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Title: An Investigation of a Method for the Separation of Labile Metalloenzymes

Abstract approved: Duane R. Baline

A method for the separation of metalloenzymes has been investigated. The activity and metal content of a metalloenzyme was measured before and after elution from a Sephadex gel chromatography column to discern whether it was eluted with its metal ion still bound. A metal spiked buffer solution was employed to stabilize the metal-enzyme complex during elution.

The results show that a stable zinc metalloenzyme (carboxypeptidase A) may be eluted without the use of a metal spiked buffer solution. However, a more labile zinc metalloenzyme (α-amylase) required the metal spiked buffer solution to prevent dissociation during elution. Ninety-eight percent or better of the metal content and enzyme activity was recovered upon elution of both enzymes. The α-amylase, the zinc content of which is less well-established, was found to have a mole ratio of Zn:enzyme of 1:2.

AN INVESTIGATION OF A METHOD FOR THE SEPARATION OF LABILE METALLOENZYMES

A Thesis

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PREFACE

The details of the manners in which metals manifest their important biological roles are still relatively unknown. It has been shown that they function in catalysis, in the synthesis and the stabilization of the structure of proteins, and in cell membrane transport -- processes in which they function in a specific manner. However, little is known of the chemical details or the mechanisms by which these processes take place. The essentiality of many metals in biological systems is now known, but the manner by which they exercise their roles is less well understood.

One of the major problems that has hampered bioinorganic research is the labile nature of many metal-enzyme complexes. Most of the information currently available is limited to the structures, mechanisms, and kinetics utilizing only the stable metalloenzymes. Similar data could be obtained for the less stable metalloenzymes if separation techniques which minimize the dissociation of the complex could be developed.

This manuscript describes in detail a proposed method and procedure for the separation of labile metalloenzymes. A procedure of this type does not exist and the goal of this work was to obtain information to aid in the development of such a technique.

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SECTION 1

INTRODUCTION

Enzymes are found in nature in complex mixtures, usually in cells which may contain a hundred or more different enzymes. The foundations of enzyme biochemistry were laid long before any pure enzymes had been obtained. In some cases, it is possible by the use of sufficiently specific test methods to study enzymes which are in an impure state; but, in order to study a given enzyme properly it must be purified. In most cases other enzymes present will interfere, either by attacking the substrate giving side reactions, transforming the product into some other substance, or attacking the coenzyme or even the enzyme itself. It is sometimes difficult, until the enzyme in question has been purified, to determine exactly which reaction it catalyzes.

For studies of enzyme specificity it is necessary to have the enzyme as pure as possible. If a reaction is found to be catalyzed by an impure enzyme, there will be some doubt whether it is due to some other substance present in the preparation.

The purification of enzymes dates from about 1922. The first crystalline enzyme, urease, was obtained by Sumner[1] in 1926. By 1940 about twenty highly purified enzymes had been isolated and the process has continued at an ever-increasing rate. At the present time a dozen or more enzyme purifications are reported each year. By no means are all of these crystalline, although some of the non-crystalline preparations may be as pure as the crystalline ones, for the crystallization of a protein gives no guarantee of its purity and it is not uncommon for the first crystals obtained to be only fifty

per cent pure. One of the major problems that has hampered bio-inorganic research is the labile nature of many metalloenzyme complexes. Most of the currently available information has been limited to the structures, mechanisms, and kinetics utilizing only the stable metalloenzymes. Similar data can be obtained for the less stable metalloenzymes if separation techniques which minimize the dissociation of the complex can be developed.

The major purpose of this study is to develop a method of separation for metalloenzymes and to analyze the elutant to determine the metals contained in specific fractions. This investigation will first focus on attempting to achieve a chromatographic separation of a stable, well-characterized metalloenzyme. This procedure will then be applied to less stable metalloenzymes of similar characteristics to investigate the possibility of a general procedure for separation.

The success of this technique will be evaluated by the measurement of enzyme activity before and after separation. If the metalloenzyme can be separated intact the enzyme activity will be approximately the same before and after separation.

If this technique can be developed it will provide a way for other researchers to gain information needed for extension of their work to animal tissues, and eventually to an attempt to understand the mechanisms by which these metals are involved in the metabolic processes in man.

SECTION 2

REVIEW OF LITERATURE

2.1 Metalloenzymes

2.1.1 Activation by Metals

The activity of an enzyme is often dependent upon the presence of a metal ion. In a metalloenzyme this ion is firmly bound as a constituent of the enzyme itself. It may be bound to ligand sites in the protein or in combination with some prosthetic group such as a porphyrin. In other cases it is necessary to add a metal ion to "activate" the enzyme. The distinction between a metalloenzyme and a metal activated enzyme is based upon the stability of the metal-enzyme complex.

Classification of metalloenzymes, in which the metal is tightly bound to the protein, and metal activated enzymes, in which a correlation between the metal concentration and specific activity is apparent, is a somewhat vague task. The normal distinction is based upon the ability to separate and purify the enzyme without dissociation of the ligand-metal complex. Zinc metalloproteins have been found to have dissociation constants of about 10^{-10} M or less. However, the dissociation of the enzyme into an equilibrium between the metal ion, apoenzyme and metal-enzyme complexes depends upon the procedures used in obtaining the purified substance.[2] In cases where the metal has to be added it is usually easily removed again, either by dialysis or by precipitation of the protein. A finite concentration of the free activating ion must then be maintained in order to hold the enzyme in the form of the metal activated enzyme. Sometimes the metal activator may combine with the substrate rather than

with the enzyme; the true substrate of the enzyme then being a metallo-substrate rather than the substance actually added.[3]

Fifteen different metal cations have been found to activate one or more enzymes. These are: Na^{+}, K^{+}, Rb^{+}, Cs^{+}, Mg^{++}, Ca^{++}, Zn^{++}, Cd^{++}, Cr^{+++}, Cu^{++}, Mn^{++}, Fe^{++}, Co^{++}, Ni^{++}, Al^{+++}, and NH_4^{+}. Molybdenum compounds also activate certain enzymes, but the ionic form of the activator is obscure. All of these metals have atomic numbers between 11 and 55 and the majority lie between 19 and 30. No metal with an atomic number greater than 55 is known to activate an enzyme by itself.[4]

One of the important factors that determines which metal ions activate an enzyme is the size of the ion. All of the metal ion activators listed have ionic radii which lie within a narrow range, in the middle of observed values of ionic radii. The ions in the list, however, are by no means all interchangeable. Some enzymes may be activated by only one, in more cases two or three, of these ions. In several cases two ions, usually of different ionic charge, are required simultaneously for activation (i.e., Pantothenate synthetase which requires Mg^{++} and K^{+} for activation) though the reason for this is not clear.

The predominance of coordination compounds formed from transition metal ions and ligand groups of porphyrins, amino acids, and proteins found in all biological species makes a basic understanding of coordination chemistry essential. The ability of a metal ion to bind to a chelating agent depends not only upon its physical size, but also upon the geometric arrangement of the bonding orbitals of the metal. There is some reason to debate whether a particular metalloenzyme has a specificity for a metal ion due to the positions of the ligating groups, or if the position of the ligating groups is established by the presence

of the metal during synthesis (template effect). Regardless of which is more important, the ability of a metal to replace another that occurs naturally in an enzyme and restore enzymatic activity appears to depend to a great extent upon the geometric orientation of the bonding orbitals of the metals.[5]

2.1.2 Zinc Containing Metalloenzymes

The importance of zinc for the normal functioning of most organisms can be anticipated due to its high concentration, as compared to other trace metals, in the cell. The only transition element that is present in the cell in higher concentrations is iron.

Purified zinc metalloenzymes show that the apoenzyme has a preference for binding zinc relative to Co II, Ni II, and Cu II ions. The binding ligands are usually nitrogen or oxygen. These Lewis bases would normally be expected to have a greater coordinating affinity for these other metals. One would expect that zinc binding sites would contain sulfur ligands, but this does not seem to be the normal complex present in most zinc metalloproteins.

There are three well established zinc binding ligands in metalloenzymes: the imidazole nitrogen, the γ-carboxyl of glutamic acid, and the sulfhydryl group of cysteine. Carboxypeptidase A and thermolysin both bind zinc in a somewhat distorted tetrahedral structure. The imidazole nitrogen of two separate histidyl residues and the γ-carbonyl of a glutamyl residue occupy three of the available coordination sites. A water molecule or hydroxide ion, depending upon pH, occupies the fourth site. Zinc is bound to sulfhydryl groups in metallothionein.

The apoenzyme of alkaline phosphatase has been synthesized in

the absence of zinc. Enzyme activity is obtained when zinc is added, indicating that the zinc binding site is present even in the absence of the metal but catalytic activity does depend upon its presence.

The function of zinc in a metalloenzyme would be expected to involve Lewis acid reactions. This has been shown to be the case in carboxypeptidase. However, in aspartate transcarbamylase the zinc ion is not located at the active site. It seems to be involved with maintaining the structure of the regulatory subunit. A similar situation exists in superoxide dismutase. The active catalytic site contains a copper ion, but a zinc ion is located in close proximity.

There are thirty-seven functionally distinguished enzymes that have been reported to contain Zn II as a component after isolation. Some of these that have been well established to be Zn II metalloenzymes are listed in Table 1. Other enzymes that have been reported to contain zinc, but for which less evidence is available, are given in Table 2. The enzymes listed in Table 1 include nine in which zinc acts as a Lewis acid during a hydrolysis or hydration of the substrate. There are two enzymes, alcohol dehydrogenase and superoxide dismutase, that involve oxidation-reduction reactions. Zinc is not involved in the actual electron transfer, but may have a role in positioning or polarizing the substrate.

The other two enzymes catalyze group transfer reactions. The formation of an enzyme-metal-substrate complex in which zinc may withdraw electrons from the substrate has been suggested as a possible mechanism for the reaction of yeast aldolase. The zinc ion is not located at the active site in aspartate transcarbamylase but is present in the regulatory subunits.[55]

TABLE 1

Well-Characterized Zinc Metalloenzymes

ENZYME	SOURCE	MW	ZINC/MOLE	COFACTOR	REF.
Carboxypeptidase A	Bovine pancreas	34,500	1		6
Carboxypeptidase B	Bovine pancreas	34,500	1		7
Neutral protease	B. thermoproteolyticus	37,500	1	4 Ca (II)	8
	B. subtilis	44,700	1	n Ca (II)	9
	Leucostoma (snake venom)	22,500	1	2 Ca (II)	10
Amino peptidase	Bovine lens	324,000	12		11
	Porcine kidney	300,000	6		12
		280,000	2		13
Alkaline phosphatase	E. coli	80,000	2		14
Carbonic anhydrase B	Human erythrocyte	26,600	1		15
	Bovine erythrocyte	31,000	1		16
Carbonic anhydrase C	Human erythrocyte	32,000	1		17
Alcohol dehydrogenase	Horse liver	87,000	4	2 NAD	18
Superoxide dismutase	Bovine erythrocyte	32,000	2	2 Cu (II)	19
Aldolase	Yeast	80,000	2		20
	E. coli	135,000	2		21
Aspartate transcarbamylase	E. coli	300,000	6		22
Transcarboxylase	P. shermanii	790,000	6-12	6 Biotin	23
DNA polymerase	E. coli	109,000	2		24

TABLE 2

Enzymes Reported to Contain Zinc[a]

ENZYME	SOURCE	MW	ZINC/MOLE	COFACTOR	REF.
RNA polymerase	T_7 phage	110,000	2-4		25
RNA polymerase	E. coli	370,000	2		26
Nucleotide pyrophosphatase	Rat liver		n Zn		27,28
5'-Nucleotidase	E. coli	52,000	1		29
Cyclic phosphodiesterase	E. coli		n Zn		30
Phosphomannose isomerase	Yeast	450,000	1		31
Phosphoglucomutase	Yeast		1		32
α-D-Mannosidase	Jack bean		n Zn		33
β-Lactamase	B. cereus		n Zn		34
5'-AMP aminohydrolase	Rat muscle	290,000	2 Zn		35
Collagenase	C. histolyticum	105,000	n Zn		36
Neutral protease	B. cereus		n Zn		37
Dipeptidase	Porcine kidney	47,000	1		38
Phospholipase C	B. cereus				39
Dipeptidase	Mouse ascites tumor	87,000	1		40
α-Amylase	B. subtilis	50,000	n Zn	3 Ca	41,42
Aminopeptidase	Porcine jejunum	280,000	2		43
D-Glyceraldehyde-3P-dehydrogenase	Porcine muscle		3	3 NAD	44

TABLE 2

(cont.)

ENZYME	SOURCE	MW	ZINC/MOLE	COFACTOR	REF.
Lactic dehydrogenase	Rabbit muscle		n Zn	NAD	45
Malic dehydrogenase	Bovine heart	40,000	1		46
Glutamic dehydrogenase	Bovine liver	1,000,000	2-4 Zn	n NAD	47
Pyruvate carboxylase	Yeast	600,000	4 Zn	4 Biotin	48
Mercaptopyruvate sulfur transferase	E. coli	23,600	1		49
Rhodanese (sulfur transferase)	Bovine liver	37,000	2		50
5-Amino levulinic acid dehydratase	Bovine liver	260,000	n Zn		51
α_2-Macroglobulin	Human serum	840,000	3-8		52
Metallothionein	Equine renal cortex	10,000	3	5 Cd (II)	53
	Human renal cortex	10,500	3	4-5 Cd (II)	54

[a]Definitive data relating zinc to the structure or function are not available.

The essential nature of zinc in metalloenzymes has been investigated by several methods. The enzymes have been dialyzed to remove the metal ion and the activity of the apoenzyme measured. Chelating agents, 1-10 phenanthroline, EDTA, and 8-hydroxyquinoline, have been used to complex the metal. These bi- and poly-dentates have been found to remove the metal from carboxypeptidase A, thermolysin, alkaline phosphatase and carbonic anhydrase. Mixed complexes of the enzyme and chelating agent have not been found, indicating a dissociation of the metal-enzyme complex occurs as the free metal ion concentration is decreased by formation of a metal-chelate complex. An exception has been noticed in the formation of a ligand-zinc-enzyme complex when 1-10 phenanthroline reacts with alcohol dehydrogenase.

In addition to the use of organic chelating agents to remove zinc from an enzyme, other metal ions have been substituted for the zinc. Table 3 shows some formation constants for these metalloenzymes (that normally contain zinc) with other divalent metal ions. One must also consider rate constants for the replacement of zinc by other metals but the formation constants listed provide a means of making some comparisons of the stability of the metalloenzyme complexes.

It can be seen from the data in Table 3 that the stabilities of the metallocarboxypeptidase complexes of Cu II and Cd II are about the same as zinc but that of Hg II is significantly greater when corrected for chloride and Tris interferences. It should also be noted that chloride may compete effectively with the enzyme in binding Hg II.

TABLE 3[a]

Formation Constants for Some Metalloenzymes

Metal	Metallocarboxypeptidases		Metallocarbonic Anhydrases	Metalloalkaline Phosphatases
	Log K Apparent	Log K Corrected[b]	Log K pH 5.5	Log K pH 7.6
Mn II	5.6	5.6	3.8	6.5
Co II	5.8	7.0	7.2	4.2
Ni II	5.7	8.2	9.5	2.9
Cu II	5.7	10.6	11.6	
Zn II	8.3	10.5	10.5	5.6
Cd II	7.9	10.8	9.2	6.5
Hg II	6.7	21.0	21.5	

[a]Reference 56

[b]Corrected for competition by chloride and Tris

In addition to the stability of these metalloenzyme complexes, it is of interest to compare the relative activities of the enzymes to determine what functional changes occur if another divalent metal ion is substituted for zinc. Table 4 summarizes the relative activity of several zinc containing enzymes after such substitutions have been made. The activity is expressed relative to that of the normal zinc metalloenzyme which is assigned the value 100.

2.1.3 Separation

The tendency of a metalloenzyme to dissociate is a mjaor problem that must be solved if one is to obtain a pure sample of the metalloenzyme as it exists in the cell.

Gel filtration chromatography can be used for the separation of tightly bound metal-ligand complexes, but this method does not work well for the separation of labile complexes. The metal-ligand complex exists in an equilibrium state, $ML \rightleftarrows M + L$. The metal ion, ligand, and complex will pass through the column at different rates. Thus, the labile metal ion will dissociate from the ligand at each successive plate in the column in an attempt to reestablish equilibrium. The resulting chromatograph is complex due to the difference between the elution times of the enzyme and apoenzyme, and a representative sample of the metalloenzyme is not obtained. This dissociation is enhanced if the column packing adsorbs the metal ion, as has been observed with some Sephadex gels.

Recently both of these problems were avoided by the use of a buffer containing zinc ions to equilibrate the column prior to using it to separate low molecular weight Zn binding proteins in human and bovine milk.[59] An ion exchange effect was observed due to the

TABLE 4

Enzyme Activity as a Function of Metal Ion
Species Substituted at the Active Sites of Zinc Metalloenzymes

ENZYME	Metal Ion[a] Mn	Fe	Co	Ni	Cu	Zn	Cd	Hg	Ref.
Carboxypeptidase A									
Peptidase	28	29	215	50	0	100	0	0	57,58
Esterase	200		130	100	0	100	210	120	
Carboxypeptidase B									
Peptidase			150			100	0		57,58
Esterase			150			100	200		

[a]Activity expressed as percent activity of the zinc protein

presence of Na, K, Ca, and Mg ions in the sample. This makes the chromatogram somewhat difficult to interpret.

2.2 Gel Chromatography

2.2.1 Sephadex

Gel filtration first became an established laboratory technique with the introduction of Sephadex in 1959.[60] Since then, gel filtration has been increasingly used both in analytical and in preparative work, as well as on a production scale in the chemical industry.

Before Sephadex was introduced, fractionation and separation of molecules according to size could only be carried out by very time consuming or expensive methods. Using the appropriate type and grade of gel, such separations can now be performed rapidly and simply. Gel chromatography is particularly useful for separation of substances of biological origin, which are often very labile.

Sepahdex is a dextran gel which is prepared by cross-linking selected dextran fractions with epichlorohydrin. Dextran is an anhydroglucose polymer produced in sucrose-containing solutions by different strains of Leuconostoc mesenteroides. Because of the high content of hydroxyl groups in the polysaccharide chains, Sepahdex is strongly hydrophilic and the beads swell in water and electrolyte solutions. It is a chromatographic material capable of separating substances according to molecular size. The separation method is most commonly known as gel filtration, gel chromatography, or exclusion chromatography. Sephadex is supplied in the form of minute beads which have considerable advantages over non-bead gels as it imparts good flow and separation properties to chromatographic

materials.[61]

A gel filtration can be described in the following manner. Molecules larger than the largest pores of the swollen Sephadex, i.e., above the exclusion limit, cannot penetrate the gel particles and therefore pass through the column in the liquid phase outside the particles. They are thus eluted first. Smaller molecules, however, penetrate the gel particles to a varying extent depending upon their size and shape. Molecules are therefore eluted from a Sephadex bed in order of decreasing molecular size.

2.2.2 Determination of Molecular Weights

One of the most striking properties of Sephadex gels as chromatographic materials is their capacity for separating substances according to molecular size. For proteins, extensive investigations have shown that the elution volumes of globular proteins are largely determined by their molecular weights. The elution volume is approximately a linear function of the logarithm of the molecular weight over a considerable range.[62] Gel filtration can also be used for the determination of molecular weights of peptides and of macromolecules other than proteins. Because the relationship between molecular weight and elution volume is different for different types of molecules, a separate calibration curve should be determined for each type of substance.

With this method even crude separations can be used to determine the molecular weight of biologically active substances such as enzymes. The enzyme activity in the column effluent is measured and thus the elution volume can be determined. The molecular weight of a substance can then be estimated by comparison to a calibration curve, produced by

chromatography of proteins of known molecular weights. This is the only method of estimating molecular weights of proteins which does not require extensive purification of the sample. Thin-layer gel filtration can be used for the determination of molecular weights of small samples of substances.

2.3 Determination of Activities

2.3.1 Carboxypeptidase A

Carboxypeptidase A was first isolated in crystalline form from beef pancreas by Anson in 1935.[63] Extensive enzymatic studies have defined this enzyme as an exopeptidase that is specific for catalyzing the cleavage of certain carboxyl-terminal peptide bonds in peptides and proteins.[64]

A method used for the determination of carboxypeptidase A activity is a modification developed by Folk and Schirmer[65] from a similar method used for the determination of carboxypeptidase B activity. Miles Biochemicals laboratories later modified the method of Folk and Schirmer to the method which is commonly used today.[66]

This method involves the measurement of the increase of absorbance at 259 nm, due to the hydrolysis of hippuryl-L-phenylalanine by the enzyme. The activity is expressed as the change in absorbance per minute. The specific activity is the activity per milligram of enzyme protein per milliliter of solution.

2.3.2 α-Amylase

Many methods for estimating serum amylase are now available to the clinical biochemist. This wide diversity of amylase methodology has resulted in the use of a variety of differently derived

units which makes comparisons difficult. The major principles upon which the various methods of determining amylase activity are based are the measurement of: 1) a decrease in viscosity of a starch solution; 2) a decrease in turbidity of a starch suspension; and 3) a decrease in intensity of the color of the starch-iodine complex. Although some of these methods have been reviewed in the past, no recent attempts have been made to appraise the entire choice of methods available or to select a standard procedure.

2.3.2.1 Viscosimetric Methods

The viscosity of a starch solution is a function of the interference between swollen granules. Thus, when α-amylase randomly hydrolyzes α-1,4-glucosidic linkages in both linear and branched fractions of the hydrated starch granule, the molecular structure is disrupted with a resultant reduction in viscosity.

The first viscosimetric method of determination of amylase activity in serum was devised by Davison.[67] This method was an adaptation of the technique of Northrup and Hussey[68] for the determination of pepsin and trypsin wherein starch was substituted for gelatin. An Ostwald viscometer was used to measure the change in flow rate and the enzyme activity was related to the time required to reduce the original viscosity of the starch solution by 20%. Elman and McCaughan[69] modified this method to avoid expressing the time dependence of the units. This modified unit is defined as the quantity of enzyme required to reduce the original viscosity by 20%. Chesley[70] demonstrated that the viscosimetric method was highly dependent upon the nature of the starch substrate.

He suggested that amylose be used as the substrate, since differences in results were attributable to the varying percentages of amylose and dextrins in different starches.

Myers and Reid[71] concluded that the viscosimetric method of Elman and McCaughan[72] was preferable to the amyloclastic and saccharogenic methods available at that time. Based upon a comparative study of various amylase assay techniques however, the technique was not suitable for serum with low amylase activity.

2.3.2.2 Turbidimetric Methods

These techniques are based upon measurement of the reduction in turbidity of stable starch suspensions after incubation with amylase-containing material. This decrease in turbidity is due to a decrease in granule size associated with starch hydrolysis. Turbidimetric methods were developed for the determination of amylase in saliva,[73] pancreatic juice,[74] and urine,[75] but it was not until 1955 that this method for the determination of serum amylase activity was described by Peralta and Reinhold.[76] This modification of Waldron's method[77] led to the development of a convenient technique involving only a five minute incubation of the serum with starch. In 1960, Guth[78] described another modification of Waldron's method for the determination of amylase activity in serum. An additional turbidimetric technique for the determination of serum amylase activity, also based upon Waldron's method, was later proposed by Ware.[79]

Peralta and Reinhold,[80] later followed by Guth,[81] devised new units for their turbidimetric methods. However, Ware[82] calibrated his technique to yield results in Somogyi units. This was achieved by the use of control serum of known amylase activity, assayed by a

saccharogenic method.[83]

2.3.2.3 Starch-Iodine Methods

According to Searcy,[84] the first quantitative method for the determination of amylase iodimetrically was introduced by Wohlgemuth in 1908.[85] This technique required a twenty-four hour incubation of six different concentrations of starch substrate. Many variations of this method have been reported. In 1938, Somogyi[86] optimized the iodine and potassium iodide concentrations used in this method and introduced a procedure based upon a standardized starch substrate. Further improvements were suggested in 1960 by Somogyi[87]. Recognizing that amylase reacted faster on "soluble, linear" starch, the improved method used a carefully standardized extract of unmodified corn starch as the substrate. Buffering the pH of the starch substrate improved the reproducibility.

Searcy,[88] in a review article, was critical of some aspects of the 1939 procedure. The 1960 Somogyi[89] method corrected a number of these objections. Beeler[90] found that the simplicity of the amyloclastic method as compared to the saccharogenic approach made the Somogyi procedure the method of choice and suggested it as the reference method in the United States.

2.4 Determination of Metal Content

The ever present need for a sensitive, specific and rapid analytical method for trace metal determination has been a challenge to the analyst. Many metals are present in biological systems in concentrations of the order of 10^{-6} M or less. Existing analytical methods of reasonable cost, such as spectrophotometry, polarography,

and spectroscopy, have in the past been coupled with solvent extraction procedures and other sample pre-concentration steps in order to increase sensitivity and specificity. Extensive work in the improvement of the sensitivity of these methods has solved many of these analytical problems.

Fifty years ago Policard[91] attempted to perform microemission spectrometry on tissues and single cells which proved beyond the then available technical facilities. The method was refined and extended subsequently for the analysis of inclusions in steel and mineral splinters in rocks.[92] In the biological field, however, repeated attempts with steadily improved equipment remained futile. This is primarily due to the low metal concentrations in the minute amounts of biological matter found in individual cells, and available analysis methods with relatively high detection limits demanding large amounts of material for analysis. The recent development of analytical atomic absorption spectroscopy followed by its current acceptance and applications has helped to solve many of the problems.

One of the first applications of atomic absorption spectroscopy to chemical analysis was made by Kirchoff,[93] who demonstrated the presence of several elements in the solar atmosphere. Later, with Bunsen, he demonstrated that atomic spectra could be used in emission or absorption analysis as the basis of a new method.[94] Liveing and DeMar[95] also made many investigations of the absorption of spectral lines by atomic vapors. However, the only routine use of atomic absorption for analysis until recent years was the determination of mercury vapor contamination in laboratory atmospheres.[96]

In 1953 Walsh[97] recognized the potential advantages of the

flame absorption method over flame emission methods and devised a simple and versatile apparatus which could be used to determine the concentration of many metals in a solution. In 1955 he published an excellent paper examining the basic principles and the theoretical factors governing the relationships between atomic absorption and atomic concentration.[98] He also demonstrated the promising application of this technique in chemical analysis and pointed out many advantages over emission methods. However, recent work has indicated that the use of the newly developed hot flames, or plasma emission methods is competitive with atomic absorption. Most recently, laser excitation spectroscopy has shown great promise, although the technical problems have not as yet been fully overcome.[99]

SECTION 3

EXPERIMENTAL

A general procedure by which a labile metalloenzyme can be eluted through a Sephadex chromatography column with its metal(s) still bound has been investigated. The unique aspect of this technique was the composition of the elutant, which was a pH 7.4 Tris/HCl buffer solution spiked with 10 µg/ml concentrations of Mg^{++}, Mn^{++}, Co^{++}, Ni^{++}, Cu^{++}, Zn^{++}, and Cd^{++}. This elutant was used to shift the equilibrium of the $ML \rightleftarrows M + L$ reaction to favor the metal-ligand complex and keep the metal bound to the metalloenzyme while it was passing through the column. The "efficiency" of this method was measured by monitoring the metal content and activity of the metalloenzyme before and after elution. If the metalloenzyme was eluted with its metal ion still bound, the metal content of the enzyme would be approximately the same before and after elution. The enzyme activity was used as a measure of the recovery of the enzyme.

3.1 Reagents and Equipment

The chemicals used in this study were A.C.S. Analytical grade reagents. All solutions were diluted with distilled, deionized water. Class A glassware was used throughout the study. The solution used as the elutant was a tris(hydroxymethyl)amino methane/HCl buffer of 10^{-3} molarity. A metal spiked buffer solution was also used in which Zn^{++}, Cu^{++}, Cd^{++}, Mn^{++}, Mg^{++}, Co^{++}, and Ni^{++} ions were added in the concentration of 10 µg/ml (4×10^{-4} M to 9×10^{-5} M) to the buffer solution.

3.2 Column

3.2.1 Reagents

Bovine pancreas ribonuclease A (5 X crystallized), bovine pancrease α-chymotrypsinogen A (6 X crystallized), chicken egg albumin (99% pure), bovine serum albumin (crystallized, essentially globulin free), porcine pancrease α-amylase (2 X crystallized), and blue dextran (molecular weight approximately 2×10^6) were purchased from Sigma Chemical Company. Sephadex G-100-120 (lot No. 48C-0402), with 40-120 μ beads and a water regain of 10.0 ± 1.0 g H_2O/g dry gel, was also obtained from Sigma Chemical Company.

3.2.2 Preparation of Column

Sephadex gel beads were suspended in enough buffer so that when it was stirred, incorporated air bubbles could escape rapidly to the surface. It was then allowed to swell for one day at 70°C to assure complete swelling. A glass tube (1.2 X 60 cm), with a plug of glass wool at the bottom, was approximately half filled with buffer and the gel suspension was carefully added to bring the liquid level to the top of the tube. When a 5 to 6 cm layer of Sephadex had settled to the bottom, more gel suspension was added and the settling continued. After the column was poured, a filter paper disk was placed on top of the gel to prevent its disturbance when the sample was added. The column was then washed with buffer for one to two days. It took this long for the column to become completely equilibrated, as determined by the elution volume for a given protein becoming a constant.

3.2.3 Calibration of Column

Unless otherwise indicated, the protein was added to the top of the gel bed in 1.00 ml of buffer solution. The protein was washed into the gel with additional buffer solution and buffer solution was then added above the gel. One milliliter fractions were collected with a Gilson Fraction Collector equipped with a drop counting device. The effluent was monitored with a Glenco U.V. monitor at 280 nm with the output connected to a Linear Instruments Company strip chart recorder. A diagram of apparatus is shown in Figure 1.

A second protein sample was placed on the column as soon as there was no danger of the two components overlapping, as the elution volume was not influenced by this procedure. The void volume, V_o, was determined each day by application of blue dextran (molecular weight = 2×10^6), which was completely excluded from the gel, to the column. Elution volumes, V_o and V , were determined from the amount of elutant collected between the initial addition of protein sample and the maximum absorbance due to the eluted protein. Elution volumes were interpolated to the nearest 0.1 ml by the triangulation method. A calibration curve of V/V_o vs. log of molecular weight was constructed using the elution volumes obtained from various molecular weight proteins.

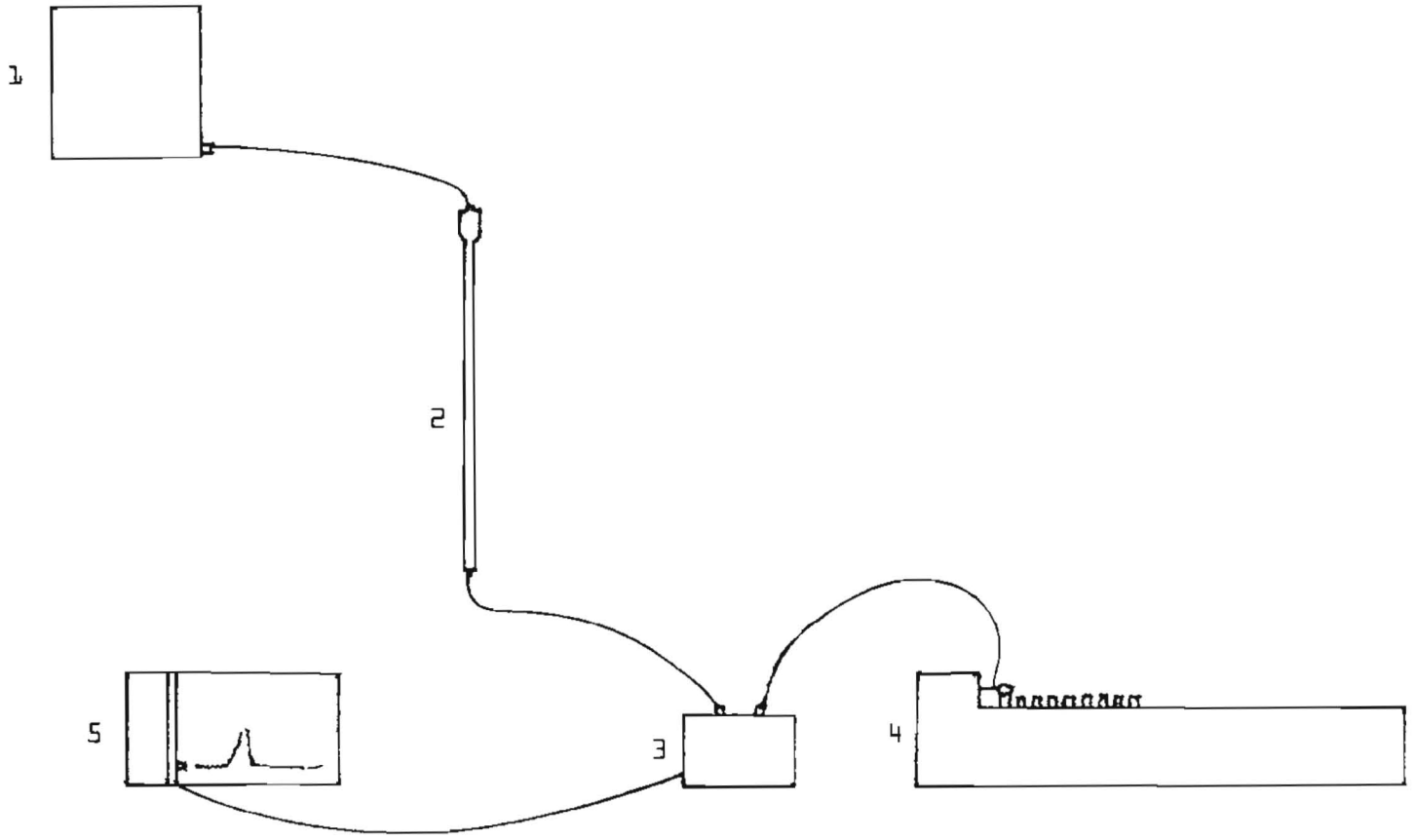
3.2.4 Elution of Enzymes

The elution of carboxypeptidase A was carried out using the buffer solution previously mentioned. The enzyme was analyzed before elution for activity and metal content. It was then applied to the column in the same manner as the proteins and allowed to pass through the column. One milliliter fractions were collected and analyzed for

Figure 1. Diagram of Apparatus.

Numbered parts include:

1. reservoir,
2. column,
3. U.V. monitor,
4. fraction collector, and
5. strip-chart recorder.


1
2
3
4
5

enzyme activity and metal content.

The elution of α-amylase was carried out in the same manner as above, except a 7.6×10^{-4} M concentration of cyanide ion was added to the buffer solution to inhibit the action of the enzyme on the column. Ribonuclease A was also passed through the column following the procedure for α-amylase elution, except that no activity measurements were made. The appropriate procedure was then repeated for each enzyme using only a pH 7.4 Tris/HCl buffer solution for comparative purposes.

3.3 Determination of Enzyme Activities

3.3.1 Reagents

Hippuryl-L-phenylalanine from Sigma Chemical Company and a Tris/HCl buffer solution (pH 7.4) were used for the determination of carboxypeptidase A activity. A Sigma Chemical Company kit no. 700 was purchased for the visual colorimetric determination of amylase. This kit contained a sterile starch solution and a 2×10^{-3} M iodine solution.

3.3.2 Carboxypeptidase A

Carboxypeptidase A catalyzes the hydrolysis of C-terminal peptide linkages of amides, peptides, and ester analogs which possess a free amino group and a L-configuration.[100] The method of activity determination used in this study was a modification of the method of Folk and Schirmer,[101] in which the increase of absorbance at 259 nm, due to the hydrolysis of hippuryl-L-phenylalanine, was measured.

$$\text{hippuryl-L-phenylalanine} \xrightarrow[H_2O]{\text{carboxy-peptidase A}} \text{hippuric acid} + \text{L-phenylalanine}$$

The activity was determined by placing 2.9 ml of substrate and 0.1 ml of buffer in each of two 10 mm quartz cells at 25°C. These were then placed in the sample and reference beams of a G.C. McPherson Spectrophotometer. The absorbance was adjusted to zero. One cell was then emptied, washed, and refilled with 2.9 ml of substrate and 0.1 ml of the enzyme. The rate of increase of absorbance at 259 nm was recorded for approximately three minutes. The values of absorbance were linear over this period of time. The activity was calculated as follows,

$$\frac{\text{units of enzyme activity}}{\text{mg of protein}} = \frac{\Delta A_{259\ nm}\ /\ \text{min}}{(0.30\ \text{mg/ml})(\text{mg enzyme/ml reaction mixture})}$$

where, 1 unit of enzyme activity = that amount of enzyme catalyzing the hydrolysis of 1 μmole of substrate per minute at 25°C. A molar absorptivity coefficient of 0.30 mg/ml for hippuryl-L-phenylalanine, determined by Miles Biochemicals Research laboratories,[102] was used in this calculation.

3.3.3 α-Amylase

Amylase hydrolyzes starch to oligosaccharides and, in turn, slowly to maltose and glucose. Measurement of the time required for the amylase to hydrolyze starch, in a carefully standardized substrate, is a quantitative expression of the enzyme's activity.

This simple procedure takes advantage of the color reaction between starch and iodine that yields an intense blue color, whereas the oligosaccharides produce a red color.

$$\underset{(\text{blue with } I_2)}{\text{starch}} \xrightarrow{\text{amylase}} \underset{(\text{red with } I_2)}{\text{oligosaccharides}} \xrightarrow{\text{amylase}} \text{maltose + glucose}$$

The color change from blue to reddish-brown is sufficiently pronounced to permit visual detection of the endpoint without use of a photometer.

The test procedure was carried out by pipetting into each of six 10 X 75 mm test tubes, 0.25 ml of iodine solution at room temperature. The cap of the bottle containing the starch substrate was swabbed with 70% ethanol to prevent contamination. The amount needed for determination, approximately 2 ml for each determination, was withdrawn using a syringe fitted with a sterile needle and then placed into a small beaker. Two milliliters of starch substrate was then pipetted from the beaker into a test tube and was placed in a 40°C water bath. After five minutes, 0.5 ml of enzyme was added to the test tube. The solution was mixed well, returned to the bath, and the timer started. After three minutes, 0.25 ml of enzyme-starch mixture was removed with a pipette, leaving the remainder of the solution in the water bath. This 0.25 ml aliquot was then added to one of the test tubes containing the iodine solution, mixed, and the color observed. If the solution was purple the hydrolysis was not complete. After another one or two minutes, a second 0.25 ml aliquot was withdrawn from the enzyme-starch mixture and the elapsed time recorded. This aliquot was added to a second tube of iodine solution and the color noted. If the purple color persisted, the incubation was continued for one or more time intervals until a reddish-brown color was observed. The total elapsed time to complete the hydrolysis of the substrate was recorded. As the reddish tint increased, aliquots were removed at more frequent intervals to avoid passing the endpoint. After some familiarity with the method, it was possible to approximate the time that would elapse between color tests by observing the amount of blue or purple in the starch-iodine mixture. Occasionally, the

color of the solution would revert to a purplish color upon standing but this did not alter the time recorded for complete hydrolysis.

The amylase activity was calculated by the following expression,

$$\text{Somogyi Units} = \left[\frac{F}{\text{time required for complete hydrolysis (min)}}\right]\left[\frac{\text{dilution factor}}{100}\right][V_t]$$

where, Somogyi Unit = that amount of amylase which will cause formation of reducing power equivalent to 1 mg of glucose in thirty minutes at 40°C when the reaction is carried out by the saccharogenic method of Somogyi,

F = 1600 at 40°C, as determined by Sigma Chemical Company,
= 1800 at 37°C, as determined by Sigma Chemical Company, and

V_t = total volume.

3.4 Determination of Metal Content in Enzymes

The metal content of the enzymes was determined by the use of a Jarrell-Ash Model 82-500 Atomic Absorption Spectrophotometer. A calibration curve was constructed using the absorbance values of 1, 5, 10, 15, and 20 µg/ml Zn standards made from quantitative dilutions of a 1000 µg/ml Zn stock solution. The Zn content of the enzyme elutant and of the enzyme fraction was determined. The difference between the Zn content of the fraction containing the enzyme and the elutant blank was assumed to be the Zn content of the enzyme. The weight of enzyme present as determined from the activity was used to calculate the mole ratio of Zn to enzyme.

SECTION 4

RESULTS

In this study the elution profile of carboxypeptidase A from a Sephadex column, using a buffer solution containing a trace quantity of metal ions as the mobile phase, has been shown to be symmetrical and reproducible. The enzyme was eluted with the zinc ion bound to it. The metal content was determined from the data in Appendix III by use of the calibration curve shown in Figure 2. Ninety-nine percent of the original activity of the enzyme was retained, as indicated in Appendix I. The metal content and activity results are summarized in Table 5. The molecular weight was determined by comparison of the data in Appendix V to the calibration curve, constructed by plotting retention volume, V_r/void volume, V_o, versus the logarithm of the molecular weight, as shown in Figure 3.

α-Amylase will hydrolyze a Sephadex column, therefore a buffer solution containing a 7.6×10^{-4} M cyanide ion concentration was used to inhibit this enzymatic action. The addition of thiosulfate ion to the fraction of elutant containing the enzyme removed the cyanide ion and restored 98% of the original activity. The metal content of the α-amylase solution was determined prior to application on the column. The metal content of the collected fraction containing the enzyme was also determined. Assuming that all of the enzyme added to the column was contained in the fraction collected, the metal content of the enzyme before and after elution can be compared. The metal content of the α-amylase and the percentage activity recovered is recorded in Appendices IV and II, respectively, and summarized in Table 6. The molecular weight of α-amylase was determined

Figure 2. Calibration Curve for Zinc.

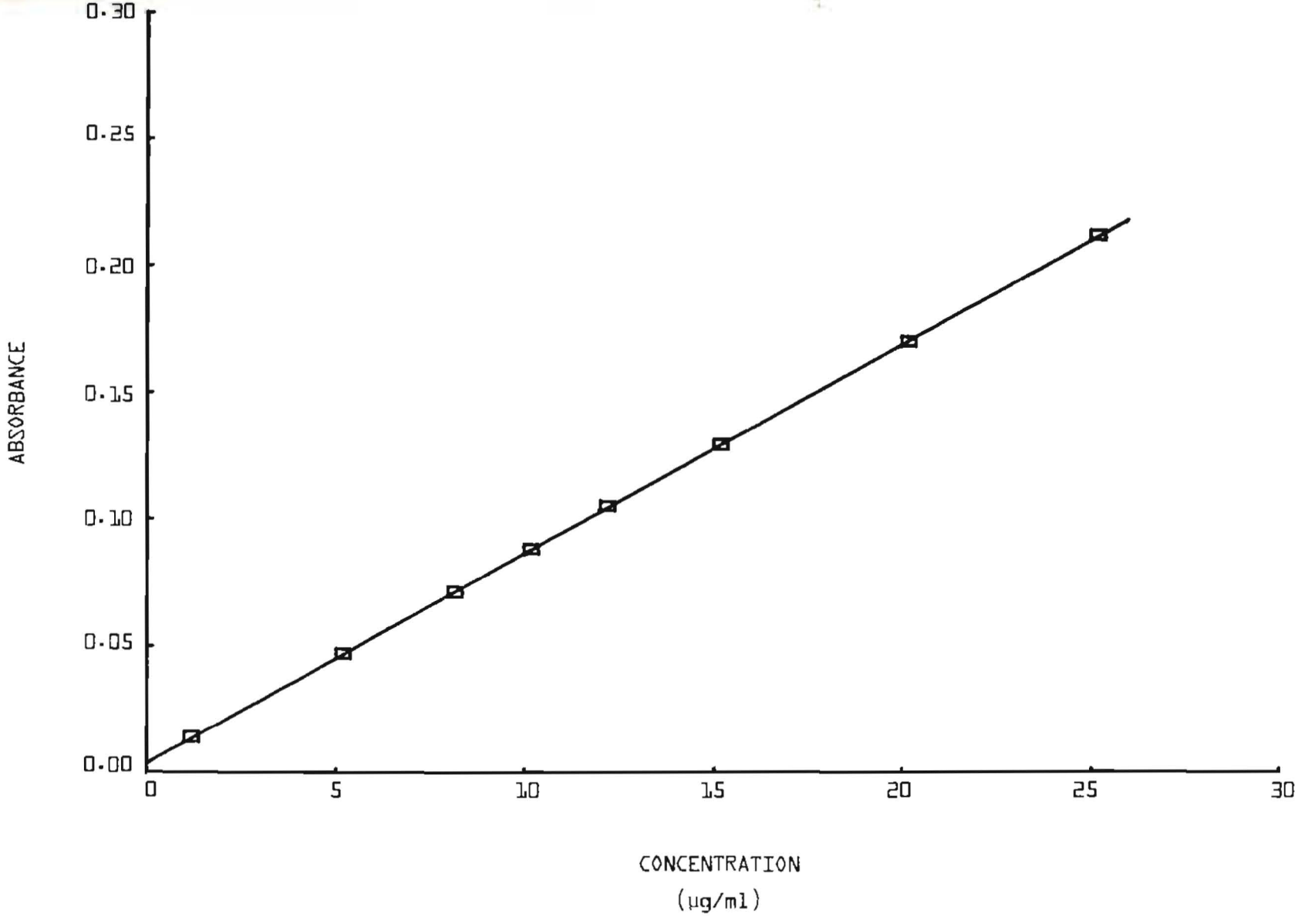
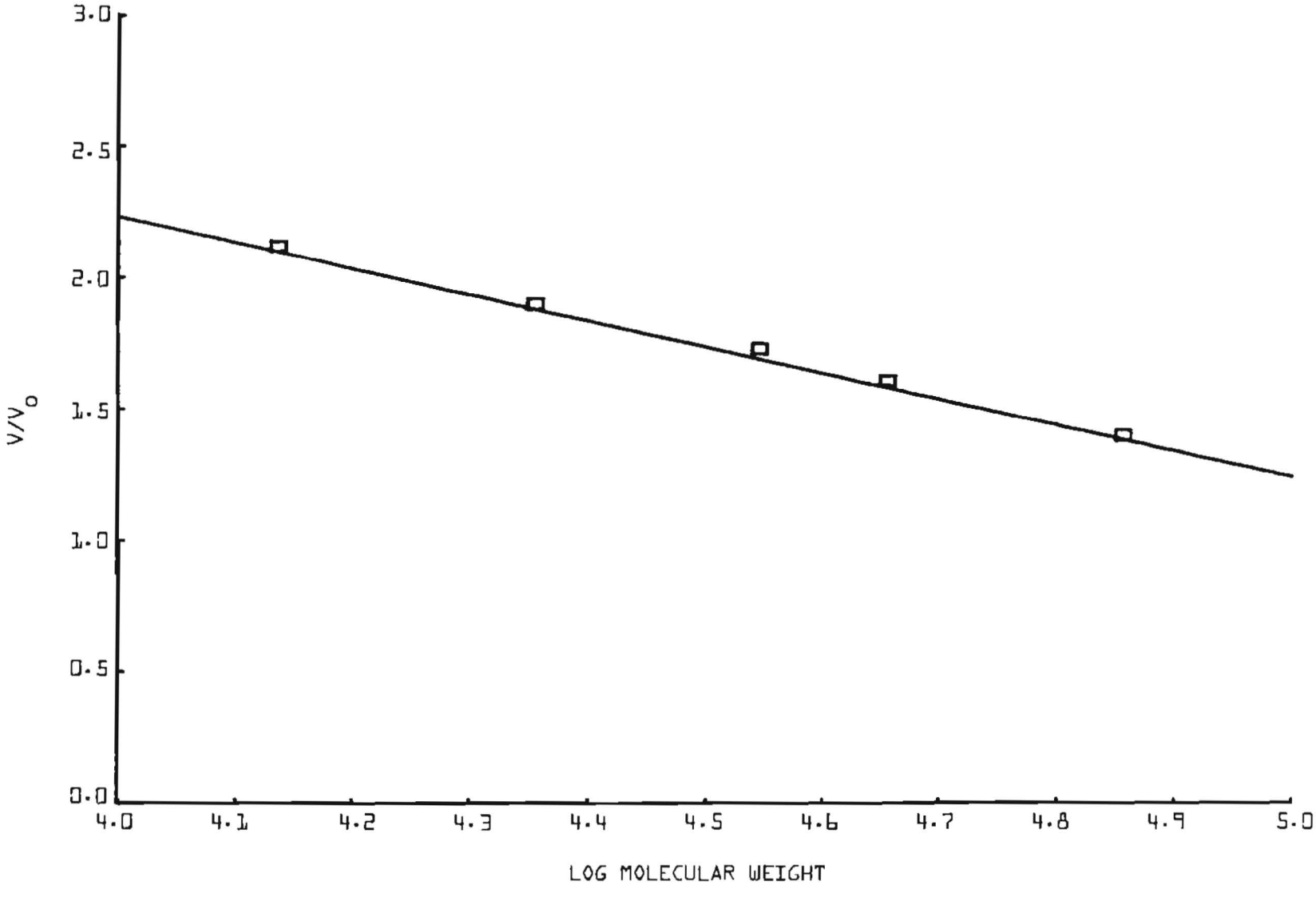

0.30
0.25
0.20
0.15
0.10
0.05
0.00
ABSORBANCE
0
5
10
15
20
25
30
CONCENTRATION
(µg/ml)

Figure 3. Calibration Curve for Molecular Weights of Proteins


3.0
2.5
2.0
1.5
1.0
0.5
0.0
V/V0
4.0
4.1
4.2
4.3
4.4
4.5
4.6
4.7
4.8
4.9
5.0
LOG MOLECULAR WEIGHT

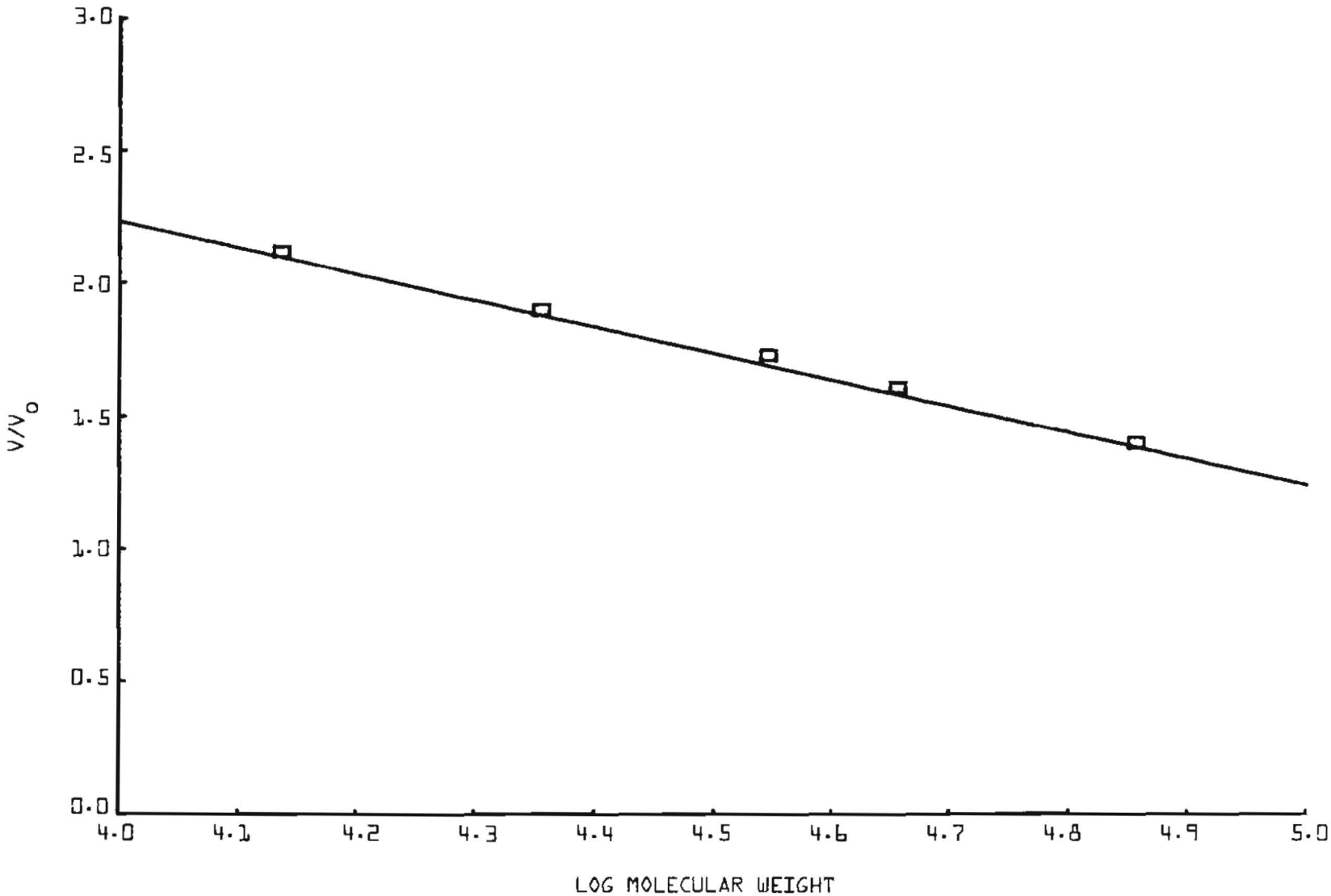

3.0
2.5
2.0
1.5
1.0
0.5
0.0
V/V0
4.0
4.1
4.2
4.3
4.4
4.5
4.6
4.7
4.8
4.9
5.0
LOG MOLECULAR WEIGHT

TABLE 5

Metal and Activity Results
for Carboxypeptidase A

ZINC CONTENT

Trial	Pre-elution (µg/ml)	Corrected Post-elution (µg/ml)	% Recovery	Conc. of Enzyme (mg/ml)	Mole Ratio Zn:Enzyme
1	9.5	9.5	100	5	1:1
2	9.4	9.8	104	5	1:1
3	9.6	9.5	99	5	1:1
4	9.5	9.5	100	5	1:1
5	9.4	9.5	101	5	1:1

ACTIVITY

Trial	Pre-elution (units/mg enzyme)	Corrected Post-elution (units/mg enzyme)	% Recovery
1	2.6	2.6	100
2	3.0	3.0	100
3	2.8	2.9	103
4	2.6	2.6	100
5	3.1	3.1	100

TABLE 6

Metal and Activity Results
for α-Amylase

ZINC CONTENT

Trial	Pre-elution (µg/ml)	Corrected Post-elution (µg/ml)	% Recovery	Conc. of Enzyme (mg/ml)	Mole Ratio Zn:Enzyme
1	6.5	6.6	101	9	1:2
2	6.3	6.5	103	9	1:2
3	6.6	6.5	98	9	1:2
4	6.5	6.6	101	9	1:2
5	6.6	6.5	98	9	1:2

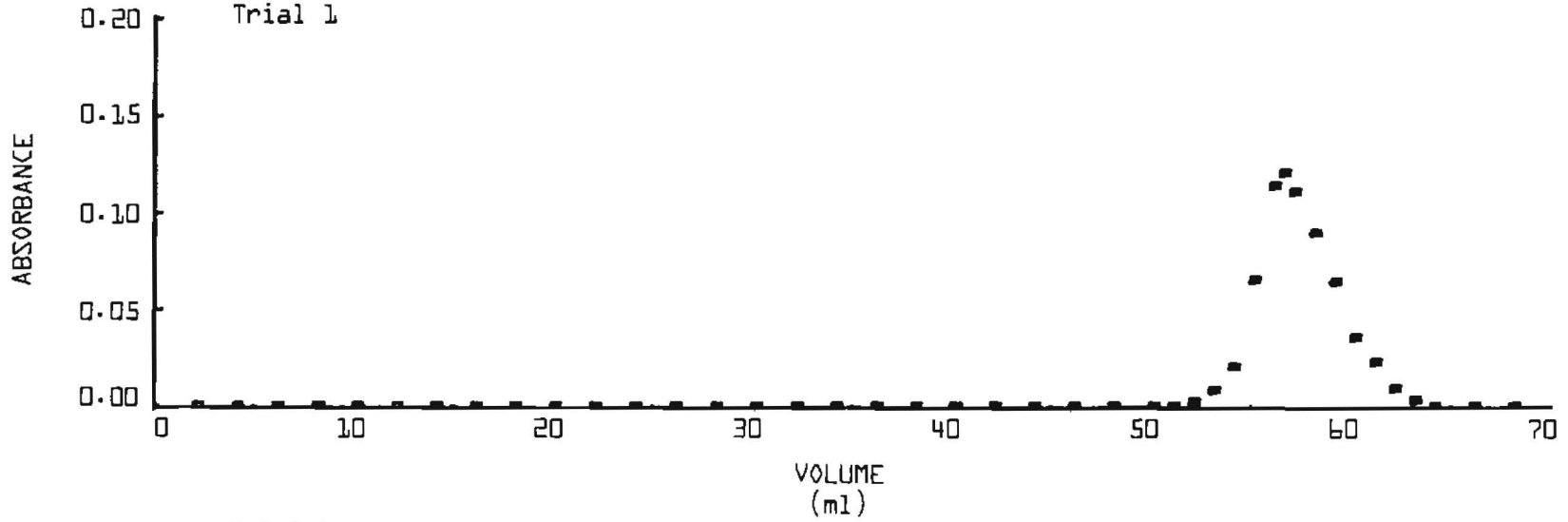
ACTIVITY

Trial	Pre-elution (Somogyi units)	Corrected Post-elution (Somogyi units)	% Recovery
1	1103	1100	100
2	1212	1168	96
3	1045	1045	100
4	1081	1078	100
5	1168	1119	96

in the same manner as for carboxypeptidase A.

The elution peaks for both enzymes, obtained by using the metal spiked buffer solution, are quite symmetrical and reproducible. Representative elution peaks for each enzyme, taken from the data in Appendices VI and VII, are shown in Figures 4 and 5. Figures 6 and 7 show elution peaks, taken from the data in Appendices VIII and IX, for both enzymes when a Tris/HCl buffer solution was used.

Figure 4. Elution Curves of Carboxypeptidase A Using a Metal Spiked Buffer Solution.


Trial 1
ABSORBANCE
0.20
0.15
0.10
0.05
0.00
0
10
20
30
40
50
60
70
VOLUME
(ml)

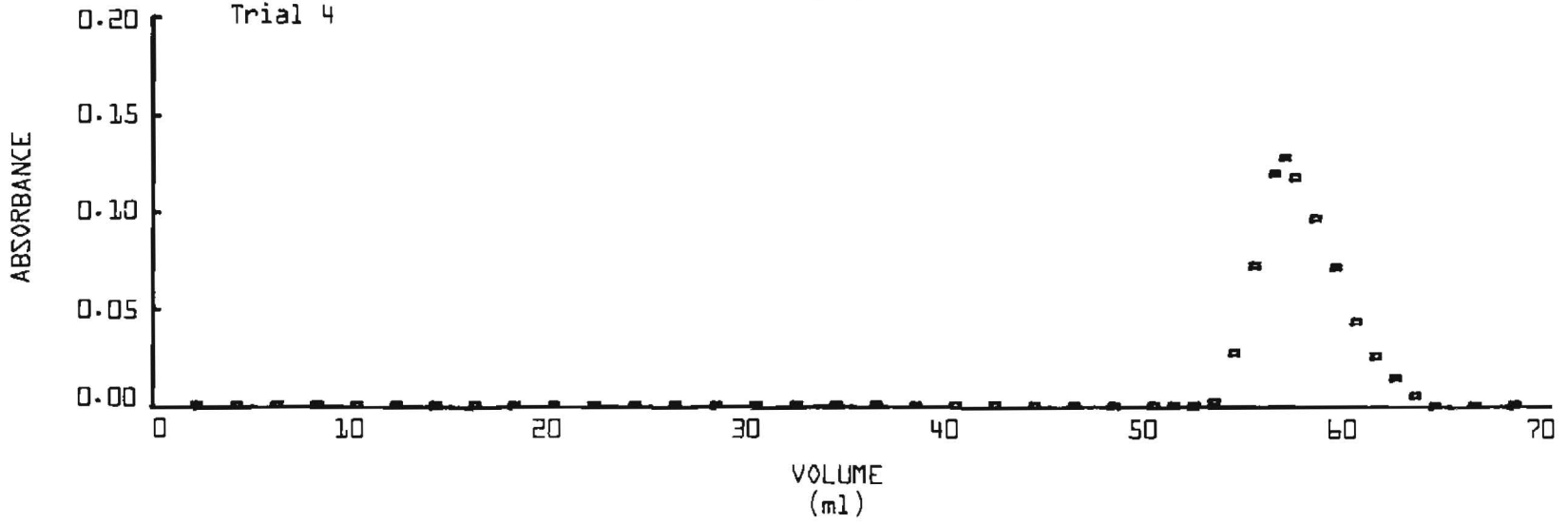

Trial 4
ABSORBANCE
0.20
0.15
0.10
0.05
0.00
0
10
20
30
40
50
60
70
VOLUME
(ml)

Figure 5. Elution Curves of α-Amylase Using a Metal Spiked Buffer Solution.

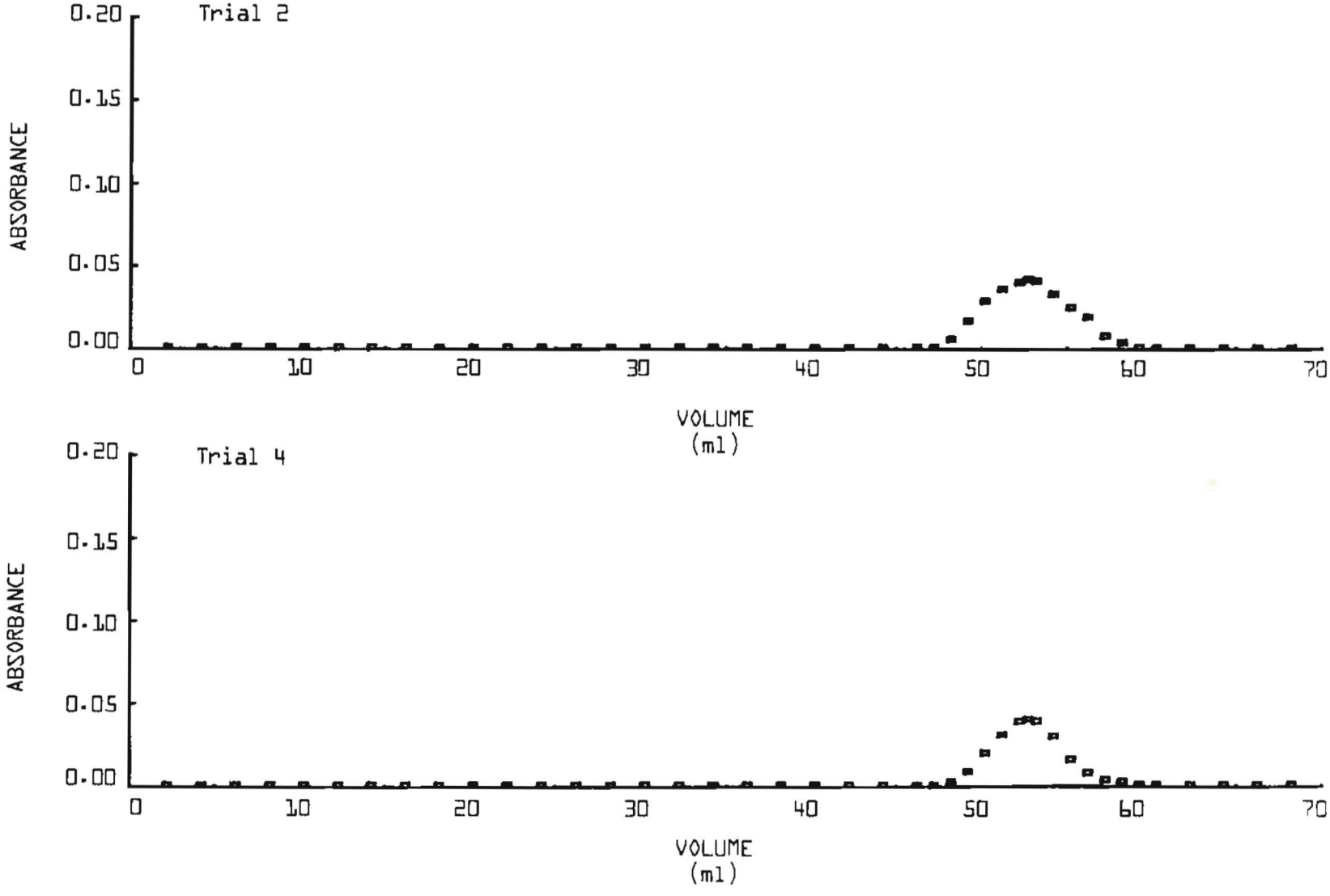
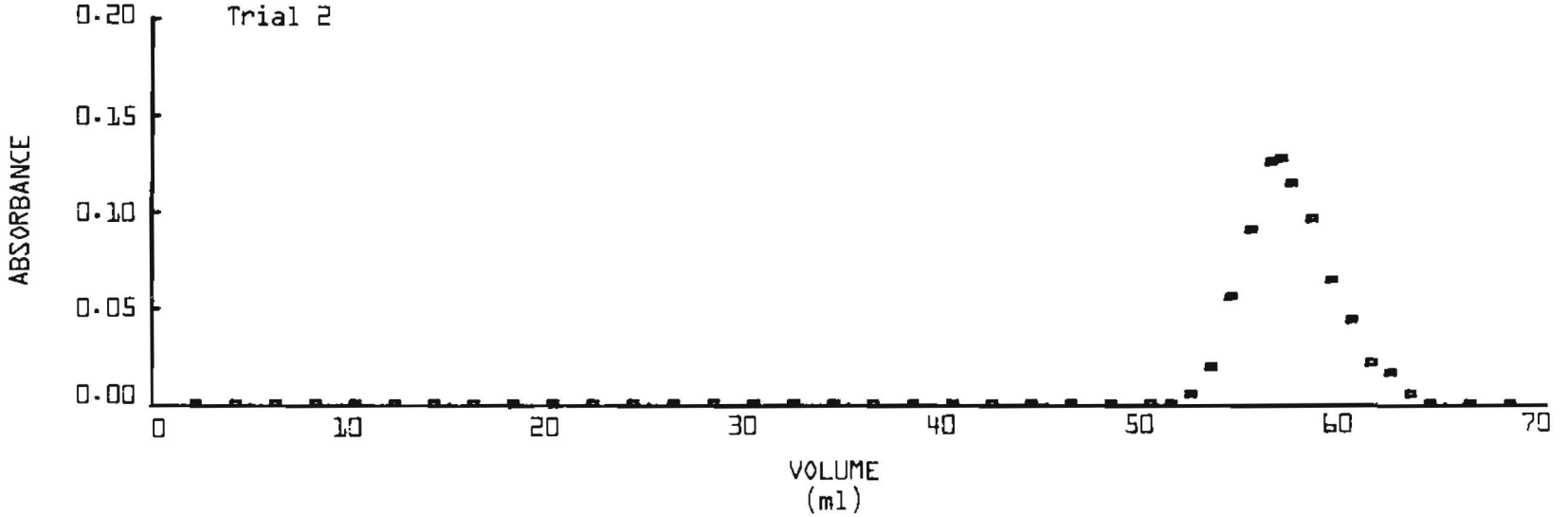

Trial 2
ABSORBANCE
0.20
0.15
0.10
0.05
0.00
0
10
20
30
40
50
60
70
VOLUME
(ml)
Trial 4
ABSORBANCE
0.20
0.15
0.10
0.05
0.00
0
10
20
30
40
50
60
70
VOLUME
(ml)

Figure 6. Elution Curves of Carboxypeptidase A Without Metal Spiked Buffer Solution.


Trial 2
ABSORBANCE
0.20
0.15
0.10
0.05
0.00
0
10
20
30
40
50
60
70
VOLUME
(ml)

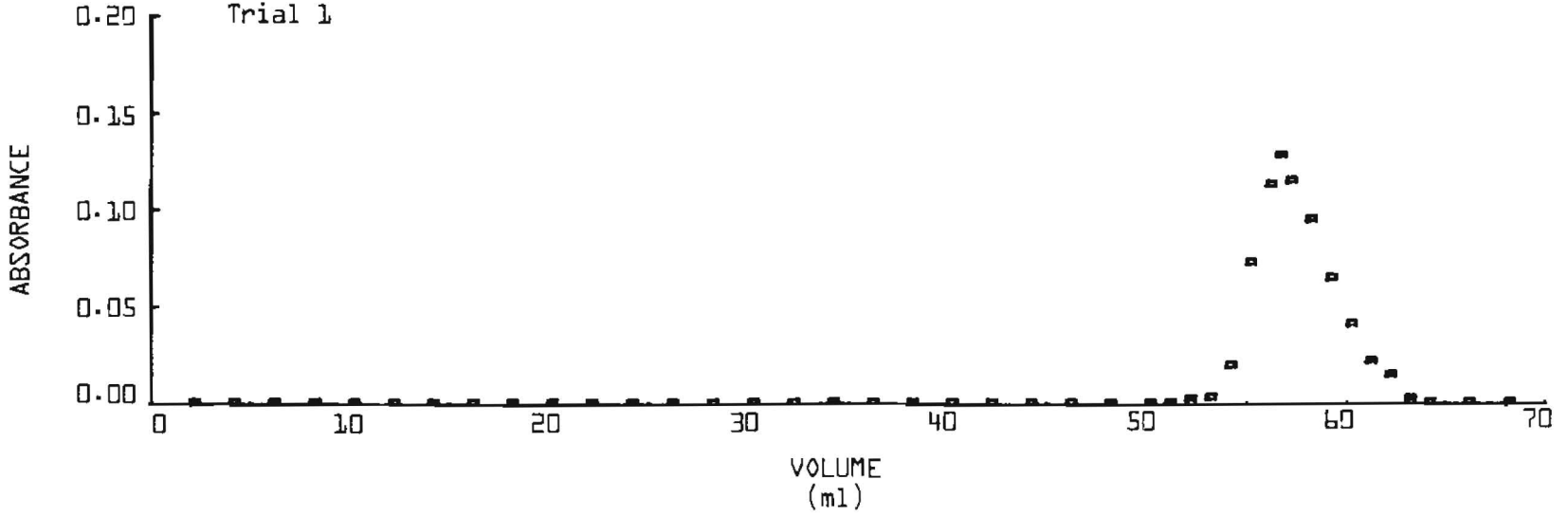
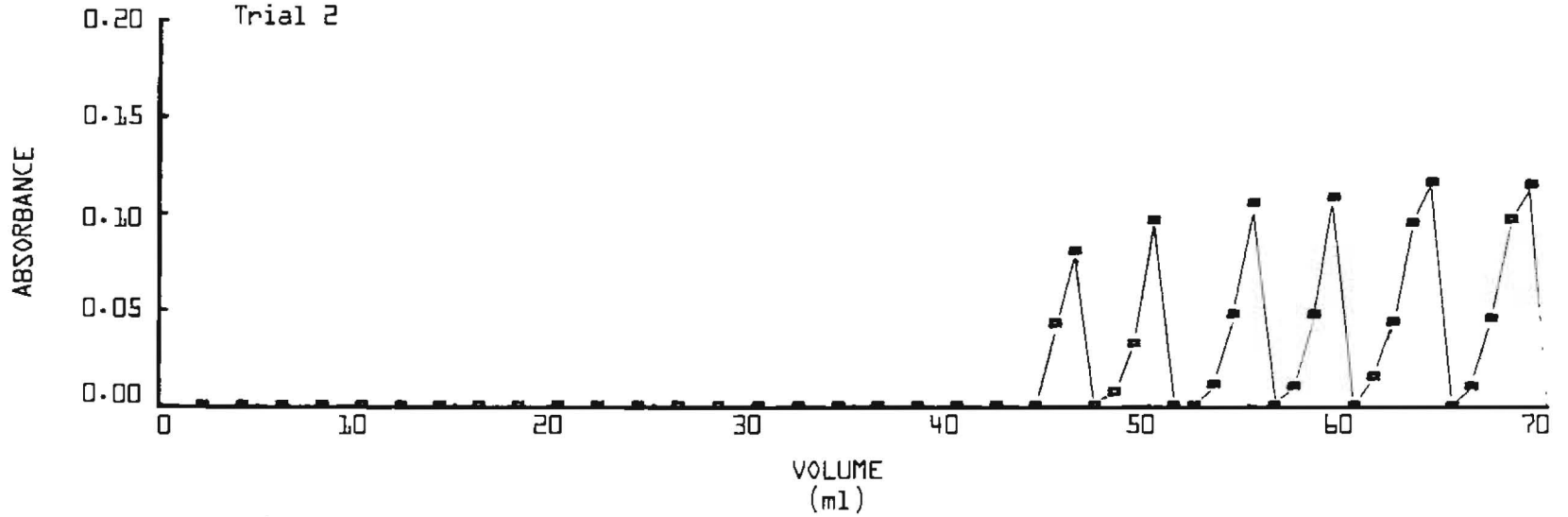
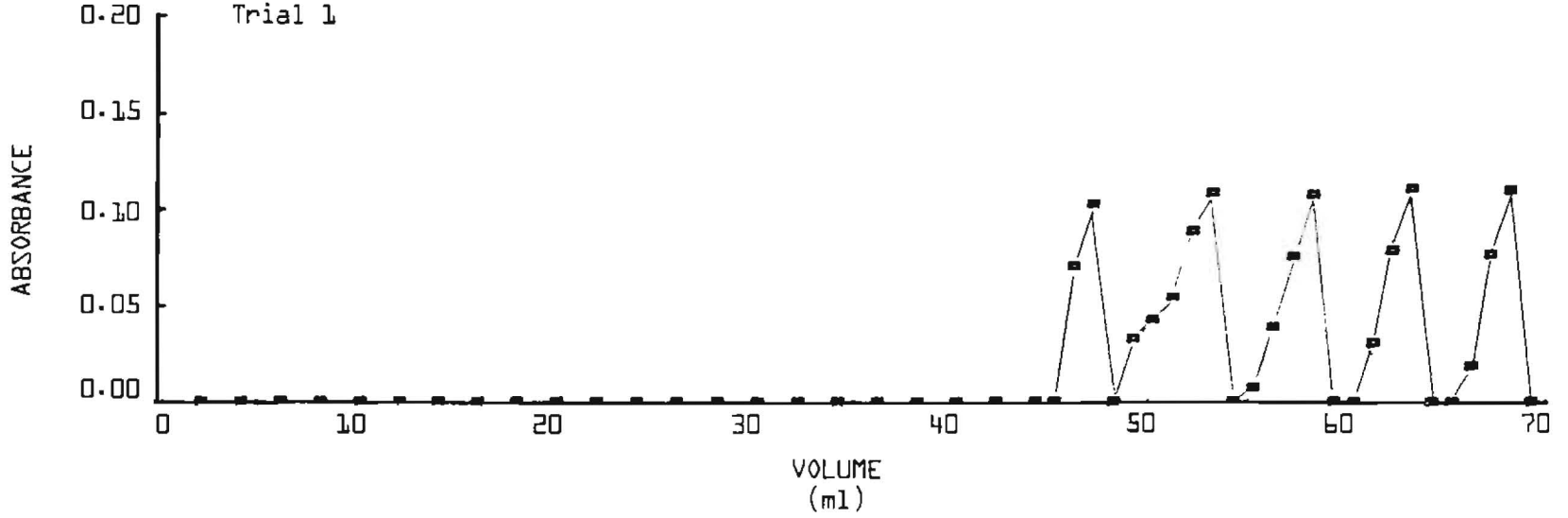

Trial 1
ABSORBANCE
0.20
0.15
0.10
0.05
0.00
0
10
20
30
40
50
60
70
VOLUME
(ml)

Figure 7. Elution Curves of α-Amylase Without Metal Spiked Buffer Solution.


Trial 2
ABSORBANCE
0.20
0.15
0.10
0.05
0.00
0
10
20
30
40
50
60
70
VOLUME
(ml)


Trial 1
ABSORBANCE
0.20
0.15
0.10
0.05
0.00
0
10
20
30
40
50
60
70
VOLUME
(ml)

SECTION 5

CONCLUSIONS

Carboxypeptidase A and α-amylase were eluted through a Sephadex chromatography column with 100% recovery of metal content and 98% or greater recovery of enzyme activity. Ribonuclease A was also eluted with no appreciable accumulation of zinc metal ion in the collected fraction containing the enzyme. This illustrates that while zinc metalloenzymes can be eluted with their zinc metal ions still bound, an enzyme with no metal associated with it does not accumulate zinc metal ions upon elution.

This proposed method takes advantage of the equilibrium reaction shown below.

$$ML \rightleftharpoons M + L$$

The metal spiked buffer solution shifts the reaction towards the metal-ligand complex during elution. Since the dissociation constants of most metal-ligand complexes are of the order 10^{-10} M, a metal-ligand complex undergoes no dissociation during elution. The stability constant of the metalloenzyme is usually the highest for its natural metal activator, therefore very little substitution of other metal ions is observed. As shown by comparison of Figure 4 to Figure 6, the elution curves of carboxypeptidase A (a stable metalloenzyme) with and without the metal ions in the elutant are the same. In contrast, by comparing Figure 5 to Figure 7 it is evident that without the metal ions present in the elutant the α-amylase (a more labile metalloenzyme) undergoes dissociation.

Another factor affecting the enzyme elution was the action of the enzyme upon the gel material. When α-amylase was applied on a

Sephadex column without an inhibitor it hydrolyzed the gel and was not eluted. Therefore, a cyanide ion was introduced into the eluant solution to inhibit this enzymatic action. After the enzyme was passed through the column, the cyanide ion was removed by the addition of thiosulfate ion. The reaction involved is postulated as follows.

$$S_2O_3^{-2} + (EM)CN^- + H_2O \longrightarrow SO_4^{-2} + SCN^- + EM + H_2\uparrow$$

The cyanide ion was chosen due to the fact that it is an inhibitor for a wide range of enzymes. Although removal of the cyanide ion is not possible for every enzyme, it was chosen so a general, rather than a specific, method for enzymatic separations could be proposed.

This proposed method of elution can hopefully be used to separate other metalloenzymes with their associated metals. This will open the door for the study of content and roles of metal ions in many metalloenzymes.

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APPENDIX I

Activity Determinations of Carboxypeptidase A

Activity Determinations of Carboxypeptidase A

TRIAL 1

Pre-elution	
Time (sec)	Absorbance
0	0.000
30	0.065
60	0.130
90	0.195
120	0.260
150	0.325
180	0.390
	$\frac{\Delta A}{min} = 0.130$

Post-elution	
Time (sec)	Absorbance
0	0.000
30	0.006
60	0.012
90	0.018
120	0.024
150	0.030
180	0.036
	$\frac{\Delta A}{min} = 0.012$

TRIAL 2

Pre-elution	
Time (sec)	Absorbance
0	0.000
30	0.075
60	0.150
90	0.225
120	0.300
150	0.375
180	0.450
	$\frac{\Delta A}{min} = 0.150$

Post-elution	
Time (sec)	Absorbance
0	0.000
30	0.008
60	0.015
90	0.023
120	0.030
150	0.038
180	0.045
	$\frac{\Delta A}{min} = 0.015$

TRIAL 3

Pre-elution	
Time (sec)	Absorbance
0	0.000
30	0.070
60	0.140
90	0.210
120	0.280
150	0.350
180	0.420

$\frac{\Delta A}{min} = 0.140$

Post-elution	
Time (sec)	Absorbance
0	0.000
30	0.006
60	0.012
90	0.018
120	0.024
150	0.030
180	0.036

$\frac{\Delta A}{min} = 0.012$

TRIAL 4

Pre-elution	
Time (sec)	Absorbance
0	0.000
30	0.065
60	0.130
90	0.195
120	0.260
150	0.325
180	0.390

$\frac{\Delta A}{min} = 0.130$

Post-elution	
Time (sec)	Absorbance
0	0.000
30	0.007
60	0.013
90	0.020
120	0.026
150	0.033
180	0.039

$\frac{\Delta A}{min} = 0.013$

TRIAL 5

Pre-elution	
Time (sec)	Absorbance
0	0.000
30	0.077
60	0.155
90	0.232
120	0.310
150	0.387
180	0.465

$\frac{\Delta A}{min} = 0.155$

Post-elution	
Time (sec)	Absorbance
0	0.000
30	0.007
60	0.014
90	0.021
120	0.028
150	0.035
180	0.042

$\frac{\Delta A}{min} = 0.014$

APPENDIX II

Activity Determinations of α-Amylase

Activity Determinations of
α-Amylase

Pre-elution			Post-elution		
Trial	mg enzyme/ml	Time for complete hydrolysis (min)	Trial	mg enzyme/ml	Time for complete hydrolysis (min)
1	9	1.45	1	0.82	16.00
2	9	1.32	2	0.82	15.07
3	9	1.53	3	0.82	16.93
4	9	1.48	4	0.82	16.38
5	9	1.37	5	0.82	15.73

APPENDIX III

Determination of Metal Content for Carboxypeptidase A

Determination of Metal Content for Carboxypeptidase A

Pre-elution

Trial	Absorbance	Blank Absorbance	Corrected Absorbance	[Zn] due to Enzyme (µg/ml)
1	0.168	0.086	0.082	9.5
2	0.167	0.086	0.081	9.4
3	0.169	0.086	0.083	9.6
4	0.168	0.086	0.082	9.5
5	0.167	0.086	0.081	9.4

Avg. = 9.5

Post-elution

Trial	Absorbance	Blank Absorbance	Corrected Absorbance	[Zn] due to Enzyme in Diluted Fraction (µg/ml)	Dilution Factor	Corrected [Zn] (µg/ml)
1	0.096	0.086	0.010	0.86	11	9.5
2	0.098	0.086	0.012	0.98	10	9.8
3	0.095	0.086	0.009	0.79	12	9.5
4	0.097	0.086	0.011	0.95	10	9.5
5	0.096	0.086	0.010	0.86	11	9.5

Avg. = 9.5

Zinc Standards

[Zn] (µg/ml)	Absorbance
1	0.012
5	0.045
8	0.069
10	0.086
12	0.103
15	0.127
20	0.168
25	0.210

APPENDIX IV

Determination of Metal Content for α-Amylase

Determination of Metal Content for α-Amylase

Pre-elution

Trial	Absorbance	Blank Absorbance	Corrected Absorbance	[Zn] due to Enzyme (μg/ml)
1	0.142	0.086	0.056	6.5
2	0.141	0.086	0.055	6.3
3	0.143	0.086	0.057	6.6
4	0.142	0.086	0.056	6.5
5	0.143	0.086	0.057	6.6

Avg. = 6.5

Post-elution

Trial	Absorbance	Blank Absorbance	Corrected Absorbance	[Zn] due to Enzyme in Diluted Fraction (μg/ml)	Dilution Factor	Corrected [Zn] (μg/ml)
1	0.093	0.086	0.007	0.60	11	6.6
2	0.092	0.086	0.006	0.59	11	6.5
3	0.092	0.086	0.006	0.59	11	6.5
4	0.093	0.086	0.007	0.60	11	6.6
5	0.092	0.086	0.006	0.59	11	6.5

Avg. = 6.5

Zinc Standards

[Zn] (μg/ml)	Absorbance
1	0.012
5	0.045
8	0.069
10	0.086
12	0.103
15	0.127
20	0.168
25	0.210

APPENDIX V

Determination of Molecular Weights of Proteins

Determination of Molecular Weights
of Proteins

Protein	V (ml)	V_o (ml)	MW	V/V_o	log MW
Bovine serum albumin	45.5	33	70,000	1.38	4.85
Chicken egg albumin	52.5	33	45,000	1.59	4.65
α-Chymotrypsinogen	62.0	33	22,500	1.88	4.35
Carboxypeptidase A	56.5	33	34,300	1.71	4.54
α-Amylase	52.5	33	45,000	1.59	4.65
Ribonuclease A	69.3	33	13,600	2.10	4.13

APPENDIX VI

Elution Curves of Carboxypeptidase A Using a Metal Spiked Buffer Solution

Elution Curves of
Carboxypeptidase A
Using a Metal Spiked Buffer Solution

Volume (ml)	Absorbance				
	Trial 1	Trial 2	Trial 3	Trial 4	Trial 5
0.0	0.000	0.000	0.000	0.000	0.000
25.0	0.000	0.000	0.000	0.000	0.000
50.0	0.000	0.000	0.000	0.000	0.000
51.0	0.000	0.000	0.000	0.000	0.000
52.0	0.002	0.000	0.001	0.000	0.005
53.0	0.008	0.003	0.003	0.002	0.010
54.0	0.020	0.025	0.019	0.027	0.035
55.0	0.065	0.070	0.060	0.072	0.080
56.0	0.113	0.117	0.114	0.119	0.112
56.5	0.120	0.125	0.120	0.127	0.117
57.0	0.110	0.115	0.114	0.117	0.111
58.0	0.089	0.094	0.088	0.096	0.087
59.0	0.064	0.069	0.061	0.071	0.064
60.0	0.035	0.040	0.042	0.043	0.035
61.0	0.022	0.027	0.021	0.025	0.022
62.0	0.009	0.014	0.010	0.014	0.008
63.0	0.003	0.008	0.005	0.005	0.002
64.0	0.000	0.000	0.002	0.000	0.000
65.0	0.000	0.000	0.000	0.000	0.000
70.0	0.000	0.000	0.000	0.000	0.000

APPENDIX VII

Elution Curves of α-Amylase Using a Metal Spiked Buffer Solution

Elution Curves of
α-Amylase
Using a Metal Spiked Buffer Solution

Volume (ml)	Absorbance				
	Trial 1	Trial 2	Trial 3	Trial 4	Trial 5
0.0	0.000	0.000	0.000	0.000	0.000
15.0	0.000	0.000	0.000	0.000	0.000
30.0	0.000	0.000	0.000	0.000	0.000
45.0	0.000	0.000	0.000	0.000	0.000
46.0	0.000	0.000	0.000	0.000	0.000
47.0	0.000	0.000	0.000	0.000	0.000
48.0	0.009	0.005	0.007	0.002	0.005
49.0	0.018	0.016	0.019	0.008	0.010
50.0	0.027	0.028	0.027	0.019	0.019
51.0	0.034	0.035	0.031	0.030	0.032
52.0	0.038	0.039	0.036	0.038	0.038
52.5	0.040	0.041	0.038	0.039	0.040
53.0	0.039	0.040	0.037	0.038	0.041
54.0	0.033	0.032	0.030	0.029	0.037
55.0	0.026	0.024	0.023	0.015	0.026
56.0	0.019	0.018	0.012	0.007	0.014
57.0	0.008	0.007	0.004	0.003	0.003
58.0	0.002	0.003	0.002	0.002	0.001
59.0	0.000	0.000	0.000	0.000	0.000
60.0	0.000	0.000	0.000	0.000	0.000
70.0	0.000	0.000	0.000	0.000	0.000

APPENDIX VIII

Elution Curves of Carboxypeptidase A
Without Metal Spiked Buffer Solution

Elution Curves of
Carboxypeptidase A
Without Metal Spiked Buffer Solution

Volume (ml)	Absorbance	
	Trial 1	Trial 2
0.0	0.000	0.000
25.0	0.000	0.000
50.0	0.000	0.000
51.0	0.000	0.000
52.0	0.002	0.005
53.0	0.003	0.019
54.0	0.019	0.055
55.0	0.072	0.090
56.0	0.112	0.125
56.5	0.127	0.127
57.0	0.114	0.114
58.0	0.094	0.096
59.0	0.064	0.064
60.0	0.040	0.043
61.0	0.021	0.021
62.0	0.014	0.016
63.0	0.002	0.005
64.0	0.000	0.000
65.0	0.000	0.000
70.0	0.000	0.000

APPENDIX IX

Elution Curves of α-Amylase Without Metal Spiked Buffer Solution

Elution Curves of
α-Amylase
Without Metal Spiked Buffer Solution

Volume (ml)	Absorbance	
	Trial 1	Trial 2
0.0	0.000	0.000
15.0	0.000	0.000
30.0	0.000	0.000
45.0	0.000	0.042
46.0	0.070	0.080
47.0	0.102	0.000
48.0	0.000	0.007
49.0	0.032	0.032
50.0	0.042	0.096
51.0	0.054	0.000
52.0	0.088	0.000
53.0	0.108	0.011
54.0	0.000	0.047
55.0	0.007	0.105
56.0	0.038	0.000
57.0	0.075	0.010
58.0	0.107	0.047
59.0	0.000	0.108
60.0	0.000	0.000
61.0	0.030	0.015
62.0	0.078	0.043
63.0	0.110	0.095
64.0	0.000	0.116
65.0	0.000	0.000
66.0	0.018	0.010
67.0	0.076	0.045
68.0	0.109	0.097
69.0	0.000	0.115