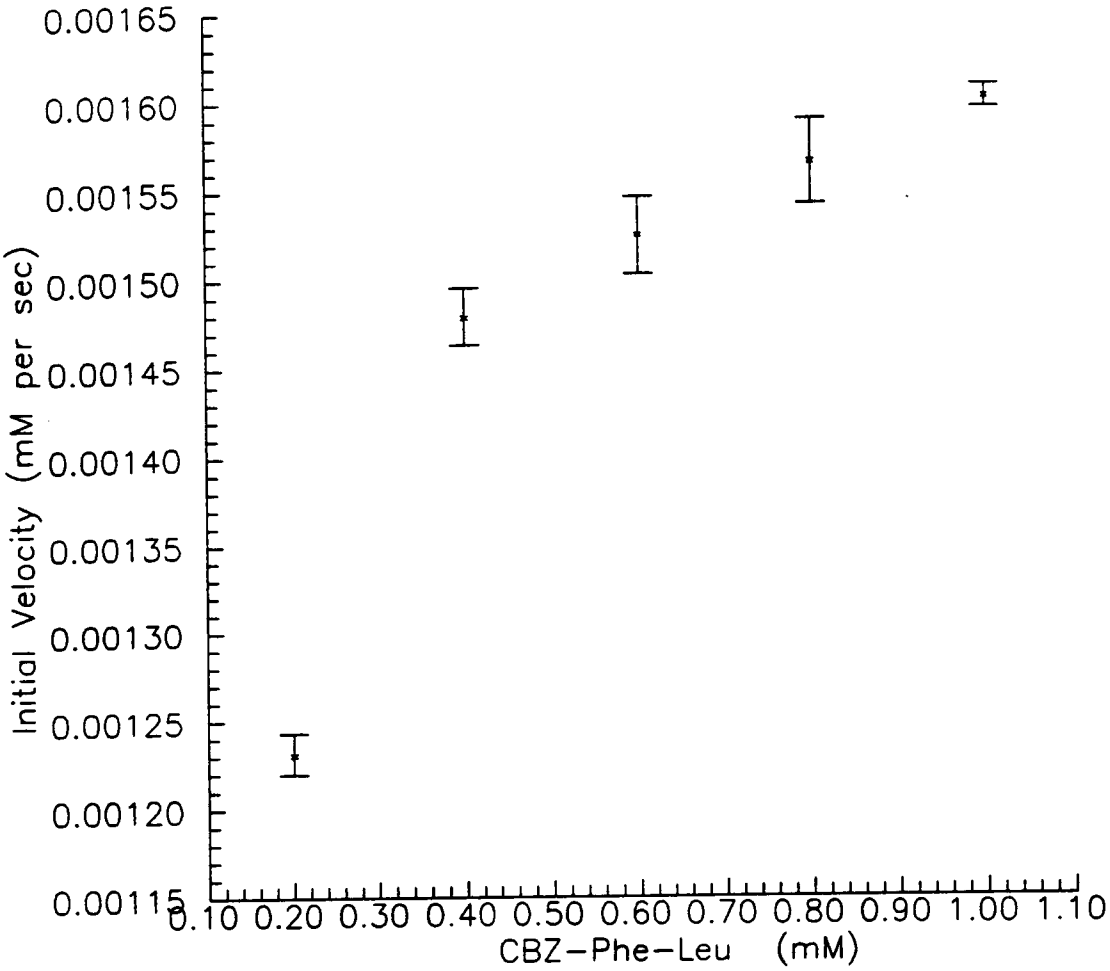

Fig 12. **Michaelis-Menten curve for CBZ-Phe-Leu in phosphate buffer. Concentration of the enzyme : 0.1 unit/ml. Error bars calculated for standard error of the mean (n = 3).**

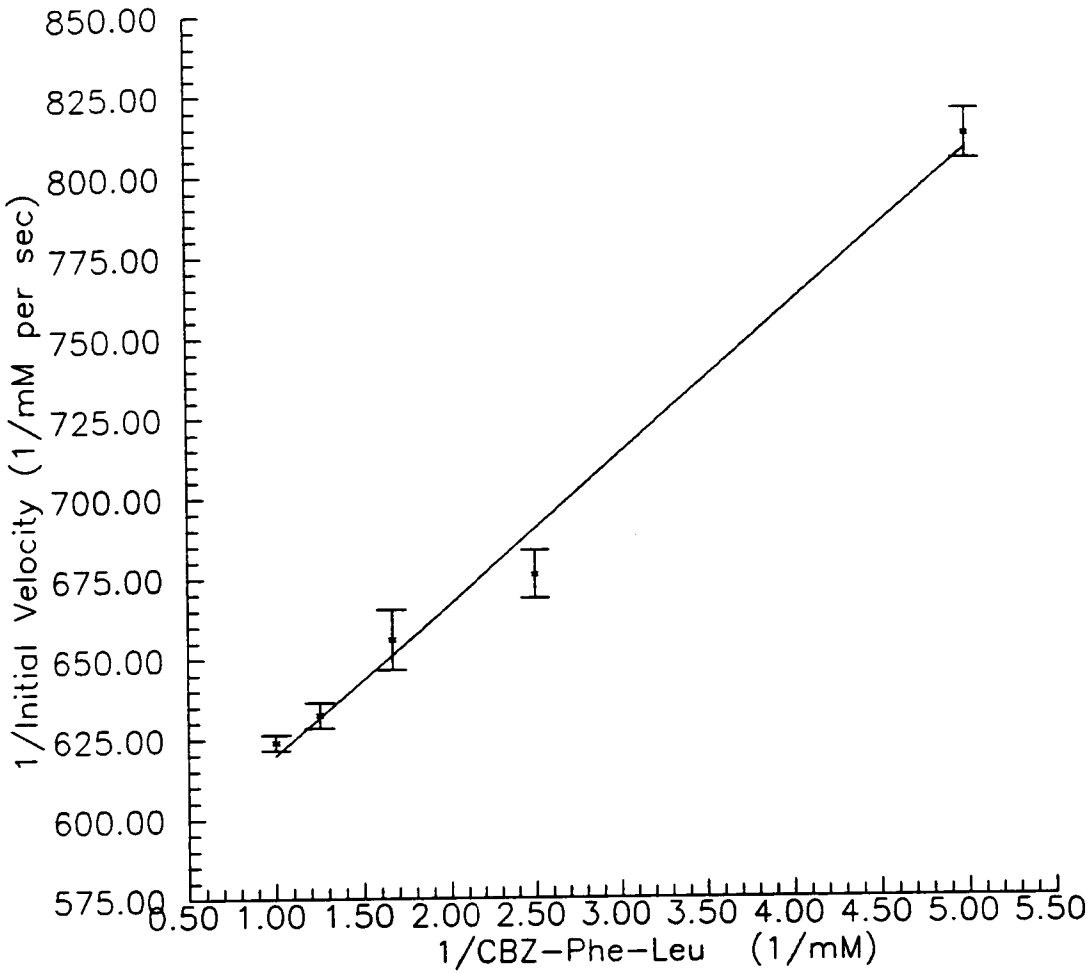

Fig 13. **Lineweaver-Burk plot for CBZ-Phe-Leu in phosphate buffer and NaCl. Concentration of the enzyme : 0.1 unit/ml. Error bars calculated for standard error of the mean (n = 3). The line shows the least squares fit.**

Table 5.

Kinetics parameters for enzymatic hydrolysis of various dipeptides in the indicated solvents

Dipeptide	Matrix	K_m[a] (mM)	V_{max}[b] (mM/min)
CBZ-Phe-Leu	Buffer	0.087±0.004	0.096±0.005
	10% TDE	0.207±0.003	0.060±0.001
	20% TDE	0.329±0.004	0.024±0.016
CBZ-Leu-Tyr	Buffer	0.110±0.002	0.091±0.004
	10% TDE	3.199±0.003	0.075±0.004
	20% TDE	12.07±0.005	0.037±0.001
CBZ-Glu-Tyr	Buffer	0.309±0.003	0.016±0.004
	10% TDE	0.680±0.004	0.005±0.001
	20% TDE	1.094±0.004	0.004±0.0005

The [a]K_m and [b]V_{max} values for dipeptides ± standard error of the mean (n = 3). Substrate concentration ranges from 0.2 mM to 1 mM in all peptides. Enzyme concentration was 0.1 unit/ml for CBZ-Phe-Leu and CBZ-Leu-Tyr and 1 unit/ml for CBZ-Glu-Tyr. Values calculated from M-M plot.

concentration suggest that the inhibition by TDE is mixed type rather than purely competitive or noncompetitive. Hayashi and coworkers reported that K_m increases logarithmically with increasing concentration of ethanol as the inhibitor in the hydrolysis. In our results the exact relationship between TDE concentration and k_m is not logarithmic, but roughly linear.

The dipeptides CBZ-Leu-Tyr and CBZ-Glu-Tyr were chosen because the penultimate amino acids in both the dipeptides are different. From the K_m values (Table 5), we can see that a basic amino acid (glutamic acid) in the penultimate position slows down the hydrolysis to a larger extent than a nonbasic amino acid such as phenylalanine. This observation is consistent with data reported by Hayashi and coworkers [21]. Inhibition by TDE was seen in both cases. In view of these observations, we can say that TDE in general reduces the activity of CPY.

Comparison of the rate constants and the kinetic parameters proves that enzyme activity was retained when the amount of TDE was as high as 20%. This suggested that 20% TDE-buffer might be a suitable matrix for EHMS.

Hydrolysis of the pentapeptide, Leu-Enkephalin was carried out in buffer, 10% TDE and 20% TDE, with 1 unit/ml enzyme. Compared to the dipeptide CBZ-Phe-Leu, this reaction was very slow, and needed 10 times more enzyme to reach

completion in a reasonable period of time. The reaction was followed until a constant absorbance was reached (Fig 14). Reaction in 40 % TDE was seen only when 16 units/ml of the enzyme was used, and took 24 hours for completion. This is very slow compared to the uninhibited reaction which was complete within approximately 3 hours with 1 unit/ml of enzyme. The rate constants for the three steps of the reaction (release of leucine, phenylalanine, and then glycine) were not found in the literature and are difficult to assess using this conventional method.

Hydrolysis of Leu-Enkephalin was modelled assuming the hydrolysis was first order. The first step involves release of leucine from the pentapeptide, to give a tetrapeptide.

Tyr-Gly-Gly-Phe-Leu $\longrightarrow$ Tyr-Gly-Gly-Phe + Leu

This reaction can be represented in the first order rate equation

$$\frac{dA(t)}{dt} = -k_1 A(t) \qquad (1)$$

where A(t) is the concentration of pentapeptide at time t, and k_1 is the first order rate constant. This equation can be solved for A(t), to give

$$A(t) = A_o e^{-k_1 t} \qquad (2)$$

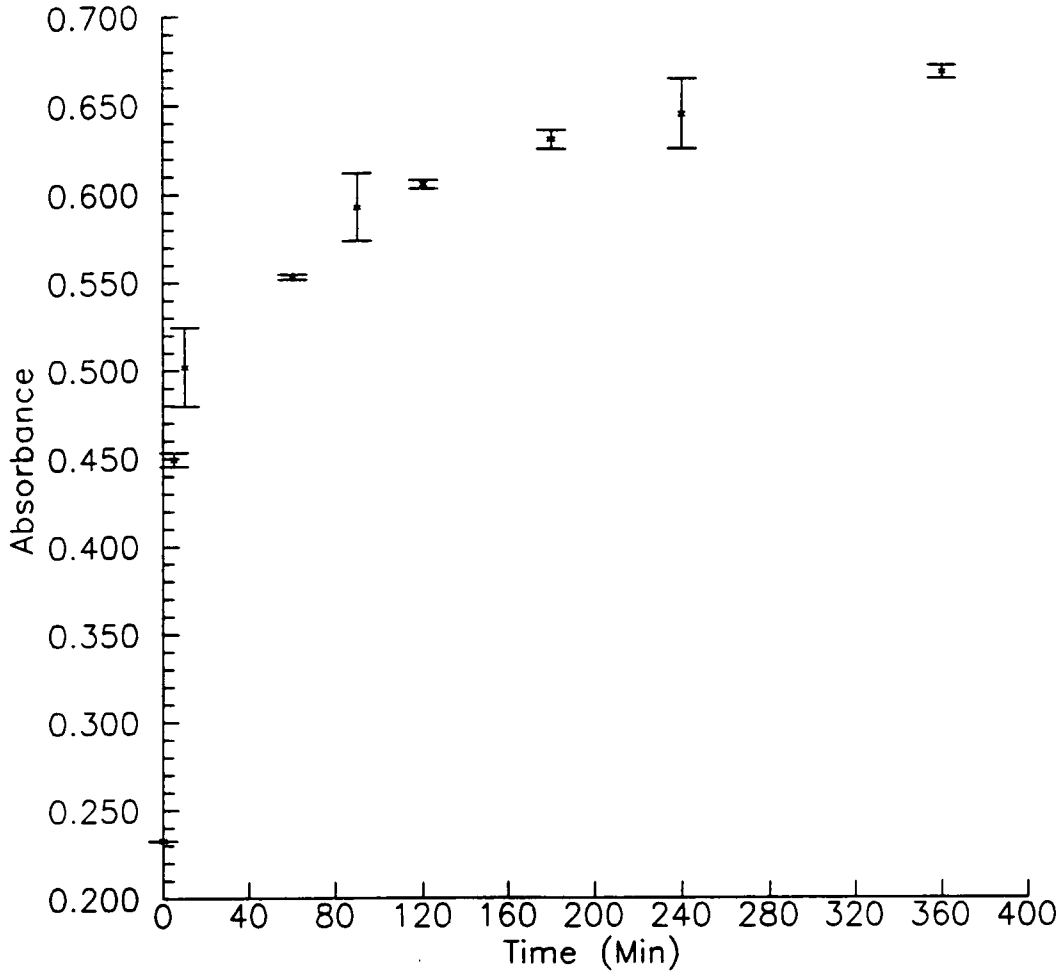

Fig 14. **Absorbance vs Time plot for Leu-Enkephalin in phosphate buffer. Substrate concentration: 1 mM; enzyme concentration: 1 unit/ml. Error bars calculated for standard error of the mean (n = 3).**

where A_o is the initial concentration of the pentapeptide. The rate of change of concentration of the tetrapeptide intermediate can be written as,

$$\frac{dB(t)}{dt} = k_1 A(t) - k_2 B(t) \tag{3}$$

where B(t) is the concentration of tetrapeptide, and k_2 is the rate constant for hydrolysis of the tetrapeptide. This is a first order separable differential equation. This can be rewritten to separate variables.

$$\frac{dB(t)}{dt} + k_2 B(t) = k_1 A(t) \tag{4}$$

Multiplying both sides of the equation by the integrating factor, and rewriting gives,

$$\frac{de^{k_2 t} B(t)}{dt} = e^{k_2 t} k_1 A_o e^{-k_1 t} = k_1 A_o e^{(k_2 - k_1) t} \tag{5}$$

Integration of left side of Eq.5 leads to Eq.6.

$$e^{k_2 t} B(t) = \int k_1 A_o e^{(k_2 - k_1) t} dt \tag{6}$$

Integration of right side of the Eq.6, and imposing the initial condition that at time equals zero, B(t) is zero, this differential equation can be solved in the following

manner to give concentration of tetrapeptide at time t (Eq. 8).

$$e^{k_2 t}B(t) = \frac{k_1 A_o}{k_2 - k_1} e^{(k_2 - k_1)t} + constant \qquad (7)$$

$$B(t) = \frac{A_o k_1}{k_2 - k_1} [e^{-k_1 t} - e^{-k_2 t}] \qquad (8)$$

The rate of change of concentration of leucine is given by

$$\frac{dP1}{dt} = k_1 A(t) \qquad (9)$$

where P1 is concentration of leucine, which can be obtained by solving Eq. 9 for P1.

$$P_1 = A_o[1 - e^{-k_1 t}] \qquad (10)$$

The rate equation for release of phenylalanine is

$$\frac{dP2}{dt} = k_2 B(t) \qquad (11)$$

where P2 is concentration of phenylalanine. Substituting the value of B(t) and solving for P2 gives concentration of phenylalanine as

$$P_2 = L1e^{-k_2T} - k_2\frac{L1}{k_1}e^{-k_1t} + k_2\frac{L1}{k_1} - L1 \qquad (12)$$

where

$$L1 = \frac{A_o k_1}{k_2 - k_1} \qquad (12a)$$

The concentration of tripeptide C(t) is obtained by solving the differential Eq.13, by using the same procedure as above, since this is also a separable equation.

$$\frac{dC(t)}{dt} = k_2 B(t) - k_3 C(t) \qquad (13)$$

where k_3 is rate constant for the hydrolysis of the tripeptide.

$$C(t) = k_2 \cdot \frac{L1}{k_3 - k_1}e^{-k_1t} - k_2 \cdot \frac{L1}{k_3 - k_2}e^{-k_2t} - k_2 \cdot \frac{L1}{k_3 - k_1}e^{-k_3t} + k_2 \cdot \frac{L1}{k_3 - k_2}e^{-k_3t} \qquad (14)$$

The hydrolysis of peptapeptide does not go to completion. The final product obtained is a dipeptide (Tyr-Gly) which is difficult to cleave. The end product concentrations D (dipeptide), and P3 (Gly), are obtained by solving the equations 15 and 16, giving the integrated rate equation (17).

$$\frac{dD(t)}{dt}=k_3C(t)\,;\,\frac{dP_3}{dt}=k_3C(t) \tag{15}$$

$$L3=\frac{L1}{k_3-k_1}\,;L4=\frac{L1}{k_3-k_2} \tag{16}$$

$$D(t)=-k_3\frac{L3}{k_1}e^{-k_1t}+k_3\frac{L4}{k_2}e^{-k_2t}+k_3\frac{L3}{k_3}e^{-k_3t}-k_3\frac{L4}{k_3}e^{-k_3t} \tag{17}$$

$$P_3=D \tag{18}$$

The important part of this reaction is that consecutive steps are happening, in a continuous manner; i.e., before the release of leucine is complete, the freshly released tetrapeptide can be hydrolysed to give the tripeptide and phenylalanine. At one point in time, more than two products and intermediates are present. The absorbance read at any time is the sum of absorbances of amino acids, and the peptides present at that time. The total concentration of amino acids present at time t was calculated by dividing the absorbance by absorption coefficient obtained from leucine calibration plot. Thus the overall equation fitted was

$$\text{sum} = A(t) + B(t) + C(t) + D(t) + P1 + P2 + P3 \tag{19}$$

A nonlinear approximation program [24] was used to estimate the kinetic parameters (Appendix). Tables 6 and 7 show the observed and model predicted data. The kinetic parameters are displayed in Table 8. Fig 15 and Fig 16 show the curve fits for observed vs model predicted data. The

Table 6.

Concentration vs time data for hydrolysis of Leu-Enkephalin with CPY in buffer system

Time (min)	[a]Conc.(observed)	[a]Conc.(predicted)[b]
0	0.077	0.081
5	0.146	0.149
10	0.167	0.161
60	0.186	0.188
90	0.197	0.198
120	0.200	0.206
180	0.213	0.215
240	0.220	0.221
360	0.224	0.224

[a]Concentration in mM; [b]Model predicted values. Sum of residuals: 7.43E-05

Table 7.

Concentration vs time curves for hydrolysis of Leu-Enkephalin with CPY in buffer & 20 % TDE system

Time (min)	[a]Conc.(observed)	[a]Conc.(predicted)[b]
0	0.077	0.103
60	0.124	0.128
120	0.149	0.148
180	0.178	0.165
300	0.185	0.189
390	0.191	0.202
600	0.220	0.224

[a]Concentration in mM; [b]Model predicted values. Sum of residuals: 9.886E-04.

Table 8.

Estimated kinetic parameters for hydrolysis of Leu-Enkephalin with CPY in indicated matrix

Matrix	$^{a}k_1$	$^{a}k_2$	$^{a}k_3$
Buffer	0.386	9.45E-03	2.57E-04
Buffer&20%TDE	4.76E-04	8.28E-04	9.98E-06

$^{a}k_1$, k_2, k_3 in min^{-1}. See text for definitions.

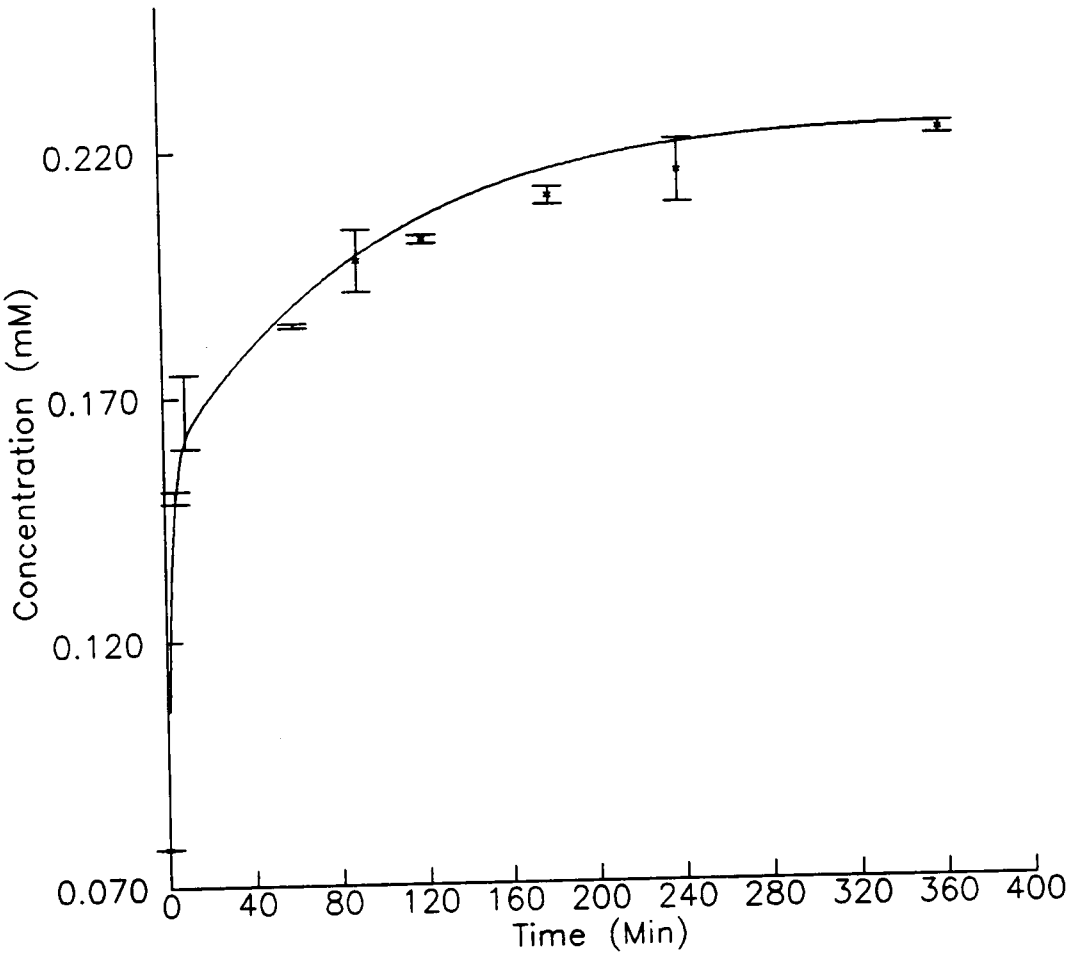

Fig 15. Hydrolysis of Leu-Enkephalin in phosphate buffer system. Curve fit obtained by using parameter values k_1 = 0.386, k_2 = 9.45E-03, k_3 = 2.57E-04. Error bars calculated for standard error of the mean (n = 3).

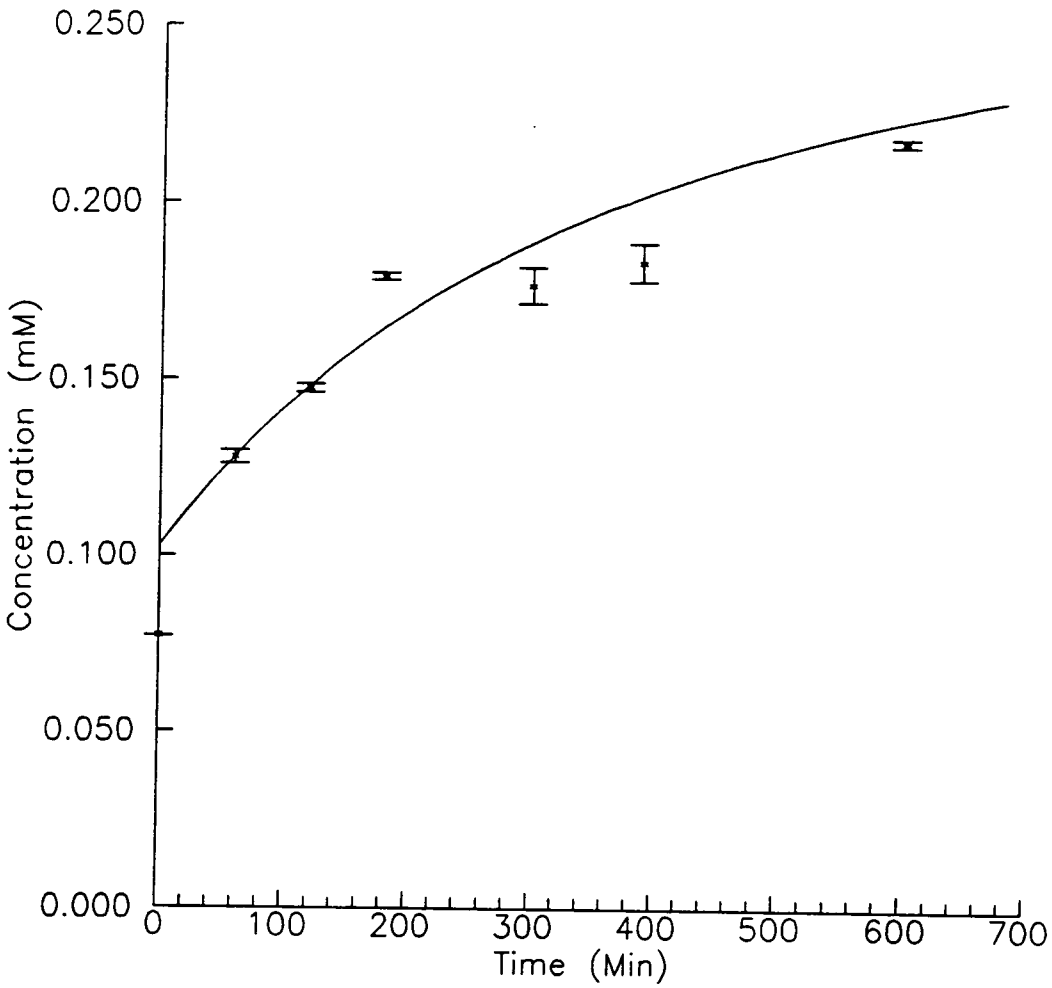

Fig 16. Hydrolysis of Leu-Enkephalin in phosphate buffer/20% TDE system. Curve fit obtained by using parameter values k_1 = 4.76E-04, k_2 = 8.28E-04, k_3 = 9.98E-06. Rate constants and error bars calculated for standard error of the mean (n = 3).

difference in the kinetic parameters with and without TDE proves that rate of hydrolysis was much slower in presence of TDE. Attempts to hydrolyse Leu-Enkephalin with a 10% TDE solution in buffer were not very successful. There was a gross scatter in the data. Hydrolysis with 40% TDE was not successful at the concentration of the enzyme used for the previous experiments. An indication of some reaction was observed at 16 unit/ml of the enzyme. These results suggest that solution of 20% TDE in pH 6.5 phosphate buffer with 0.5 M sodium chloride might work for EHMS. The results from EHMS were not available at the time of this writing, to compare the conventional technique with EHMS.

5. SUMMARY AND CONCLUSIONS

Summary

The present investigation deals with an attempt to estimate rate constants for the hydrolysis of a few peptides so that the versatility of EHMS could be compared with the conventional method. Three dipeptides, CBZ-phe-leu, CBZ-leu-tyr, and CBZ-glu-tyr, and a pentapeptide leu-enkephalin were hydrolysed with the enzyme carboxypeptidase Y, with and without the nonvolatile viscous solvent thiodiglycol (TDE), 0.5 M sodium chloride, and pH 6.5 phosphate buffer. The absorbances of the released amino acids were monitored by a UV/VIS detector. The kinetics of hydrolysis with and without TDE were compared, for the dipeptides, assuming Michaelis-Menten kinetics. The rate constants for pentapeptide hydrolysis were estimated by a nonlinear approximation method, assuming first order kinetics at each step.

Conclusions

Enzyme hydrolysis by CPY follows Michaelis-Menten kinetics. The rate parameters for hydrolysis of dipeptides,

k, K_m, and V_{max} differed with the substrate used, and reflect the activity of the enzyme, under the reaction conditions. The inhibition seen in the presence of TDE was of mixed type, as evidenced by the decrease in the parameters k, K_m, and V_{max}.

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APPENDIX

The nonlinear least squares program used for parameter estimation was written for Basic-I, and was written for desk top use. The inputs for the program are number of data points, number of parameters to which the data needs to be fitted, the initial guess for the parameters(ps(i)), total number of tries (TT), the step size (dp), and the number elapsed tries (TE) for each iteration. The output is the sum of residuals (ES) at each step, and when the convergence criterion is met, the program prints out the standard deviation and the sum of residuals. The original program has the data points x(i), and y(i) input at the screen. More details can be found in the original reference.

A few changes were made to the original program to fit our needs. For the present use, input data file was a text file, called "indat.dat". The output was written to the screen. The total number of tries was 100,000, the step size was 40, and the number of elapsed tries was 40. The time and the concentration at each time were stored in variables x(i) and y(i) respectively. The rate constants k_1, k_2, and k_3 were stored in the variables ps(i), and were input at the screen. The necessary equations were input from line 9000, and the sum was calculated for each x(i), and stored in the variable

"yr", which was the variable used for the function value, by the original program. The initial guesses used were such that the $k_1 > k_2 > k_3$. The best fit was obtained, when there was no change in the sum of residuals. The source code used is given below.

Nonlinear least squares program used for curve fitting

```
1000 REM===R EVOL===
1050 DIM X(50), Y(50), P(10), PH(10), PR(10)
1062 PM = 10: NM = 50: Z = 0.4711
1200 OPEN "INDAT.DAT" FOR INPUT AS #1
1280 INPUT "NUMBER OF DATA PAIRS", N: PRINT
1300 IF N > NM OR N < 2 THEN GOTO 1280
1330 FOR I = 1 TO N : INPUT #1, X(I), Y(I)
1340 PRINT : NEXT i: PRINT
1370 INPUT "NUMBER OF PARAMETERS", NP: PRINT
1390 IF NNP>N OR NP>PM OR NP<1 THEN GOTO 1370
1410 PRINT "PARAMETER STARTING VALUES": PRINT
1450 FOR J = 1 TO NP: INPUT "PS= ", P(J): NEXT J: PRINT
1500 INPUT :PARAMETER VARIATION", DP :PRINT
1530 DP = DP/100: IF DP = 0 THEN GOTO 1450
1570 INPUT "TOTAL TRIES LIMIT ", TL: PRINT
1580 INPUT "TOTAL ELAPSE TRIES ", TE: PRINT: TT=1
1640 PRINT "DP = ", DP:PRINT:TF=1
1700 FOR J = 1 TO NP
1710 PL(J) = (1-DP) * P(J):PH(J)=(1+DP)*P(J)
1720 NEXT J: GOSUB 5000: FS = ES
1760 PRINT "START ES = ", ES, "START TT = ', TT:PRINT:GOSUB
6000
1850 TT = TT + 1
1890 J = INT(NP*RND(Z)+1):P0 = P(J)
1940 P(J) = PH(J) - PL(J)*RND(Z)*PL(J)): GOSUB 5000
2020 IF ES>FS THEN P(J)=P0:TF=TF+1:GOTO 2170
2040 FS=ES:TF=1:PRINT :"DP=", DP:PRINT
2080 FOR J = 1 TO NP: PRINT J, P(J): NEXT J
2120 PRINT "ES= ", ES TAB(20);"YREG", TAB(30); "YRES"
2170 IF TT>TL THEN GOTO 2300
2210 IF TF<TE THEN GOTO 1850
2240 DP = DP * 0.67: GOTO 1640
2300 GOSUB 6000: PRINT: PRINT " S  T O P " : STOP
2330 PRITN " I X Y "; TAB(20); "YREG";TAB(30);"YRES"
2360 FOR I = 1 TO N GOSUB 7000
2390 PRINT I, X(I); Y(I); TAB(15);YR,TAB(25);Y(I)-YR:NEXT I
2430 FOR J = 1 TO NP: PRINT J, P(J): NEXT J
2480 PRINT "ES= ", ES : PRINT
2520 INPUT "RADEX RANGE (Y/N) ", YN$
```

```
2530 IF YN$ = "Y" THEN GOSUB 7000
2560 GOTO 1500
3000 '
5000 ES = 0
5070 FOR I = 1 TO N: GOSUB 9000
5110 IF YR = 0 THEN GOTO 5150
5130 DY = Y(I)/YR-1 : ES = ES + DY*DY
5150 NEXT I
5210 RETURN
5500 '
6000 PRINT "BOUNDARY PARAMETER RATIOS"
6080 FOR J = 1 TO NP: P0 = P(J)
6120 P(J) = PL(J) : GOSUB 5000: EL = ES/FS
6140 P(J) = PH(J) : GOSUB 5000: EH = ES/FS
6160 PRINT J, EL, EH: P(J) = P0: NEXT J : PRINT ES = FS
6210 RETURN
6900 '
7000 PRINT " J            P(J)            RADEXRANGE"
7080 GOSUB 5000: FS = 2 * ES
7110 FOR J = 1 TO NP
7120 FOR K = 0 TO 1 : DP = K = 0.5
7160 P0 = P(J)* ( 1-DP) : GOSUB 5000
7200 IF ES<FS THEN GOTO 7180
7220 DP = -0.1 * DP: IF ABS(DP)<+EPS THEN GOTO 7340
7260 P(J) = P(J) * (1 + DP) : GOSUB 5000
7280 IF ES > FS THEN GOTO 7260
7300 GOTO 7180
7340 PR(K) = P(J) : P(J) = P0
7370 NEXT K: PRINT J, P0, RR(0), RR(1)
7410 NEXT J
7430 RETURN
8000 '
9000 A0 = 0.07
9001 EK1 = EXP(-P(I) * X(I))
9002 EK2 = EXP(-P(2) * X(I))
9003 AL1 = A0 * P(1) / P(2) - P(1))
9004 AL11 = P(2) * AL1/P(3) - P(1))
9005 AL12 = P(2_ * AL1/P(3) - P(2))
9006 A = A0 * EK1
9007 B = AL1 * (EK1-EK2)
9008 ALEU = A0 * (EK1-EK2)
9009 EK3 = EXP(-P(3)*X(I))
9010 C = AL11*EK1 - AL12*EK2 - AL11*EK3 + AL12*EK3
9020 APHE = AL1*EK2 - (AL1(P(2)*EK1/P(1) + (AL1* P(2)/P(1))
- AL1
9030 D = -(P(3)*AL11*EK1/P(1)) + (P(3)*AL12*EK2/P(2)) +
(P(3)*AL12*EK3/P(3)) - (P(3)*AL12*EK3/P(3))
9040 AGLY = D
9050 YR = A + B + C + D + APHE + ALEU + AGLY
9080 RETURN
9980 CLOSE #1
9999 REM======SAVE EVOL======
```

VITA

K. Someswari Rao was born in Hyderabad, India on March 11, 1949. She attended Osmania University from 1967 to 1976. She came to U.S.A. in 1976. In 1979, she received a doctorate degree from Osmania University, with a major in organic chemistry. She worked in U.T. Memorial Hospital from 1980-82. From 1982 to 1988, she was a full time mother, while attending evening school at University of Tennessee, taking courses in numerical mathematics. In 1988 she entered U.T. chemistry department, while working at Oak Ridge National Laboratory, Health and Safety Division, where she worked until April 1991. She joined Integrated Laboratory Systems in April 1991.

She will be working at ORNL again from January, 1992. She will also be graduating from U.T with a Master's degree in analytical chemistry.