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PEPTIDASE ACTIVITIES IN SACCHAROMYCES CEREVISIAE:
PURIFICATION AND CHARACTERIZATION OF A DIPEPTIDASE

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PEPTIDASE ACTIVITIES IN *SACCHAROMYCES CEREVISIAE*: PURIFICATION AND CHARACTERIZATION OF A DIPEPTIDASE

by

BRUCE ROSE

A dissertation submitted to the Graduate Faculty in Chemistry in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

1979

This manuscript has been read and accepted for the Graduate Faculty in Chemistry in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

7 December 1979
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Abstract

PEPTIDASE ACTIVITIES IN *SACCHAROMYCES CEREVISIAE*: PURIFICATION AND CHARACTERIZATION OF A DIPEPTIDASE

by

Bruce Rose

Adviser: Professor Fred Naider

At least four distinct aminopeptidase activities and a single dipeptidase activity were found in cell-extracts of a leucine, lysine auxotroph of *Saccharomyces cerevisiae*. The assay for peptidase activity involved polyacrylamide gel electrophoresis followed by an enzyme-coupled activity staining procedure. The aminopeptidases have largely overlapping specificities but can be distinguished from one another by their electrophoretic mobilities, and activities toward different peptide substrates. Substrates tested included both free and blocked di- and tripeptides and amino acid derivatives.

The dipeptidase was obtained in an electrophoretically homogeneous state and its substrate specificity characterized. Preferential hydrolysis of Met-X dipeptides was observed in the absence of added modifiers. Dipeptidase activity was extremely sensitive to changes in pH and the addition of divalent metal ions. Presumably, these observations were a direct result of conformational purterbations in the active site of the enzyme. The dipeptidase is a

metalloenzyme having optimal activity at pH 8.0 and an isoelectric pt. of 6.5. Molecular weight of the dipeptidase by gel filtration was estimated to be 141,000 daltons and it is composed of four equal subunits. This enzyme is inhibited by PCMB, EDTA, o-phenanthroline, amino acids, iodoacetic acid, and dithiothreitol. Dipeptidase activity toward most of the dipeptides studied followed Michaelis-Menten kinetics. Substrates not adhering to this kinetic scheme were better described by allosteric considerations using the Hill equation.

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This thesis is dedicated to the two most important people in my life. First, my wife Marcie without whom I would not have survived my graduate years and to my mother Rose who made sure that my education came before all other things.

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ABBREVIATIONS

The abbreviations used throughout are as follows: Ac, acetyl; OMe, methyl ester; Cbz or Z carbobenzoxy (benzyloxycarbonyl); Tris, tris (hydroxymethyl) aminomethane; NH_2, amide; alanine, Ala; leucine, Leu; proline, Pro; methionine, Met; glycine, Gly; serine, Ser; glutamic acid, Glu; lysine, Lys; histidine, His; phenylalanine, Phe; threonine, Thr; EDTA, ethylendiamine-tetracetic acid (supplied as its sodium salt); DFP, diisopropylfluorophosphate; PMSF, phenylmethanesulfonyl fluoride; PCMB, p-mercuribenzoate; SDS, sodium dodecyl (lauryl) sulfate; BSA, bovine serum albumin, sarcosine, Sar.

I. INTRODUCTION

A. Peptide Hydrolases

Enzymes are biologically active proteins responsible for catalyzing the myriad of chemical reactions which occur in all living organisms. The International Union of Biochemistry has classified enzymes into six main classes depending upon the type of reaction catalyzed. One class, the Hydrolases (EC 3), participate in the hydrolysis of various types of chemical bonds including ester and peptide bonds. Enzymes involved in the hydrolysis of peptide bonds (peptide hydrolases, EC 3.4) are commonly called proteases. The various modes of protease action has resulted in several sub-classifications. Proteases may first be divided into endopeptidases (proteinases, EC 3.4.21-3.4.24 and 3.4.99) and exopeptidases (peptidases, EC 3.4.11-3.4.15). It should be noted that in much of the Biochemical Literature the term protease is used to mean proteinase rather than its original broader meaning as used here. Also, many proteases and some peptidases possess esterolytic activity.

Endopeptidases act upon internal peptide bonds usually associated with proteins but may also cleave smaller peptides. These proteinases are further sub-classified on the basis of their catalytic action. Throughout this text the term proteinase will be used to describe this entire class of enzymes.

Exopeptidases are responsible for the cleavage of terminal amide linkages from both large and small peptides,

yielding the free terminal α-amino acid and in most cases a shortened peptide. Exopeptidases are further sub-grouped into five divisions. The amino peptidases (α-aminoacylpeptide hydrolase, EC 3.4.11) cleave amino acids from the free amine terminus whereas carboxypeptidases (peptidylamino-acid or acylamino-acid hydrolases, EC 3.4.12) cleave the free carboxyl terminal amino acid. In an analogous manner there are two types of peptidases which cleave dipeptides from either the amino (dipeptidylpeptide hydrolase, EC 3.4.14) or carboxy (peptidyldipeptide hydrolase EC 3.4.15) terminus. Dipeptidases (dipeptide hydrolase EC 3.4.13) are more limited in their action and act only upon dipeptides yielding free α-amino acids. In general, the dipeptide must not be blocked at either terminus, should be composed of L amino acids, and possess an unsubstituted peptide bond. [1] The term peptidase will be used to describe this class of enzymes.

B. Functions of Peptide Hydrolases

There is an extremely large literature dealing with the proposed functions of proteinases and peptidases and several reviews have appeared.[2-7] In this section some of these findings will be discussed and an essential biochemical role for these hydrolases illustrated. It should be kept in mind that some of the products obtained from proteinase action will necessarily be susceptible to peptidase action. Therefore, the functions of proteinases in many instances may be coupled with those of peptidases and much of what is discussed with regard to the former infers a probable role for the latter.

It is well established that various microorganisms[8-14] contain a variety of intracellular peptide hydrolases. One probable role for these enzymes is nutrition. A number of researchers have established that bacteria, yeast and other microorganisms are able to utilize exogenous peptides for cell growth.[11, 15-17] These workers employed amino acid auxotrophs lacking extracellular peptidase activity and supplied the required amino acids as peptides. A positive growth response was indicative of intracellular peptide hydrolysis followed by utilization of amino acid(s) released. Using these methods Naider et al[11], Becker et al[18] and Marder et al[19] have demonstrated a nutritional role for peptidases in several amino acid auxotrophs of Saccharomyces cerevisiae. Earlier work on yeast by Matile and coworkers[20, 21] also showed that yeast could grow on peptides. More direct evidence is obtained from the report of Kessel and Lubin[22] who studied a glycine requiring auxotroph of Escherichia coli which had lost its peptidase activity toward diglycine. This organism failed to grow on diglycine whereas prior to mutational loss of peptidase activity diglycine did serve as a growth source.

Peptides may serve as superior nutrients when compared with supplying the required amino acid itself.[7, 23] One possible explanation is that competition between several amino acids for transport into the cell may cause a decrease in the uptake of the required amino acid. However it has been clearly demonstrated that in Escherichia coli[24]

and in Saccharomyces cerevisiae[19] amino acids and peptides possess separate transport systems and would therefore not compete with one another for transport into the cell. In addition there may exist a cellular mechanism which prevents the utilization of certain amino acids. Such a case was reported by Cascieri and Malletti[25] using a histidine auxotroph of Pseudomonas putida. They found that cell yields were 5-10 times greater when grown on histidine containing peptides than when histidine itself was used. It was found that an L-histidine ammonia-lyase was present which attacked L-histidine but did not act on histidine containing peptides.

There are several factors which suggest that peptide hydrolases play a much larger role than that of nutrition alone. In E. coli it has been found that peptidases are constitutive enzymes[26], they are not inducible but may be partially latent[26] and they are present in excess of that required for nutrition.[14] In addition, it is known that peptidases having overlapping specificities are present within the same organism and this suggests a broader range of cellular functions.[10, 14] Collett[27] found that in adult Calliphora there is a storage pool of small peptides which began as proteins, and these peptides are utilized as a source of amino acids via peptidase action. Similarily it was demonstrated in barley grain[28] that there is a 10% reserve of insoluble proteins. During germination these reserve proteins are hydrolyzed to soluble peptides and then further cleaved to yield amino acids. These findings imply that peptide hydrolases are

necessary to replenish the metabolic amino acid pool.

Protein turnover is a term given to a general mechanism describing the intracellular hydrolysis of proteins to free amino acids and the re-incorporation of these amino acids into newly synthesized proteins. The existence of this phenomenon as a regular part of the cell cycle involving proteinases and peptidases is well documented and several reviews have appeared regarding it in microorganisms[4, 6, 29] and in mammalian cells[2, 3]. In addition to the regularly occurring process of protein turnover there are several related processes involving this phenomena. For example, in the rat, proteins having short half-lives tend to be those involved in metabolic pathways and therefore afford the cell a means by which to adapt readily to new environmental conditions.[3] Wieanders et al[30] found a decrease in protein turnover with increasing age of the rat using rat livers. However, the exact reasons for this were not established although possible causes include a decrease in specific proteolytic activity and the reduced digestibility of proteins.

Closely related to the above is the degradation of non-functional proteins. Non-functional proteins may arise for the following reasons: premature termination of polypeptide chains, spontaneous denaturation, and mutations resulting in translational errors in proteins or the incorporation of amino-acid analogues.[2,4,23] The accumulation of such proteins could prove detrimental to the cell and their removal is essential. In a similar manner certain peptides

may also inhibit growth if accumulated and peptidases may be necessary to protect against this occurrence.[7]

The discussion up to now has dealt with the total degradation of proteins and peptides to their constitutive amino acids. Peptide hydrolases are also involved in limited proteolysis and functions which fall into this class usually involve the action of a specific peptide hydrolase. It is well established that many enzymes are initially present as inactive precursors (zymogen form) which undergo limited proteolytic action to yield active enzymes.[31, 32] For example, in yeast, proteinase B is responsible for the conversion of prechitin synthase to its active form, chitin synthase.[33] Proteinases A and B play a role in the inactivation of several yeast enzymes involved in either the first step of a metabolic pathway or steps initiating new pathways or cycles.[4, 34] These observations suggest metabolic regulatory functions for these proteinases in yeast.

Several enzymes from bacteria and yeast have been shown to undergo specific modification via proteinase action which resulted in the partial loss of the enzymes specificity.[3,4,6,34] Rouget and Chapeville[35] demonstrated that proteolytic modification of leucyl-tRNA synthetase in _E. coli_ led to loss of the enzymes ability to transfer activated leucine to RNA but this modified form was still able to catalyze the leucine-dependent pyrophosphate-ATP exchange.[36] Similarly yeast aldehyde dehydrogenase is degraded into two homogeneous forms differing in specific activity.[4] The

biological significance of these modifications is still unclear.

Polypeptide synthesis in prokaryotes[37] and chloroplasts[38] of eukaryotic organisms is initiated by N-formylmethionine. However, examination of finished polypeptides in E. coli showed that they did not possess formate residues[37] and only approximately 40% of these proteins still contained N-terminal methionine.[39] It is believed that the formyl group is removed by a highly specific deformylase followed by cleavage of the N-terminal methionyl residue by an aminopeptidase. To date this aminopeptidase has not been isolated.

C. Peptide Hydrolases of Saccharomyces cerevisiae

1. Proteinases A and B

S. cerevisiae contains two proteinases, A and B, both of which are located in the vacuole.[40] Both proteinases have specific protein inhibitors which are located in the cytoplasm.[40] These inhibitors may serve as the cell's policing force to prevent any proteinase activity from leaving the vacuole. Proteinase A has a molecular weight of about 60,000 and an isoelectric point of about 3.7.[41] It is considered an acid proteinase being inactive at basic pH values. It does not act on synthetic peptide or ester substrates [41,42] or on Azocoll.[42] It is not inhibited by metal chelating agents, sulfhydryl agents or the typical "serine-proteinase" inhibiting reagents.[41,42,43] However, it has been established that pepstaten can inhibit proteinase A activity.[44]

Several workers have provided evidence that proteinase A is involved in the activation of both proteinase B and carboxypeptidase Y by inactiviating their specific inhibitors.[44,45,46]

Proteinase B is quite unlike proteinase A. It has a molecular weight of about 80,000 and an isoelectric point of about 5.0.[47] Proteinase B is inhibited by PCMB,* DFP, PMSF and chymostaten but not by EDTA.[42,43] It is active toward proteins, Azocoll and esters but inactive toward Cbz-dipeptides.[42,43,49] Matern et al[46] found that proteinase B is involved in the inactivation of the inhibitor of carboxypeptidase Y and Saheki et al[44] found a similar behavior regarding the inhibitor of proteinase A. Proteinases A and B have been studied in detail by many researchers but covering this material in detail is beyond the scope of this work and the reader is referred to several of those articles.[40-51]

2. Early Peptidase Literature

It has been demonstrated by several researchers[9, 21, 52-58] that yeast contains a variety of peptide hydrolases. The most extensively studied yeast genus is Saccharomyces cerevisiae and this section will be devoted to the intracellular peptide hydrolases found in this strain. Most of the pioneering work in this field was carried out by Grassman and co-workers[56,59-62] during the late 1920's and early 1930's. In 1928 Grassman and Dyckerhoff[61] resolved yeast autolysate into dipeptide and polypeptide active fractions. The dipeptidase fraction was found to hydrolyze only dipeptides and had no activity toward acylated peptides, polypeptides,

* See page x for all abbreviations

dipeptide amides or amino acid amides. Thus, substrates for the dipeptidase must possess both free amino and carboxy terminii. However, in 1938 Schneider[63] found that yeast dipeptidase was able to cleave l-alanylaminomalonyl diamide, which does not have a free carboxyl group. These conflicting results were both obtained using impure dipeptidase fractions, although presumably lacking in polypeptidase activity, and therefore the discrepancy was not resolved.

The polypeptidase fraction was active toward polypeptides, polypeptide amides and esters, dipeptide and amino acid amides, and dipeptide esters. It failed to cleave free dipeptides and acylated peptides but acquired dipeptide splitting activity upon the addition of chloride ions. It is presumed that this indicates the presence of more than one polypeptidase. The failure of this enzyme(s) to cleave acylated peptides along with its activity profile suggests that it is an aminopeptidase(s).

3. Aminopeptidases

The first homogenous yeast aminopeptidase was obtained by Johnson.[57] This enzyme was shown to require Zn^{+2} and Cl^{-1} for maximal activity toward dl alanyldiglycine and dl-leucyldiglycine although the addition of Zn^{+2} alone to the latter substrate resulted in a decrease in activity. pH optimums for these two substrates were 7.0 and 7.9 respectively. The Zn^{+2} could be partially replaced by Co^{+2} but not by Mn^{+2} or Mg^{+2}. The aminopeptidase was able to hydrolyze dl-leucylglycine at one-eighth the rate of cleavage for dl-leucyldiglycine whereas Grassman[56] found a 500

fold difference for these two peptides. Johnson[57] stated and many researchers believed[64-66] that Grassmans and Johnsons preparations represented different enzymes. However, more recent work[58] suggests that these enzymes are identical and the differences observed earlier were due to differences in assay procedures and the sensitivity of the aminopeptidase to the presence of activators. It should be noted that as early as 1939, Berger and Johnson[67] found that the polypeptidase activity of crude cell-extracts of S. cerevisiae toward different tripeptides varied in a random fashion even when using the same divalent metal ion. Thus the addition of Zn^{+2} as zinc sulfate caused almost a doubling of the rate for dl-alanyldiglycine hydrolysis whereas the rate for dl-leucyl-diglycine cleavage was reduced by approximately 12%. This effect may have been caused by the activation of different peptidases or to more subtle effects regarding interaction between metal, substrate, and a single peptidase activity. The latter explanation, although not necessarily true in this particular case, has been shown for several purified yeast peptidases.[58,64,68]

In 1966 during purification of a yeast dipeptidase Cordonnier[64] also partially characterized an impure aminopeptidase fraction. Using dl-Leu-Gly-Gly and $(Gly)_3$ as substrates he found a pH optimum of 6.5-7. EDTA caused a total loss of activity showing a metal ion dependence for this enzyme. The

cleavage of dl-Leu-Gly-Gly was slightly activated by Mg^{+2} but inhibited by several other metal ions including Zn^{+2}. Hydrolysis of dl-Leu-Gly-Gly was five times faster than the rate obtained for dl Leu-Gly. These findings are similar to those of Johnson.[57]

Although some evidence existed regarding the occurrence of more than one aminopeptidase in yeast, it was not until the work of Matile et al[21] that this was actually confirmed to some extent. Using starch-gel electrophoresis and an activity staining procedure Matile et al[21] was able to demonstrate the existence of four aminopeptidases in cell-free extracts of S. cerevisiae. The assay was based upon activity toward leucyl-p-nitroanilide, a typical substrate used to determine leucine aminopeptidase activity. (The use of such substrates is subject to question as will be described in the Results section.) The number of peptidase activities appeared to vary with the growth stage of the cell. Four bands of activity were found for stationary cells but only three for exponentially growing cells. The missing activity was found to be the only one located in the vacuole. However, gel filtration of freshly prepared cell-extracts from exponentially growing cells showed the existence of low levels of this latter aminopeptidase. The failure of the activity staining procedure to detect the fourth aminopeptidase was due to the lability of this enzyme and the limited sensitivity of the technique. This aminopeptidase has a pH optimum of 7.6

and was not inhibited by EDTA nor activated by the addition of Co^{+2}. It had the highest molecular weight of all of the aminopeptidase activities based upon its elution behavior from a Sephadex G-150 column. In this respect it may seem similar to the aminopeptidase of Johnson[57] but the failure of EDTA to inhibit this aminopeptidase clearly showed that this was a different aminopeptidase activity.

Masuda et al[9] characterized two aminopeptidases found in the acidic fraction of S. cerevisiae autolysate which they named APase I and APase II. APase I was purified to electrophoretic homogeneity (cellulose acetate membrane electrophoresis) and shown to be a metalloenzyme based upon inactivation by EDTA and o-phenanthroline. It was further characterized as a zinc metallo-enzyme based upon the activating effect of Zn^{+2} ions after total loss of activity due to EDTA. The substrate used for this study was Leu-p-nitroanilide. Zn^{+2} was able to restore approximately 70% of this enzymes activity whereas other divalent metal ions failed completely or restored less than 15% of its original activity. APase I was active toward several free dipeptides, glucagon, and derivatives of amino acids including amides, naphthylamides and p-nitroanilides. APase II on the other hand was found to hydrolyze dipeptides and a tetrapeptide but not Leu-p-nitroanilide or other amino acid derivatives. This impure enzyme had a molecular weight of approximately 34,000 and was inhibited by EDTA and o-phenanthroline. No information is given regarding any attempts to reactivate this enzyme by the addition of metal ions.

Masuda et al [9] also studied the effects of other inhibitors on the aminopeptidases. They found that APase I was inhibited by PCMB and monoiodoacetate, suggesting that a sulfhydryl group is essential for its activity. L-cysteine was also inhibitory, probably due to its metal chelating power, since β-mercaptoethanol had no affect. Neither of the aminopeptidases was affected by DFP.

Metz and Rohm[58] have purified to homogeneity and characterized a yeast aminopeptidase similar to that of Grassman[56] and Johnson.[57] It is a zinc metalloenzyme having a molecular weight of approximately 640,000,[69] an isoelectric point of 4.7 and is extremely stable in solution.[58] Furthermore it appears to be a glycoprotein (~12%) as demonstrated by polyacrylamide gel electrophoresis of the purified aminopeptidase followed by treatment with periodate and Shiff's reagent. Carbohydrate bands were seen to comigrate with the aminopeptidase band, the latter being revealed by protein staining. The zinc content determined by atomic absorption spectrophotometry was equal to 1.8 ± .05 moles of zinc per mole of enzyme. The enzyme was activated by adding Zn^{+2} and Cl^- or Br^- ions and inhibited by metal ion chelating reagents. The effect of EDTA could be overcome by adding Zn^{+2} to the enzyme but other metal ions failed to do so. The mechanisms of Zn^{+2} and Br^- (or Cl^-) activations were studied by their effects on kinetic parameters.[58] Non-hyperbolic kinetics were obtained at pH 7.8 for the substrate Ala-Thr-Ala-OMe in the absence of added Zn^{+2} or Br^- with a Hill coefficient of 1.7. As the

concentration of Zn^{+2} was increased V_{max} increased whereas K_{app} and n (the Hill coefficient) were relatively unaffected. The addition of Br^- in increasing concentrations restored Michaelis-Menten kinetics even in the presence of Zn^{+2} and caused a lowering of K_{app}. The authors concluded that Zn^{+2} and substrate were bound to the enzyme in a random fashion and that the effect of Br^- is allosteric in nature. The aminopeptidase has an apparent pH optimum range of 7-8.5 depending upon the substrate tested in the presence of Zn^{+2} and Cl^- ions. However, in the absence of these activators the optimum pH range drops to 6.0-6.5. SDS polyacrylamide gel electrophoresis yielded a molecular weight of approximately 53,000 for each of 12 subunits found. This result was in agreement with amino acid analysis and the carbohydrate content of the enzyme.[69] Further studies, on the quaternary structure of this aminopeptidase were conducted by Metz et al[69] and Marx et al.[70] These workers found that at neutral pH the active enzyme was in equilibrium with two inactive subfragments (molecular weights 320,000 and 110,000) and also higher molecular weight aggregates. The active enzyme was reported to be a dodecamer having pseudo-D_3 symmetry with the dimer being the smallest symmetrical unit.

This yeast aminopeptidase, in the presence of Zn^{+2} and Cl^- (Br^-) ions, cleaves tripeptides, dipeptides and many types of amino acid and peptide derivatives providing that a free amine terminus is present. The best substrates are those containing hydrophobic amino acids at the amine terminus

whereas glycine or charged amino acid residues in this position result in slower cleavage rates. Kinetic parameters were also determined for several substrates and both K_{app} and V_{max} were shown to depend on the position occupied by an amino acid and its respective side chain.

Recently, Frey and Rohm[55] have attempted to ascertain the number of peptidases in yeast and selective methods to assay for their activities. Homogenates of S. cerevisiae were shown to possess three aminopeptidases and a single dipeptidase. These activities were partially separated from one another. The dipeptidase activity will be discussed shortly and only the aminopeptidases will be discussed now. The aminopeptidase activities were selectively monitored by using their different substrate specificities and their sensitivities to Zn^{+2} activation. The three aminopeptidases are denoted by the Roman numerals, I, II and III and have molecular weights of 640,000, 85,000 and 30,000 respectively. Aminopeptidase I is identical to that already described by Metz and Rohm.[58] Aminopeptidase II is similar to aminopeptidases described by Masuda et al,[9] Cordonnier[52] and Matile[21] whereas Aminopeptidase III is similar to an aminopeptidase described by Masuda et al[9] and an amino acid naphthylamidase.[71]

As previously stated, Aminopeptidase I requires both Zn^{+2} and Cl^- (Br^-) ions for maximum activity. In contrast, Aminopeptidase II is not activated by Zn^{+2} but is slightly activated by lowering the concentration of Zn^{+2}. This aminopeptidase is also characterized by its rapid action

toward lysyl-derivatives and specifically its ability to hydrolyze Lys-p-nitroanilide at an appreciable rate whereas the other aminopeptidases are unable to do so. Very little information is given about Aminopeptidase III. It rapidly hydrolyzes Ala-Thr-Gly-OMe and it is much more active toward dipeptides than toward p-nitroanilide derivatives. It has not been purified.

Aminopeptidase I is the only aminopeptidase located in the vacuole. Its biosynthesis is repressed by both the presence of glucose and amino acids in the growth medium. Aminopeptidase II levels did not change appreciably with changes in the growth phase. However, activity increases of 50 - 100% were found for cells growing on a rich nitrogen source like peptone. It was also suggested that Aminopeptidase II may have an external location in yeast, mainly outside the plasmalemma. This was similar to the earlier contention of Matile et al[20] for one of the aminopeptidases which he found in cell-extracts of S. cerevisiae. However, several other authors[11,18,19] have not found any evidence for extra cellular peptidase activity. This question still remains a matter of controversy and the answer to it may well lie in differences encountered with various strains of S. cerevisiae.

4. Carboxypeptidases

In 1970 it was demonstrated by Hayashi et al[72] that yeast proteinase C had carboxypeptidase activity. In the years that followed it became clear that yeast proteinase C was actually a

carboxypeptidase with broad specificity and is now known as carboxypeptidase Y.

Carboxypeptidase Y has been purified to homogeneity and characterized by many researchers.[4,44,73-80] Its broad specificity includes both peptidase and esterase activities but it is devoid of endopeptidase activity. It is active toward Cbz-dipeptides and proteins at acid pH's of 5.5-6.5 whereas its esterase and amidase activity is usually found at the higher pH values of 6-8. Carboxypeptidase Y is also very special because it can liberate C-terminal proline from peptides and proteins.[81] Therefore it is very useful for performing sequence studies on polypeptides and proteins.[82] Carboxypeptidase Y is not a metal ion dependent enzyme and also does not require its substrate to have a free carboxyl group.

The native enzyme is a glycoprotein having a molecular weight of approx. 60,000, is monomeric and has an isoelectric point of 3.6.[76,80] It requires no cofactors for its activity and is inhibited by DFP, PMSF, PCMB and various divalent metal ions.[42,76] Carboxypeptidase Y has been shown to contain essential serine[78] and histidine[83] residues.

An interesting aspect of carboxypeptidase Y is that it must be activated prior to any assay procedure. Activation is necessary in order to remove a naturally occurring inhibitor of the enzyme. In intact yeast cells carboxypeptidase Y is active and located in the vacuole.[40] A naturally occurring inhibitor is located outside this structure perhaps in order

to contain carboxypeptidase Y's activity from running rampant in the cell.[40] When the cell is broken the inhibitor binds to carboxypeptidase Y thus rendering it inactive. Several methods for activating carboxypeptidase Y have been reported.[76,84,85]

For a time, many researchers suggested that there was only one carboxypeptidase (CPY) in S. cerevisiae. In 1977 Wolf and Weiser[86] clearly demonstrated the existence of a second carboxypeptidase (carboxypeptidase S). A double mutant which lacked carboxypeptidase Y and was auxotrophic for leucine, grew on Cbz-X-Leu substrates. Further proof was also given in that carboxypeptidase S is sensitive to EDTA whereas carboxypeptidase Y is not. The former could be reactivated by the addition of Zn^{+2}. It is not clear whether or not a specific inhibitor for carboxypeptidase S exists although 70% of its activity is masked in crude extracts as compared to activated extracts.

5. Dipeptidase(s)

Some of the very early work concerning dipeptidase activity in yeast has already been given. It was generally believed that microorganisms contained several specific dipeptidases[65,66] but now it is well established that almost all known peptidases have fairly broad specificities.[1,13,64,68]

In 1958 and 1960 Nishi[87,88] published papers regarding a supposed yeast glycylglycine specific dipeptidase. His first paper[87] dealt with the effects of metal ions on the dipeptidase activity using only one substrate, Gly-Gly.

Some of the conclusions arrived at were that the dipeptidase specifically required Co^{+2} ion as an activator (other divalent metal ions had no effect or deactivated the dipeptidase by competitive inhibition), the pH optimum was 7.7-8.0 and inhibition was caused by SH-poisons. Nishi[87] also proposed a mechanistic scheme for the dipeptidase consistent with his observations. The later paper dealt with inhibition of the yeast dipeptidase by amino acids.[88] This effect was originally noted by Grassman et al.[89] Nishi's results showed that leucine was a competitive inhibitor for Gly-Gly but not for Co^{+2} ion. The main problem with both of these elegant studies is that only one substrate, Gly-Gly, was tested. It will be shown presently that the idea of having a "specific Gly-Gly yeast dipeptidase" is in error and that the modulating effects of metal ions vary both with the conditions of assay and the particular substrate under investigation.

Cordonnier[64] characterized a dipeptidase activity which he obtained in a semi-purified state. The characterizations were carried out on crude extract as well as on various partially purified fractions. Some of his findings for the purified dipeptidase may be summarized as follows: the dipeptidase is a metalloenzyme, of strongly anionic character possessing active site thiol groups, and its molecular weight is approx. 100,000 as determined by gel filtration experiments. The purified dipeptidase activity was active toward several dipeptides and did not cleave dl Leu-Gly-Gly (the only tripeptide tested under most conditions). However, this

preparation showed some activity toward several blocked dipeptides like Cbz-Ala-Gly and Ala-Gly-NH_2. Therefore, it is likely that traces of carboxypeptidase and aminopeptidase activities were present. Generally a pH optimum of 7.5-8.0 was observed depending upon the substrate tested. The effects of various metal ions with respect to different substrates was also investigated. Most divalent metal ions including Co^{+2} caused an increase in the rate of Gly-Gly hydrolysis whereas the same metal ions usually caused decreases in rates of cleavage of other dipeptides.

The metal ions effect was perhaps one of the main reasons that Cordonnier postulated the existence of more than one dipeptidase. Other reasons for his view were that during purification the ratio of Ala-Gly/Gly-Gly activities varied considerably and the activities toward Ala-Gly and Gly-Gly (as well as their ratios) varied upon exposure to various adsorbents. Although these observations cannot be discounted they may be explained in terms of a single dipeptidase if the following items are considered. The dipeptidase preparation was not pure and the observed effects may be due to a peptidase(s) other than a dipeptidase. Also differences in peptidase activity due to metal ions may depend upon the the substrate and metal ion used. Furthermore, Cordonnier[64] was not able to separate the alleged different dipeptidase activities. One last point which is not clear cut but may be important concerns the yeast itself. Cordonnier used S. cerevisiae variety ellipsoideus.[64] This species is com-

prised of many wine yeasts having a great variability regarding expression of their taxonomic properties.[90] It is possible therefore, that slight alterations in even the same enzyme may exist in these various wine yeasts.

Rohm[68] has found a single dipeptidase activity in S. cerevisiae from brewers yeast and has purified the dipeptidase to homogeneity based upon the results of polyacrylamide gel electrophoresis. Rohm and co-workers[55,68,91,92] have also characterized this enzyme in detail. The dipeptidase is a zinc metalloenzyme (3.1ug Zn/mg protein) having a molecular weight of 130,000, an isoelectric point of 6.3, a pH optimum of 7.8-8.3 and is comprised of four subunits.[68] However, in a later paper Rohm states that the dipeptidase is comprised of at least two subunits with identical molecular weights of 65,000.[92] This apparent contradiction was not explained by the author.

The substrate specificity of the dipeptidase is characterized by its action on only free dipeptides possessing an unsubstituted peptide bond and having the l configuration.[68] Thus, substrates like Gly-Pro, Gly-Sar, Gly-Leu-NH_2, Gly-D-Leu and N-Ac-Ala are not cleaved. The requirement for a free carboxyl group is absolute whereas the need for a terminal ammonium ion (NH_3^+) is less stringent since both Pro-Gly and Formyl-Gly-Gly are cleaved, the latter being a very poor substrate.[91] Generally, the best substrates contained uncharged hydrophobic amino acids and the worst substrates contained charged amino acids.

Kinetic studies[91] with various dipeptides led to the conclusion that the portion of the active site in contact with the carboxy terminus of the dipeptide is an open structure of moderate hydrophobic character whereas that portion in contact with the amine terminus is of more limited dimensions and more hydrophobic in nature. The effect of different metal ions on the activity of the dipeptidase varied with the substrate under investigation. It was also noted that the dipeptidase was sensitive to subtle alterations in specificity on exposure to various physical stresses. Therefore, even freezing and thawing of the dipeptidase may produce slight alterations in specificity.

Rohm and co-workers[91,92] demonstrated biphasic kinetic behavior between pH 6 and 7 presumably due to local isomerizations affecting the geometry of the active site. Both above and below these pH values the kinetic behavior was usually better described by fitting the data to the Hill equation rather than to hyperbolic kinetics. This was especially true for substrates containing N-terminal leucine where n_H values exceeded 1.4. The inhibition of dipeptidase activity by amino acids also exhibited a complex behavior and depended upon the particular combination of substrate and amino acid investigated. Based upon this and other kinetic evidence it was proposed that the dipeptidase acted on substrates by an ordered Uni-Bi mechanism.[91] Table I summarizes the properties of various yeast peptidases which have been characterized to date.

TABLE 1. PROPERTIES OF YEAST EXOPEPTIDASES

Type	M.W.	Inhibitors	Activators or Cofactors	Ref.
Aminopeptidase	640,000	EDTA	Zn^{+2}, Cl^{-}(Br^{-})	56-59
Aminopeptidase	200,000	Dithiothreitol PCMB, EDTA	Zn^{+2}	9
Aminopeptidase	34,000	EDTA	Divalent Metal Ions	9,55
Aminopeptidase	85,000	-	-	55-58
Dipeptidase	130,000	EDTA, PCMB Amino acids	Zn^{+2} & other Divalent Metals	59,64, 68,87,
Carboxypeptidase Y	60,000	DFP, PCMB Divalent Metal Ions	None	42,76, 80
Carboxypeptidase S	-	EDTA	Divalent Metal Ion Zn^{+2}	86

D. Dipeptidases from several sources

Dipeptidases purified and characterized to varying degrees from bacteria, yeast and mammalian sources have several common features. Contrary to earlier beliefs[66] it is now well established that most dipeptidases have broad substrate specificities[1,13,68] although they may hydrolyze certain dipeptides faster than others.[39,68] This preferential hydrolysis may be due to the assay conditions employed. In contrast, X-Pro dipeptides are not cleaved by the broad specificity dipeptidases[68,93] but rather by aminopeptidases[94,95] and/or X-Pro specific dipeptidases called imidodipeptidases.[8,96] The presence of charged amino acid residues in dipeptides usually results in extremely low hydrolysis rates if the substrate is cleaved at all.[68,93,97] However, large hydrophobic residues in dipeptides elicit the opposite response and such molecules are good substrates.[39,68,93] The positions of amino acid residues in dipeptides will of course influence their rates of hydrolysis but this varies among the dipeptidases surveyed and no trend is observed. By definition dipeptidases should only hydrolyze dipeptides but in two instances, one involving a dipeptidase from human kidney[98] and the other from cod muscle,[99] this requirement seems to be violated. In both cases it was found that a tripeptide $(Leu)_3$ or Leu-Gly-Gly respectively, was cleaved although several other tripeptides were not. It is not clear from these studies whether the tripeptide hydrolysis might be caused by some small amount of oligopeptidase contaminant or

to the dipeptidase acquiring a specific tripeptidase activity, the latter being extremely doubtful. Also, since these authors did not report the purity of the substrates examined the results obtained might be due to a dipeptide contaminant.

One striking similiarity among dipeptidases is that most of them are divalent metalloenzymes[8,64,68,99-104] and that several of them seem to specifically require Zn^{+2} or Mn^{+2}.[8,68,100-104] Most metal ion studies yield a series of complex results regarding substrate and metal ion combinations.[64,68,103,104] Specifically, a particular metal ion may be an activator toward the cleavage of $(Met)_2$ whereas it is deactivating for Met-Ala, Leu-Met, and $(Ala)_2$ hydrolysis. However, several studies show that Co^{+2} usually is an effective activator for the hydrolysis of Gly-X dipeptides, where X≠Pro.[68,87,100,103,104] The reason for the activating effect of Co^{+2} on dipeptidases toward Gly-X dipeptides is not well understood.

Consistent with the above discussion is the fact that metal ion chelators like EDTA and o-phenanthroline are potent inhibitors of dipeptidase activity.[8,68,100-104] Dipeptidases are sometimes susceptible to other enzyme inhibitors but the one reagent that is found to be inhibitory in most cases when tested is PCMB.[8,12,64,97,99,101,105] Thus, it is possible that dipeptidases possess active center sulfhydryl groups. In contrast to this it is found that DFP is not inhibitory and therefore an active serine residue is not involved in catalysis by dipeptidases.[64,101,105a] An interesting characteristic of several dipeptidases is that amino acids are found to be inhib-

itors.[91,93,99,102,105] The specific type of inhibition varies with the amino acid chosen and the composition and sequence of the dipeptide substrate. In most cases, the large hydrophobic amino acids like leucine are the best inhibitors.[91,99,102,105a] The significance of this is not clear although it suggests that under physiological conditions dipeptidase activity may be regulated by certain amino acids.

Two other properties which dipeptidases have in common are their molecular weights and pH activity profiles. The molecular weights of dipeptidases range from as low as approx. 47,200[100] to as high as 200,000[98] but most have molecular weights around 100,000.(Table 2)Their pH-activity profiles are characterized by activities over a wide pH range, usually 4.5-11.0 (Table 2), having optimum pH values between 7.5 and 9.0. (Table 2) The observed pH optimum may vary with the assay conditions and the substrates used in the assay.[64,68,92,93] A summary of the properties of several dipeptidases from various sources are given in Table 2.

TABLE 2. PROPERTIES OF DIPEPTIDASES FROM VARIOUS SOURCES

Source	M.W.	Inhibitors	Activators or Cofactors	pH opt.	Ref.
Rat liver	120,000	Monoiodoacetate	Dithiothreitol	4.5	106
	90,000	Dithiotreitol EDTA, Mn^{+2}, Cl^{-1}, Co^{+2}	-	6.5	
Hog Kidney (Particulate Renal Dipeptidase)	47,200	o-phenanthroline	Zn^{+2}	-	100
Streptococcus Diacetilactis	51,000	EDTA, PCMB Mg^{+2}, Mn^{+2}	Zn^{+2}, Co^{+2}	7.5-8.0	101
Human Milk	76,800	EDTA, PCMB Heavy Metals	Zn^{+2}/Phosphate	7.8-8.3	97
Hog Kidney (Renal Dipeptidase)	90,000	Inorganic Phosphate & Phosphate Esters o-phenanthroline amino acids	Zn^{+2}	-	102

TABLE 2 (continued)

Source	M.W.	Inhibitors	Activators or Cofactors	pH Opt.	Ref.
Neurospora Crassa	112,000	EDTA PCMB	Mn^{+2} and Others	8.5	8
Cod Muscle Dipeptidases					
Peak I	-	PCMB Amino acids Zn^{+2}	Mn^{+2}	9.2	99
Peak II	-	PCMB Amino acids Zn^{+2}	EDTA	8.2	99
Pig Intestinal Mucosa	114,000	Amino acids EDTA, PCMB 2-mercapto-ethanol Heavy metals	-	8.0-8.4	105 a,b
E. coli B	100,000	Dithiothreitol 2-mercapto-ethanol Iodoacetate EDTA Amino acids Metals	Zn^{+2} and others (Mn^{+2}, Co^{+2})	8.0	93, 103

TABLE 2 (continued)

Source	M.W.	Inhibitors	Activators or Cofactors	pH Opt.	Ref.
Mouse Ascites Tumor Cells	85,000	EDTA, Amino acids	Zn^{+2}	8.3	93, 104
Streptococcus Thermophilus	50,000	EDTA PCMB DFP	Divalent Metal Ions	7.5	12
Human Kidney Dipeptidase					
F	135,000	Co^{+2} and others EDTA cysteine	Cd^{+2} PCMB	9.0	98
S	200,000	Co^{+2} and others EDTA cysteine	Cd^{+2} PCMB	9.0-11.0	98
S. cerevisiae	130,000	EDTA Amino acids PCMB	Zn^{+2} and others	7.8-8.3	64, 68

E. Proposal

The proposed investigation will attempt to do two things. The first objective is to clarify the situation in yeast, specifically for S. cerevisiae, with regards to the number and types of intracellular peptidase activities present. This will be accomplished by characterizing various peptidase activities as to their electrophoretic mobilities and substrate specificities using a polyacrylamide gel electrophoresis-coupled peptidase staining technique. The second and main thrust of this investigation will be directed toward the purification and characterization of one or more intracellular dipeptidase activities. Purification of the dipeptidase(s) will be conducted using classical biochemical techniques. The homogeneous dipeptidase(s) will then be characterized as to its physical and chemical properties. These characterizations will include the following; molecular weight, isoelectric point, sub-unit analysis, pH profile, the effects of various metal ions, the effects of several typical enzyme inhibitors, storage and thermal stabilities, and a detailed kinetic analysis of the action of the dipeptidase(s).

Both of the above studies will be facilitated by using a well defined yeast strain, S. cerevisiae Z1-2D. This strain is auxotrophic for leucine and lysine and lacks extracellular peptidase activity.[19] Consequently, this study will be directed toward only intracellularly located peptidases. Also, it will not suffer from the possible problem associated with wild type yeast strains and therefore the possibility of

having slight alterations in even the same enzyme due to variations in yeast strains. Furthermore, both investigations will be conducted using mainly true peptide substrates, thus diminishing the possiblity of complicating the results due to esterase activity.

II. MATERIALS AND METHODS

A. Growth of *S. cerevisiae* Z1-2D

Saccharomyces cerevisiae Z1-2D a leucine, lysine double auxotroph was obtained from Nasim Khan, Brooklyn College, City University of New York. This haploid strain was derived from a cross between strains KC-372 (R. Mortimer, University of California, Berkeley) and 1323-1B (D.C. Hawthorne, University of Washington, Seattle). *S. cerevisiae* Z1-2D was maintained at 4°C on 2% agar slants containing 1% yeast extract, 2% peptone and 2% dextrose (YEPD). Starter cultures (5ml) were prepared from a minimal growth medium containing 0.67% yeast nitrogen base without amino acids (Difco Laboratories), 0.50% dextrose, leucine (60 ug/ml) and lysine (20 ug/ml). This will be called the supplemented minimal medium. The Difco medium plus dextrose was autoclaved at 121°C for 15 minutes at 15 psi and cooled slowly to room temperature. Filter sterilized amino acids were then added. The starter culture was inoculated from a fresh slant using a sterile loop under aseptic conditions, and allowed to grow at 30°C with agitation for approximately 24 hours to 160-190 Klett units. Klett units, a measure of turbidity, were determined with a Klett-Summerson photoelectric colorimeter equipped with a blue filter (400-420 nm).

Cells were harvested by centrifugation, washed twice with sterile distilled water, and resuspended in 2.5 ml of sterile distilled water. To 125 ml Erlenmeyer flasks, equipped with a side arm, containing 25 ml of supplemented minimal medium

was added o.1 ml of cell suspension containing 1-2x10^7 cells/ml. Cells were grown to late log phase (~ 140 Klett) at 30°C with shaking (100-200 rpm) and cell growth monitored by the increase in Klett units. Klett readings and cell numbers were correlated by using a Neubauer Hemacytometer to count the cells. The entire culture was then used to inoculate two liter flasks containing one liter of supplemented minimal medium. These cells were then grown at room temperature with shaking (New Brunswick Model VS) for 28 hours to late log phase. Cells grown in this manner were used for the experimental work dealing with characterization of the crude cell extract. Cells grown for the purification of the dipeptidase will be described below. For large scale growth of S. cerevisiae Z1-2D,eight 250 ml flasks containing 50 ml of supplemented minimal medium were inoculated with 0.2 ml of the starter culture cell suspension and grown to late log phase. These were then used as the inocula for eight two liter flasks containing 1 liter of supplemented minimal medium and grown to mid/late log phase (140-180 Klett). The contents of these eight flasks served as the inoculum for a 100 liter fermentation.

The 100 liter fermentation of S. cerevisiae Z1-2D was accomplished using the Fermetron FM-150 (New Brunswick Scientific). Cell growth was carried out at 30°C with agitation and cells harvested at late log phase. The growth medium used was a supplemented Vogels N Medium. Vogels N Medium was prepared as a 50-fold concentrate with the exception of biotin which was added later. For each growth

experiment the following were added to the fermentation tank: 2 liters of Vogels N Medium concentrate, 2 liters of freshly prepared biotin ($100^{mg}/l$), 2 liters of inositol (1.8g/l), and 2 liters of a vitamin solution containing calcium pantothenate ($100^{mg}/l$), thiamine hydrochloride ($200^{mg}/l$) and pyridoxine hydrochloride ($200^{mg}/l$). The volume was then adjusted to approximately 88 liters and sterilized for one hour in situ. All further additions or removals from the tank were done under aseptic conditions. The batch was allowed to cool to 30°C and 4 liters of previously autoclaved dextrose (50%) and 0.2 liters of a filter sterilized amino acid solution containing leucine ($30^{gr}/l$) and lysine hydrochloride ($12.5^{gr}/l$) were added. Fermentation was initiated using the 8 liter inoculum described above. As the cells in the 100 liter fermentation approached late log phase, (180 Klett) 8 liters were removed and stored at 4°C for use as a future inoculum. Cells were harvested at late log phase using a Schnells-Zentrifuge Cepa Type 4(Carl Padberg, Lahr/Baden) and stored at -30°C until needed. The average yield of cell paste was 344g per 92 liter run. A total of 6 runs were made.

All growth experiments were checked for revertants by plating out directly from the growth container onto minimal media plates. Four types of plates were used. The general composition of the sterile plates was o.67% Difco yeast nitrogen base without amino acids, 2% dextrose and 2% agar and this represents a type 1 plate. Types 2, 3 and 4 are the

same as type 1 with the addition of either leucine (30$^{ug}/_{ml}$) or lysine (30^{ug}/ml) or leucine and lysine (30^{ug}/ml each) respectively. The maintenance of auxotrophic integrity was judged acceptable if after 48 hours at 30°C only growth on a type 4 plate was observed. Growth media were also routinely checked for contamination by plating out onto sterile plates of the same composition as the stock slants and incubated for 48 hours at 30°C.

B. <u>Preparation of S.cerevisiae Z1-2D cell-extracts</u>

Cell-extracts of <u>S</u>. <u>cerevisiae</u> Z1-2D were prepared by two different methods depending upon the quantity of cells to be broken. Cell breakage on a small scale was performed by a grinding technique using an Omni-Mixer (DuPont Chemical, Sorvall Div.) whereas large scale cell breakage was accomplished by pressing a liquid cell suspension through a narrow orifice using a French Pressure Cell (American Instrument Co).

For small scale work frozen cell paste was thawed at 4°C, washed twice with cold distilled water, and the cells collected by centrifugation at 3000 RPM, 4°C using a Sorvall RC2-B centrifuge (Ivan Sorvall, Inc.). The pellet obtained was white in appearance and was resuspended at 4°C in either potassium phosphate buffer (40mM, pH 7.0) or a tris buffer (50mM, pH 8.3) to 50 mg cells/ml. 100ml of this suspension was added to a 500 ml Omni-Mixer cup containing 200 g of acid washed glass beads (50-70 um in diameter, Sigma Chemical Co.). The cup was immersed in an ice-water bath, and allowed to cool for 15-20 minutes. Cells were disrupted by grinding at an instrument setting of

8 for 5 minutes. The cups' contents were filtered through a coarse sintered glass funnel into a filtering flask which was immersed in an ice-water bath. The glass beads retained in the funnel were washed several times with small amounts of cold buffer to increase protein recovery. The resulting grayish filtrate was centrifuged at 12,100 x g at 4°C in a Sorvall RC2-B centrifuge (SS-34 rotor) for 10 minutes to separate cell-extract from unbroken cells and cellular debris. The supernatent (cell-extract) was collected and dialyzed at 4°C against the buffer of choice. Dialysis consisted of four 2-liter changes of buffer during a 48 hour period. The cell-extracts were frozen at -30°C pending future use. It was determined based on a cell counting technique using a Hemacytometer that approximately 90% of the cells have been homogenized.

Cells from the large scale growth experiment were thawed at 4°C washed twice in cold distilled water, pelleted by centrifugation and resuspended to 50% $^{w}/v$ in potassium phosphate buffer (40mM, pH 7.0). The cells were disrupted using a French Pressure Cell fitted with a one inch **piston** set at the high position. The cell suspension was pumped through at 10,000-12,000 psi and the resulting mixture immediately cooled and collected by attaching tubing to the outlet and allowing this tubing to sit in an ice-water bath. This mixture was then spun at approximately 11,000 xg at 4°C using a J-10 rotor in a Beckman Model J-21B centrifuge. The supernatent was removed and immediately frozen at -70°C. The pellet containing broken as well as unbroken cells was

resuspended to 50% $^{w}/v$ and passed through the press a second time to try to maximize the yield of cell extract which was again frozen at -70°C. Finally the cell extract was stored at -30°C until needed.

C. Determination of Protein Concentration

Quantitative protein measurements were performed by the method of Lowry et al,[107] using BSA as the calibration standard. Chromatographic column fractions which were assayed for peptidase activity had their relative protein concentrations measured by their ultraviolet absorbance at 280 nm. Both the quantitative protein determinations and the monitoring of column fractions was accomplished by the use of a Cary 118 UV-VIS double beam spectrophotometer (Varian Instrument Co.). Chromatographic column eluates were continuously monitored for protein at 280 nm with the use of a Uvicord III monitor (LKB Instruments) equipped with flow cells having optical path lengths of either 3 mm or .05 mm.

D. Dipeptidase Activity

Dipeptidase activity was assayed using the fluorometric method of Roth.[108] The buffered fluorescence reagent was prepared by mixing 1.5 ml of o-phthalaldehyde ($10^{mg}/ml$ in ethanol) with 90 ml of borate buffer (o.05 M sodium tetraborate decahydrate, adjusted to pH 9.5 with NaOH) followed by the addition of 1.5 ml of 2-mercaptoethanol ($5^{ul}/ml$ in ethanol). The above reagents were prepared as stock solutions and the borate buffer stored at room temperature whereas the other two were store refrigerated. The o-phthalaldehyde solution had

a yellowish tint and was allowed to stand refrigerated for 2-3 days prior to use during which time the solution became clear. The buffered fluorescence reagent was usually stable for one day at room temperature but should be periodically checked with a standard amino acid solution. Fluorescence measurements were performed on an Aminco Bowman Spectrophotofluorimeter Model #4-8202, using an excitation wavelength of 340 nm and an emission wavelength of 450 nm. The slit arrangement was either 3-2-3 or 3-1-3 depending upon the substrate and the excitation source was either a xenon or mercury-xenon lamp.

For the standard dipeptidase assay, 1.0 ml of a solution of dipeptide in potassium phosphate buffer, 40 mM, pH 7.0 was incubated at 30°C in a Thermolyne Dri-Bath (Sybron Corp.). Cleavage of the peptide bond was initiated by the addition of 5-25ul of enzyme solution which was kept in an ice-water bath. The volume of enzyme solution added depended upon the concentration of protein and the stage of protein purification. At appropriate time intervals aliquots ranging in size from 5-200 ul were withdrawn and added directly to either 1.5 or 3 ml of buffered fluorescence reagent and mixed rapidly using a Vortex Genie (Scientific Industries). This procedure terminated the hydrolysis reaction and the resulting solution was allowed to stand at room temperature for 5-10 min. depending upon the substrate under investigation, and its fluorescence intensity measured relative to a quinine sulfate standard (1^{ug}/ml in 0.1N H_2SO_4). For reasons to be explained shortly, it was occasionally necessary or desirable to quench the reaction aliquot in an equal or greater volume of ice-cold ethanol.

Then a portion of this was pipetted into the fluorescence reagent and the analysis performed as described. Regardless of the method chosen equivalent results were obtained when appropriate corrections were made for dilutions. Identical peptide solutions without added protein were monitored as controls along with the assay mixtures in order to establish blank values and to insure that non-enzymic hydrolysis did not occur. Each relative fluorescence measurement was obtained as the product of the meter multiplier and the meter reading. Meter multiplier settings varied between 1.0 for highly fluorescent solutions and .01 for solutions having low levels of fluorescence, such as the reagent blank.

Relative fluorescence was correlated with the amounts of amino acid released from the hydrolysis of dipeptides using standard calibration curves which were constructed prior to the actual assays. Calibration curves were obtained as linear plots of relative fluorescence vs various concentrations of dipeptide and amino acids which would correspond to specific percentages of hydrolysis of the dipeptide. Such plots were determined for 0 to 12% cleavage. The actual assays were never permitted to exceed 10% hydrolysis and were usually restricted to less than 5%. Rate curves were plotted (amounts of amino acids released vs time) and velocities determined by a linear least squares procedure using a Wang desk calculator. Rates determined in this manner were linear and reflected true initial rates. The specific activity is defined as the number of umoles of amino released per minute per mg of

protein under assay conditions. As stated by Roth[108] the volume of sample may be increased at the expense of buffer without changing the final volume. Therefore 100 ul of a 10^{-4}M amino acid solution added to 3ml of fluorescent reagent has the same relative fluorescence intensity as does 200 ul of a 5×10^{-5}M of the same amino acid solution if added to 2.9ml of reagent. This allows one standard calibration curve to be used for more than one initial concentration of dipeptide. When high substrate concentrations are employed problems of quenching and reproducibility occur. These may be circumvented by using smaller sample aliquots or by pipetting the assay aliquot into ice cold ethanol thereby stopping the reaction. This solution is then used directly or diluted to the proper value and a portion of it used in the fluorescence assay. The use of ethanol has another advantage for certain substrates. The time of incubation of the assay aliquot with the fluorescence reagent is always at least 5 minutes but may be extended in some cases to as long as 15 minutes without any significant change in the fluorescence intensity. However, for some peptides, especially those containing glycine, the 5 minute incubation time is critical as the fluorescence intensity decreases rapidly and in a non-linear fashion after this time. For those peptides which fit into this category and are rapidly hydrolyzed, aliquots must be taken every 15-30 seconds and it is impossible to measure these samples exactly 5 minutes after mixing. However, the use of ice-cold ethanol to stop the reaction allows one to measure the

relative fluorescence at exactly 5 minutes after mixing the ethanolic assay aliquot with buffered reagent.

E. Oligopeptidase Activity

Oligopeptidase activity toward tripeptides, tetrapeptides and amino acid derivatives was measured qualitatively by two methods. The first method was as described for dipeptidase activity. The second method involved measuring the decrease in the concentration of peptide bonds at 235nm using the UV-VIS spectrophotometer described earlier. These methods are suitable to establish that a substrate is hydrolyzed but difficult to quantitate when more than one type of peptidase activity is present which is capable of acting on the substrate or its hydrolysis products.

F. Purification of the Dipeptidase

1. Preliminary Preparation

The cell extract was thawed at 4°C and dialyzed against 8 changes of 13 liters each of potassium phosphate buffer (40mM, pH 7.0) over a 3 day period. The solution thus obtained was brownish in color. This buffer is the standard buffer used throughout the purification and in most of the characterization studies unless otherwise stated. All further purification steps were carried out at 4°C.

2. Ammonium Sulfate Precipitation

The amounts of ammonium sulfate used in the salting out of proteins was determined by the method of DiJeso[109] and

corrected for temperature variation. The cell extract (2360ml, 9^{mg}/ml) was brought to 50% saturation by the addition of solid ammonium sulfate (303.2^{g}/l) over a 3 hour period with constant stirring. The suspension was allowed to stand for 22 hours and the precipitate was removed by centrifugation in a Sorvall RC2-B centrifuge equipped with a GSA rotor (16,300xg) for 20 min. at 4°C. The precipitate was dissolved in buffer, dialyzed and held for further study. The supernatent was then brought to 80% saturation with ammonium sulfate (197.7^{g}/l) over a 4 hour period and allowed to stand for 26 hours. The solution was spun down as before except that the time was increased to 30 minutes. The supernatent was discarded and the precipitate dissolved in buffer and dialyzed against 4 changes each of 3.5 liters of buffer over a 2 day period. The resulting murky yellowish-brown solution was used for further purification.

3. Gel Filtration

Gel filtration was performed using Sephacryl S-200 Superfine in a K 50/100 column (5x100 cm) equipped with flow adaptors (products of Pharmacia Fine Chemicals). Deaerated buffer was used throughout. The preswollen suspension of Sephacryl (at 4°C) was diluted with buffer to a consistency suitable for pouring into a column. The column was poured and then packed using downward flow elution. Packing was performed by passing 1.5 column volumes of buffer through the Sephacryl using a peristaltic pump to give a flow rate of

7^{ml}/min. Equilibration was carried out using 4 column volumes of buffer at a controlled flow rate of 3^{ml}/min and the final column height was 90 cm. A void volume determination was not performed because under the conditions employed blue dextran-2000 binds to the Sephacryl to some extent. The protein solution from the salting out step was applied and eluted in the downward flow mode with the aid of a peristaltic pump at 1.4^{ml}/min. and 6.1ml fractions were collected. Fractions containing activity were combined and this solution had a light yellow tint. Similar results at lower elution rates were obtained using Sephadex G-150 instead of Sephacryl S-200.

4. Ion Exchange Chromatography

DEAE-Sephadex A-50 was purchased from Pharmacia Fine Chemicals as a free flowing powder with a chloride counter ion. Due to the standard phosphate buffer used throughout, it was desirable to exchange the chloride counter ion for phosphate ion prior to use as follows: 30 g of ion exchange resin was added to 1.5 liters of 0.8 M potassium phosphate buffer, pH 7.0. The resin was allowed to swell in a boiling water bath for 4 hours and then cooled slowly to room temperature. The swollen gel was washed five times with 500 ml of deaerated standard buffer to exchange buffers and to remove any fine particles which were present. The suspension was then cooled overnight to 4°C.

Excess buffer was decanted and the gel resuspended in enough buffer to provide the proper consistency for pouring

a column. A K26/40 column (2.6x40cm, Pharmacia Fine Chemicals) was poured with the use of a gel and eluant reservoir. The column was packed and equilibrated under a constant pressure head of 36 cm using 600 ml of buffer contained in a mariotte flask. The flow rate thus obtained was 0.5 $^{ml}/min.$ and the final bed height was 38 cm. The protein sample from the gel filtration step was applied and eluted at 0.35$^{ml}/min.$ using a peristaltic pump and 5ml fractions collected. Elution was actually performed in three stages. The column was first developed with 400 ml of starting buffer in order to remove or partially elute any loosely bound proteins. Next, a 400 ml continuous linear gradient of starting buffer containing sodium chloride (0-0.2M) was passed through the column using a gradient mixer, GM-1 (Pharmacia Fine Chemicals), attached to the peristaltic pump. The first gradient was followed by a continuous linear salt gradient from 0.2-0.5M. During gradient elution the column height decreased as the salt concentration increased. This led to slight alterations in the flow rate which were compensated for by readjusting the peristaltic pump. Dipeptidase activity appeared in the fractions eluted between 0.04 and .1M sodium chloride. Salt concentrations were determined by conductance measurements made with a YSI Model 31 Conductivity Bridge equipped with a YSI #3418 conductivity cell having a 0.1 cell constant (Yellow Springs Instrument Co.). Fractions containing dipeptidase activity were combined and dialyzed against the standard

buffer to remove any salt present. The resulting solution was divided into smaller volumes and held frozen at -30°C pending further purification.

5. Hydroxylapatite Chromatography

15 g of spheroidal hydroxylapatite (Gallard-Schlesinger Chemical Mfg. Corp.) was washed four times with 50ml portions of 0.2 M sodium hydroxide, followed by four 50 ml washings in distilled water to remove any fines, and then allowed to cool to 4°C. A K9/15 column (0.9x15 cm, Pharmacia Fine Chemicals) was half-filled with potassium phosphate buffer (4mM, pH=7.0) and a slurry of hydroxylapatite poured into the column thus displacing the buffer. A column packed in this manner must be done in stages in order to achieve the proper bed height.

The packed column was equilibrated with 4 column volumes of 4mM potassium phosphate buffer pH 7.0 at a flow rate of $0.25^{ml}/min$. A portion of the dipeptidase fraction (15.5 ml, $1.23^{mg}/ml$) from the ion exchange step which had been dialyzed against equilibrating buffer was then loaded onto the column at $0.25^{ml}/min$. Protein elution was performed at $.13^{ml}/min$. and 1.5 ml fractions collected. The column was first developed with 2 column volumes of equilibration buffer to remove loosely bound proteins. Under these conditions a considerable amount of protein was eluted which contained oligopeptidase activity but only extremely low levels of dipeptidase activity. The bulk of the dipeptidase activity

was eluted with 20ml of 10mM potassium phosphate buffer, pH 7.0. Finally, any remaining protein was eluted with 40ml of the standard buffer.

6. Preparative Polyacrylamide Gel Electrophoresis

Preparative polyacrylamide gel electrophoresis was performed at 4°C in a cylindrical gel system using Model PAG 15 water cooled unit (Savant Instruments, Inc.) and a Model 400 Power Supply (Bio-Rad Laboratories). The cylindrical gel cell measured 1.5 x 10 cm. and when in use was enclosed in a water cooled jacket. The unit is equipped with an elution cell assembly which permits continuous protein elution and collection.

All solutions used to prepare polyacrylamide gels were freshly prepared in distilled water and deaerated for 5 minutes with a water aspirator, cooled to 4°C, mixed and polymerized. The separation gel was prepared as follows: 1 part of an acrylamide solution (36%^{w}/v acrylamide, 0.5%^{w}/v N, N' methylene-bisacrylamide) was mixed with 1 part of Tris buffer (0.8M, pH 8.3) containing 0.3%^{v}/v of TEMED and the polymerization initiated by the addition of 2 parts of ammonium persulfate (0.3%^{w}/v). The solution was pipetted into the gel tube, overlaid with diluted gel buffer (0.2M, pH 8.2) and allowed to polymerize undisturbed for 4 hours although polymerization appeared to be complete within 45 minutes. The upper gel surface was rinsed four times with cold distilled water. A spacer gel was prepared by

mixing the following solutions: 1 part of an acrylamide solution (28% $^{w}/v$ acrylamide, 3% $^{w}/v$ N, N′ methylene bis-acrylamide), 1 part Tris buffer (0.5 M, pH 7.3) containing 0.7% $^{v}/v$ TEMED, 1 part distilled water, 1 part riboflavin (6 $^{mg}/ml$) and 4 parts of a sucrose solution (40% $^{w}/v$). The top of the separation gel was rinsed twice with spacer gel solution and spacer gel added to the desired level. The spacer gel solution was then overlaid with distilled water. Polymerization was photo initiated using a fluorescent lamp placed at a distance of 15cm from the gel tube. Photopolymerization was allowed to proceed for 1½ hours and terminated by removing the light source. The final gel heights obtained were 5.5 cm for the separation gel and 2.0 cm for the spacer gel.

A portion of the dipeptidase preparation from the hydroxylapatite chromatography step was dialyzed against Tris buffer (0.05M, pH 7.3) and then concentrated to 1.5 ml (1.2 $^{mg}/ml$ protein) using an Immersible Molecular Separator (Millipore Corp.). The solution was then made 6% $^{v}/v$ in glycerol and 1 drop of 0.05% $^{w}/v$ bromophenol blue dye marker added. This final solution was applied to the top of the separation gel. The upper tank buffer was composed of 1 g of Tris and 2.88 g of glycine per liter of solution, pH 8.5. The lower reservioir and the elution cell contained Tris buffer 0.2M, pH 8.2 which is identical to the separation gel. Electrophoresis was carried out at a constant current of 3 ma

until the dye marker had penetrated approximately 1 cm into the separating gel. The current was then raised to 5 ma and continued at this value as long as the total power did not exceed 2 watts. The current may be lowered slightly in order to accomplish this. As the dye marker approached the elution chamber the original buffer contained therein was replaced with a continuous flow of 0.4M Tris buffer, pH 8.0. The flow rate was $0.11^{ml}/min.$ and 3.8 ml fractions were collected. Dipeptidase activity was located by the qualitative UV method described earlier and portions of all fractions containing dipeptidase activity were tested for purity by analytical polyacrylamide gel electrophoresis. The fractions were dialyzed against the standard buffer, made $20\%^{v}/v$ in glycerol, divided into 25 or 50 ul portions and stored frozen at $-30^{\circ}C$.

G. Analytical Polyacrylamide Gel Electrophoresis

Polyacrylamide gels (0.5 x 6.5 cm, 7% acrylamide, 0.186% bisacrylamide) were prepared in a manner similar to that described by Davis[110] with the following exceptions; sample and stacking gels were omitted in most cases and the running gel was 50 mM in tris buffer, pH 8.3. All electrophoretic runs were carried out using the Bio-Rad Model 151 tube gel electrophoresis unit with cooling supplied by a circulating ice-water bath and the Bio-Rad Model 400 power supply. Gels were subjected to pre-electrophoresis using running gel buffer in the buffer tanks for two hours at three milliamps per tube

and either allowed to cool for three hours at 4°C or overnight at 4°C in a solution of running gel buffer. The preparation of samples for electrophoresis varied depending upon the stage of protein purification being investigated and will be discussed under the appropriate sections. Both upper and lower buffer reservoirs contained tris-glycine buffer (0.6 g tris, 2.88 g glycine, per liter, pH 8.3). The upper buffer reservoir (approx. 600 ml.) contained 4-6 drops of a 1% aqueous solution of bromophenol blue. Electrophoresis was performed at one milliamp per tube and the electrophoresis was terminated when the dye marker neared the end of the gel. The gels were immediately removed from their glass tubes and stained for either peptidase activity or protein.

H. Protein Staining of Polyacrylamide Gels

Gels were stained for protein by the method of Chrambach et al.[111] using Coomassie blue. Although this procedure is rapid (1 hr.) and does not require extensive destaining it was found that if the gels were allowed to stain overnight at room temperature and then destained slowly in 10% trichloroacetic acid the band intensities increased and minor bands became visible.

I. Peptidase Activity on Polyacrylamide Gels

Polyacrylamide gels were prepared and run as previously described. Protein samples for electrophoresis were prepared as follows: To 100 ul of dialyzed crude cell extract in running gel buffer was added 50 ul of distilled water and

15 ul of a 30% v/v glycerol solution containing .0004% bromophenol blue. To the top of each gel was applied 25 ul of the above mixture which contained approximately 300 ug of protein as determined by the method of Lowry et al.[107]

Immediately after electrophoresis the gels were removed from their tubes and stained for peptidase activity. Peptidase activity toward peptides, Ac-Phe and leucineamide were stained for using the method of Lewis and Harris.[112] Freshly prepared solutions contained 2.25 ml of potassium phosphate buffer (0.2M, pH 7.5), 0.3 ml of L-amino acid oxidase (5^{mg}/ml), 0.3 ml of horseradish peroxidase (8^{mg}/ml), 0.1 ml of o-dianisidine dihydrochloride, 5 mg of substrate and 0.05ml of manganous chloride. This solution was prepared by dissolving the substrate in the phosphate buffer followed by the random addition of the other reagents except for the L-amino acid oxidase which was added last. The reaction mixture was mixed with an equal volume of 2% agar at 50°C and poured over a polyacrylamide gel placed in a test tube. Peptidase activity was determined within 90 minutes by the development of brown bands in the agar overlay. Only compounds capable of yielding L-amino acids upon hydrolysis which are substrates for L-amino acid oxidase may be tested by this assay. The band development time was limited to 90 minutes due to background color and band broadening which increased with time and eventually made it impossible to discern bands.

Hydrolytic activity toward leucyl-2-naphthylamide and leucyl-p-nitroanilide was determined as described by Miller and MacKinnon.[10] Leucyl-2-naphthylamide was dissolved in N, N-dimethylformamide ($10^{mg}/ml$) and 0.1 ml of this solution was mixed with 5 ml of Tris buffer (0.2M, pH 7.5) containing 10 mg of Fast Garnet GBC salt. The solution was poured over a gel placed in a test tube and incubated at room temperature for up to 4 hours. Activity bands appeared as red rings on the gels surface. Activity toward leucyl-p-nitroanilide was detected as faint yellow bands on the gels surface. The reaction mixture was prepared by dissolving leucyl-p-nitroanilide in dimethylformamide ($20^{mg}/ml$) 5 ml of Tris buffer (0.2M, pH 7.5) and proceeding as described for leucyl-2-naphthylamide. Both of these assays were also performed in the presence of 3mM Co^{+2}. R_f values for the three procedures described were calculated as the ratio of the distance travelled by the activity band to that of the bromophenol blue dye marker.

J. Peptide Purity Determination

All peptides and their derivatives were shown to be homogeneous by tlc and/or high voltage paper electrophoresis. Thin layer chromatography was performed on silica gel plates in butanol-acetic acid water(4:1:1) or in chloroform-methanol (2:1). The peptides were detected using iodine vapors or ninhydrin. High voltage paper electrophoresis was carried out in a model LT-36 electrophoresis tank with E.C. 123 coolant and a HV-5000 power supply (Savant Instruments Inc.).

Pyridine-acetate buffer (pH 3.5) was prepared from glacial acetic acid-pyridine-water (10:1:189, vol./vol.). For some peptides a pH 1.9 buffer of formic acid-glacial acetic acid-water (6:24:270, vol./vol.) was used. Peptides (50-100 ul of a 2 mg/ml solution) were applied to Whatman 3 MM paper and run at a gradient of 37 V/cm for 1.5 to 3 h. After electrophoresis, the paper was dried, dipped in a solution of ninhydrin (0.5%, wt./vol.) in 95% aqueous acetone, and developed in a heated and ventilated chromatography oven. The ninhydrin stains were occaisionally fixed by dipping the paper in a solution of $Cu(NO_3)_2$ (1%) and HNO_3 (0.2%) in acetone-ethanol (2:1).

K. Molecular Weight Determination

The molecular weight of the dipeptidase was determined by its gel filtration behavior on Sephadex G-150 using a 1.6 x 90 cm column equipped with flow adaptors (products of Pharmacia Fine Chemicals). The Sephadex G-150 was obtained as a dry powder and was subsequently swollen in the standard buffer for 6 hours on a boiling water bath. The resulting suspension was allowed to cool to 4° C and fines removed by several washings with deaerated standard buffer (4° C). The consistency of the slurry was adjusted to allow for proper pouring into the column. The column was poured with the aid of a gel and eluant reservoir. Packing and equilibration of the column for downward flow elution was accomplished by passing four column volumes of standard buffer through the column at a flow rate of 12.5 ml/hr. and a constant

pressure head of 14 cm of water. The final height of the Sephadex bed was 49 cm. Blue Dextran 2000 (0.50ml, $1.0^{mg}/ml$) was applied to the top of the column and eluted ($10^{ml}/hr.$, using a peristaltic pump) in order to check column packing and to determine the column's void volume (the elution volume of a substance which is totally excluded from the gel matrix). Elution of Blue Dextran 2000 as well as that of the proteins was monitored by their OD_{280nm}. After agreement of three successive void volume determinations the column was deemed suitable for determining estimates of molecular weights. Marker proteins obtained from Pharmacia Fine Chemical were applied to the column (0.5ml of protein solution containing $10^{mg}/ml$ of protein), eluted with a peristaltic pump at $10^{ml}/hr.$, and their elution volumes determined. It should be noted that although there were 4 marker proteins (M.W. 13,700 - 158,000) only two of them at a time were run through the column as per manufacturers instructions (see Calibration Kit, Instruction Manual for Protein Molecular Weight Determinations by Sephadex Gel Filtration, Pharmacia Fine Chemicals). The elution volume of the dipeptidase was determined in a similar manner using a portion of the dipeptidase preparation from the ion exchange step which had been concentrated prior to use ($25^{mg}/ml$, applied 0.5ml). The elution volume of the dipeptidase was monitored by both protein OD_{280mn} and by the activity profile of the dipeptidase toward Met-Met.

L. Sub-unit Molecular Weight Determination

The number and molecular weights of the sub-units of the dipeptidase were determined by SDS-polyacrylamide gel electrophoresis. The procedure employed is given in a booklet entitled "Molecular Weight Markers for SDS Polyacryamide Gel Electrophoresis" which is distributed by Gallard-Schlesinger Chemical Mfg. Corp. The procedure is based on the procedure of Weber et al.[113] The molecular weight protein markers used were also a product of this company. The polyacrylamide gels (10% acrylamide, 0.27% N,N' methylene bisacrylamide) were prepard in 12.5 cm long glass tubes (0.50cm diameter) yielding a final gel length of approx. 9cm. Electrophoresis was performed at 8 milliamps per tube at room temperature using sodium phosphate tank buffers (0.1M, pH 7.2) containing 0.1% sodium lauryl sulphate (SDS). This buffer is identical to the gel buffer. Typical protein loads for the dipeptidase were 2,5 and 7.5 ug. Dissociation of the dipeptidase into its sub-units was accomplished by treating the dipeptidase with a 0.01M sodium phosphate buffer pH 7.0, containing 1% SDS and 0.1% 2-mercaptoethanol at 100°C for 3 minutes. After electrophoresis protein staining was performed using the method of Chrambach et al.[111] It should be noted that protein samples must be free of potassium prior to treatment with SDS because potassium will cause the SDS to precipitate.

M. Isoelectric Focusing

Isoelectric focusing using 7% acrylamide gels containing ampholine (LKB Instruments Inc.), pH 5-8 was performed in glass tubes (0.5 x6.5 cm) as described by Wrigley.[114] The dipeptidase (3-10ug in standard buffer) was applied to the top of each gel under a protecting layer of 1% ampholine in 5% sucrose. Electrophoresis was performed with cooling (4°C) at a constant current of 1.5 milliamps per tube and a maximum total voltage of 400 volts. It was determined from previous experiments using a semi-purified dipeptidase fraction that the time required for isoelectric focusing was 2.5 hours. Therefore, the purified dipeptidase was subjected to isoelectric focusing for 3.5 to 5 hours without measurable change in the isoelectric point of the dipeptidase. After isoelectric focusing the gels were removed from their tubes and cut in half the long way. One half was immersed in 5% trichloracetic acid for 30 minutes. Protein bands appear as white bands in the gel. In order to insure that all proteins in the gel are seen a more laborious staining procedure was also performed. The sectioned gel from the 5% trichloracetric acid was permitted to remain in this solution for another 1.5 hours with shaking and then the solution replaced 3 times with an identical solution every 2 hours. The gels were then stained by the method of Chrambach et al.[111] Identical results were obtained from the

two methods for the dipeptidase. The isoelectric point of the dipeptidase was determined using the other half of the gel by measuring the pH of the gels surface with a surface electrode (Brinkman Instrument Co.) and comparing this with the position of the stained protein band.

N. Determination of Kinetic Parameters

Initial rate data for dipeptides was obtained at various dipeptide concentrations as described under Dipeptidase Activity. The purified dipeptidase which had been stored frozen in glycerol (20% $^{v}/_{v}$) was thawed in an ice-water bath and 5ul portions were used to initiate the reactions.

Preliminary data obtained was fit to two linearized forms of the Michaelis-Menten equation. It became apparent that deviations from linearity for both Lineweaver-Burk and Eadie-Hofstee plots occurred for some substrates. Due to the observed deviations the rate data was fit directly to the conventional Michaelis-Menten equation ($V = \frac{Vmax\ S}{Kapp + S}$) and to the Hill equation with a constant Hill coefficient ($V = \frac{Vmax\ S^{nH}}{Kapp + S^{nH}}$). The latter equation was employed since it was possible that the observed deviations might be due to an allosteric effect. It must be kept in mind that the value of Kapp is equal to the Michaelis-Menten constant Km in the conventional equation but not in the Hill equation when $n_H \neq 1$. In the latter case Kapp is related to the substrate concentration at half saturation by $S_{0.5}^{nH}$ = Kapp.

Computer methods were used to evaluate the kinetic

parameters, Vmax, Kapp, and n_H directly from saturation data. The computer program (BMDP 3R) was obtained from the Biomedical Computer Programs book (BMDP), which is a compilation of various computer programs by the Health Sciences Computing Facilility at the University of California, Los Angeles, California. The program involves a nonlinear regression analysis technique using the method of least squares to determine optimal values for Vmax, Kapp and n_H (Hill equation only) by alternately minimizing the residual sums of squares of these kinetic parameters by an iterative search procedure. The user must supply the following information; the partial derivatives of each kinetic parameter to be determined, values of the initial velocity with the corresponding substrate concentrations, and initial estimates for the parameters to be determined. The initial estimates for Vmax and Kapp were obtained from Lineweaver-Burk plots and the starting value for n_H was always 1.0. However, the computer program was routinely run by setting Vmax=30 U/ml and Kapp = 0.2mM. The final kinetic parameters obtained with or without more precise starting values were identical, and the only difference being the number of iterations required.

Routinely, each saturation curve was plotted from data obtained for 7 to 12 different substrate concentrations each of which was performed in duplicate. Computer programs were run with and without weighting factors. The weighting

factor of choice was one over the observed velocity squared $(^1/v)^2$ which resulted in greatly reduced values for the residual sums of squares without significantly altering the kinetic parameters. Due to the small number of data points used to plot the curves it was not possible to obtain a rigorous statistical evaluation regarding comparisons of goodness of fit to either the Michaelis-Menten or Hill equations. Therefore, judgement regarding the fit of experimental data to each of these equations was made by comparison of their correlation coefficients (r).

O. Chemicals

Ac-Met-Met-Met, Ac-Met-Met, and Met-Met-Met-OMe were gifts of Dr. Fred Naider, The College of Staten Island, C.U.N.Y. Glu-Met was a gift from Dr. Khan at the same address.

Met-Gly, Gly-Met, Met-Ala, Met-Gly-Met, Met-Ala-Met, and Gly-Met-Gly were purchased from Schwarz/Mann.

Gly-Pro and Ser-Met were purchased from Vega-Fox.

Ac-Phe, Leu-NH_2, Leu-p-nitroanilide, Leu-2-naphthylamide, Tris Buffer, Glycine and the reagents used in the polyacrylamide gel electrophoresis-coupled peptidase staining techniques were purchased from Sigma Chemical Co.

All other peptides, their derivatives and amino acid derivatives were purchased from Bachem.

Reagents used for polyacrylamide gel electrophoresis were purchased from Bio-Rad Laboratories.

Ampholine was purchased from LKB Instruments Inc.

Sephacryl S-200, DEAE-Sephadex, Sephadex G-150, and the protein markers used for molecular weight estimations by gel filtration were purchased from Pharmacia Fine Chemicals.

All other reagents were of ACS reagent grade.

III. RESULTS

A. Activity Gel Staining

The results of the polyacrylamide gel electrophoresis-coupled peptidase staining method are tabulated in Table 3. A photograph of an actual staining pattern for Leu-Leu-Leu and a schematic representation is shown in Figure 1. The true activity band-widths are more closely represented by those shown in the schematic. The discrepancy between those seen in the photograph and those depicted in the schematic is due to differences in the times required for each activity band to develop. Bands which appear first tend to diffuse while other bands are still developing. There are at least four distinct aminopeptidase activities in *S. cerevisiae* Z1-2D as demonstrated by their ability to cleave several unblocked tripeptides, Met-Met-Met-OMe, Leu-Gly-NH_2 and their inability to cleave Ac-Met-Met-Met, and Ac-Phe. The only dipeptidase activity found resides in band 3. Since activity in band 3 was found toward both dipeptides and tripeptides it was suspected that this band was composed of at least one aminopeptidase and one dipeptidase. The dipeptidase and aminopeptidase were subsequently separated by hydroxylapatite chromatography. Both activities had the same R_f as band 3 when examined by the enzyme-coupled activity stain on polyacrylamide gels.

No carboxypeptidase activity was found under the standard assay conditions as described in Materials and Methods. It is well known however, that cell-extracts of

TABLE 3 - PEPTIDASE SPECIFICITY PATTERNS

Cell-extracts of S. cerevisiae Z1-2D were electrophoresed in polyacrylamide gels and stained for peptidase activity as described under Materials and Methods. The formation of brown bands in the agar overlay was indicative of a positive (+) response. Bands 1-4 correspond to those shown in Fig. 1.

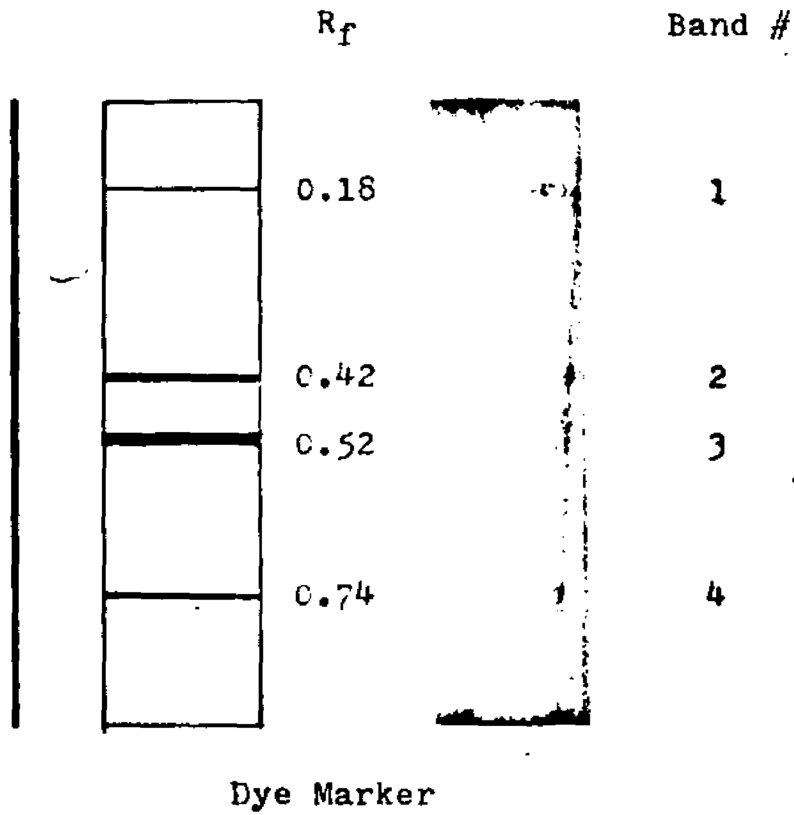
Substrate	Peptidase Activity Gel Bands			
	1	2	3	4
Met-Met-Met	+	+	+	+
Met-Gly-Met	+	+	+	+
Leu-Leu-Leu	+	+	+	+
Met-Ala-Met	+	+	+	+
Met-Gly-Gly	+	-	-	+
Gly-Met-Gly	+	-	+	-
Gly-Leu-Gly	+	-	+	-
Ac-Met-Met-Met	-	-	-	-
Met-Met-Met-OMe	+	+	+	+
Met-Met	-	-	+	-
Met-Leu	-	-	+	-
Leu-Met	-	-	+	-
Met-Gly	-	-	+	-
Gly-Met	-	-	+	-
Met-Ala	-	-	+	-
Leu-Leu	-	-	+	-
Leu-Gly	-	-	+	-
Gly-Leu	-	-	+	-
Met-Pro	-	-	-	-
Pro-Met	-	-	+	-
Leu-Pro	-	-	-	-
Pro-Leu	-	-	+	-
Ac-Met-Met	-	-	-	-
Ac-Gly-Leu	-	-	-	-
Ac-Phe	-	-	-	-
Leu-Gly-NH_2	+	+	-	-
Leu-NH_2	-	-	-	-
Cbz-Phe-Leu	-	-	-	-
Cbz-Leu-Leu	-	-	-	-
L-Leu-D-Leu	-	-	-	-
D-Leu-L-Leu	-	-	-	-

Figure 1

Peptidase Staining Pattern for Leu-Leu-Leu

$$R_f = \frac{\text{Distance traveled by the activity band}}{\text{Distance traveled by the dye marker}}$$

Figure 1



S. cerevisiae contain carboxypeptidase activity, which must be activated prior to assay.[85] Activation of cell-extracts and demonstration of carboxypeptidase activity toward Z-Phe-Leu was accomplished in solution using the procedures described by Hayashi et al. and Hayashi.[84,85] The activated cell-extract was then dialyzed against the tris buffer used for preparing protein samples for electrophoresis and the assay was carried out. However, no carboxypeptidase activity was observed on the polyacrylamide gels. The exact reason for the discrepancy between solution and gel assays is not known. It is suspected, however, to be a pH related problem since yeast carboxypeptidase activity toward amine-terminus blocked dipeptides is usually maximum at pH values below 7.0[76,77]

The peptidase activities in bands 1 through 4 each cleaved Met-Met-Met, Met-Gly-Met, Leu-Leu-Leu, and Met-Met-Met-OMe thus demonstrating overlapping substrate specificities. More important however, is the fact that they can be distinguished from one another by their electrophoretic mobilities and their action on Met-Gly-Gly, Gly-Met-Gly and Leu-Gly-NH_2. The peptidase activity found in band 1 cleaves all of these substrates and seems to have the broadest specificity. Aminopeptidase activity in bands 2, 3 and 4 each cleaves only one of these substrates and in each case it is a different one. It is believed that each of these peptidase activities belongs to a different and distinct aminopeptidase.

The response toward Gly-Met-Gly represents a very interesting case. In order for this peptide to yield an activity band it must undergo a two step reaction because glycine is not a substrate for the L-amino acid oxidase contained in the activity staining mixture. Therefore, Gly-Met-Gly must first be broken down to glycine and a dipeptide, followed by cleavage of the dipeptide to liberate methionine. In light of this, one must explain the positive result for bands 1 and 3. For band 3, the idea of two separate hydrolysis reactions by two different peptidases is plausible since this band contains both amino- and dipeptidase activities. However, this does not explain the activity observed for band 1 which under assay conditions does not cleave any of the dipeptides tested including Met-Gly. It is proposed that a positive response for Gly-Met-Gly from band 1 involves a two step hydrolysis reaction by the same aminopeptidase. The first step involves cleavage of the tripeptide to glycine and Met-Gly followed by hydrolysis of the dipeptide by the aminopeptidase itself. It is well known that yeast aminopeptidases are capable of cleaving dipeptides[9,53,58] The negative response of the aminopeptidases here to the dipeptides tested may be due in part to the assay conditions employed and the time required for the assay. The unexpected positive response of activity band 1 toward Gly-Met-Gly may be explained by assuming that the active site for both di- and tripeptide hydrolysis are identical. Cleavage of Gly-Met-Gly produces Met-Gly at the active site and then the dipeptide, which

is present at a high local concentration is cleaved to liberate glycine and L-methionine. The L-methionine released is responsible for the positive response observed. Similar results were also observed with Gly-Leu-Gly. In order to check our hypothesis for the activity in band 1, the dipeptides Met-Gly and Leu-Gly were used in the peptidase assay at 5 times, 10 times and 30 times the standard concentration (5mg peptide per assay). There was still, however, no cleavage of either dipeptide by the activity in band 1. These results do not necessarily negate our hypothesis since it may not be possible to achieve both the high concentration and proper orientation of the dipeptide in the active site of the enzyme by merely supplying it at a high concentration in the assay mixture.

A single dipeptidase activity was found for S. cerevisiae Z1-2D and resides in band 3. All free dipeptides which were tested except Met-Pro, Leu-Pro, L-Leu-D-Leu, and D-Leu-L-Leu are cleaved to their constituent amino acids. The failure of the dipeptidase to cleave X-Pro and L, D or D, L dipeptides is due to this enzymes requirement for an amide bond and its stereospecificity. This specificity has also been observed for several dipeptidases in bacteria[6,8,12] and for a purified yeast dipeptidase.[68] All blocked dipeptides tested are not cleaved by the activity in band 3 illustrating the requirement for both a free amino- and carboxy-, terminus. This specificity is expected for true dipeptidase character.[1] The above observations have been confirmed on gels and in solution for

a purified form of this dipeptidase as will be described shortly.

Matile *et al.*[21] reported four aminopeptidase activities for *S. cerevisiae* cells grown to stationary phase. These activities were measured by starch-gel electrophoresis coupled with an activity stain using leucyl-2-naphthylamide, a non-peptide substrate, in the presence of 3 mM Co^{+2}. The use of substrates other than peptides to monitor peptidase activity may lead to erroneous conclusions because these substrates are susceptible to hydrolysis by enzymes which are not true peptidases. A case was recently cited by Payne[115] regarding the alleged occurrence of an inducible periplasmic aminopeptidase in *Escherichia coli* based on activity toward p-nitroanilide and 2-naphthylamide amino acid derivatives. Although a recent report by Lazdunski and coworkers[116] shows that a number of di- and higher peptides are cleaved by this periplasmic enzyme, extremely high substrate concentrations were necessary for the cleavage of dialanine. It is thus not clear whether activity toward amino acid derivatives will always correlate with peptidase activity.

In order to investigate this point further, we tested the activity of cell-extracts of *S. cerevisiae* Z1-2D against leucyl-2-naphthylamide and leucyl-p-nitroanilide, in the presence and absence of 3 mM Co^{+2}, using the procedure of Miller and MacKinnon.[10] Only two activity bands, having R_f values similar to bands 1 and 2 as determined by the polyacrylamide gel electrophoresis-coupled activity staining procedure,

were observed. These results suggest that at least two of the aminopeptidase activities found in this study do not cleave amino acid derivatives. It is reasonable to conclude, therefore, that some of the activities reported by Matile and coworkers[21] differ from the aminopeptidase activities in bands 1 through 4.

B. Purification

Crude cell-extract was prepared as described in the Materials and Methods section. The cell-extract was purified by several methods and the results summarized in Table 4. The activities shown refer to Met-Met hydrolysis under standard assay conditions (see Materials and Methods). Results from the various purification procedures are illustrated in Figs. 2-5. Dipeptidase activity was monitored by following the quantitative hydrolysis of Met-Met, and Leu-Gly as outlined in the Materials and Methods section. The hydrolysis of Met-Met-Met was also measured in the vicinity of the dipeptidase activity by the same fluorescence method used for dipeptides. No attempt was made, however, to correlate the fluorescence intensities with the hydrolysis products of Met-Met-Met. None of the purification procedures was able to separate the dipeptidase activity toward the two dipeptides tested suggesting the presence of only one dipeptidase. The one problem associated with the purification is the rather large loss of activity units following chromatography on hydroxylapatite. The reason for this is not clear, but may be due to the loss of a metal ion cofactor. However, the

TABLE 4 - PURIFICATION OF THE DIPEPTIDASE

Procedure	Vol. (ml)	Protein (mg/ml)	Total Protein (mg)	Activity (U/ml)	Total Activity (U)	Specific Activity (U/mg)	Yield (%)	Purification
Dialyzed Crude Extract	2360	9	21240	9	21240	1	100	1
Ammonium sulfate precipitate	222	30.5	6771	90	19980	3	94	3
Sephacryl S-200	370	10	3700	50	18500	5	87	5
DEAE-Sephadex	110	1.23	135	150	16500	122	78	122
Hydroxyapatite*	24	.145	3.45	49	1180	342	51***	342
Preparative Polyacrylamide gel electrophoresis** Fraction 16	4	.04	.16	21.2	85	530	26***	530
Other active fractions from the last step above	20	Varies w/r fraction #	.66	7.5	150	Varies w/r fraction #	45***	Varies w/r fraction #

* Applied 15.5 ml from DEAE-Sephadex step (2320 U)

** Concentrated proteins from hydroxyapatite step to 1 mg/ml and applied 1.5 ml (330 U)

*** Recovery as a % of the amount of units applied

Figure 2

Gel Filtration on Sephacryl S-200

222 ml from the ammonium sulfate step was applied to a 5x90 cm column of Sephacryl S-200 equilibrated with 40mM potassium phosphate buffer, pH 7.0 and eluted at 1.4 ml/min. 6.1 ml fractions were collected and assayed for activity toward Met-Met (· — · —), Leu-Gly (o — o —), and Met-Met-Met (x — x —). Protein elution (solid line) was monitored continuously at 280nm.

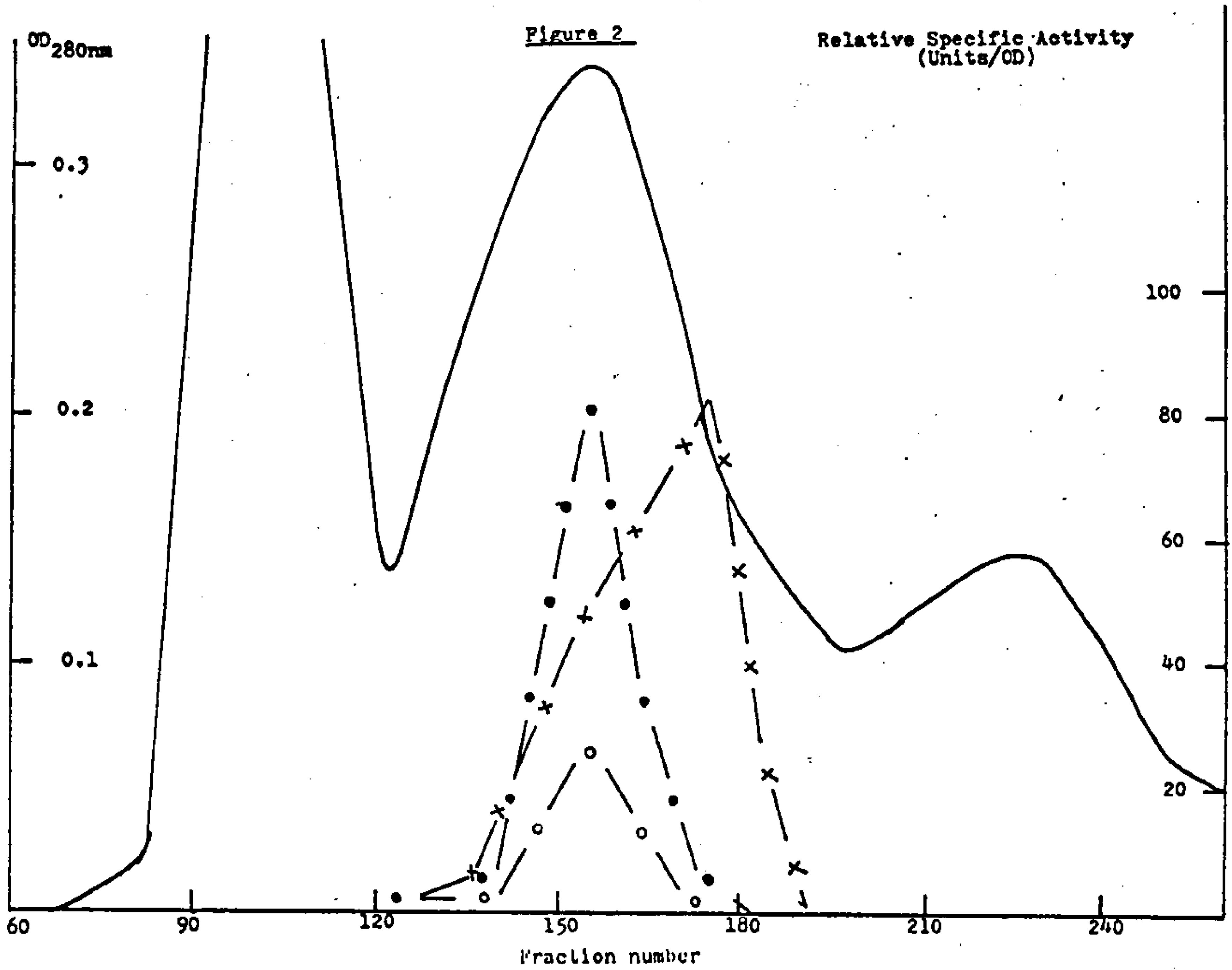


Figure 2
OD280nm
0.3
0.2
0.1
Relative Specific Activity
(Units/OD)
100
80
60
40
20
60
90
120
150
180
210
240
Fraction number

Figure 3

Chromatography on DEAE-Sephadex A-50

The combined dipeptidase fractions from the gel filtration step (370ml) were applied to a 2.6x38cm column of DEAE-Sephadex A-50 equilibrated with standard phosphate buffer at 0.35 ml/min. and elution performed in stages using linear NaCl gradients (— — —) as described under Materials and Methods. 5 ml fractions were collected and assayed for activity towards Met-Met (• — • —), Leu-Gly (o — o —) and Met-Met-Met (x — x —). Protein measurements (solid line) were performed continuously at 280nm.

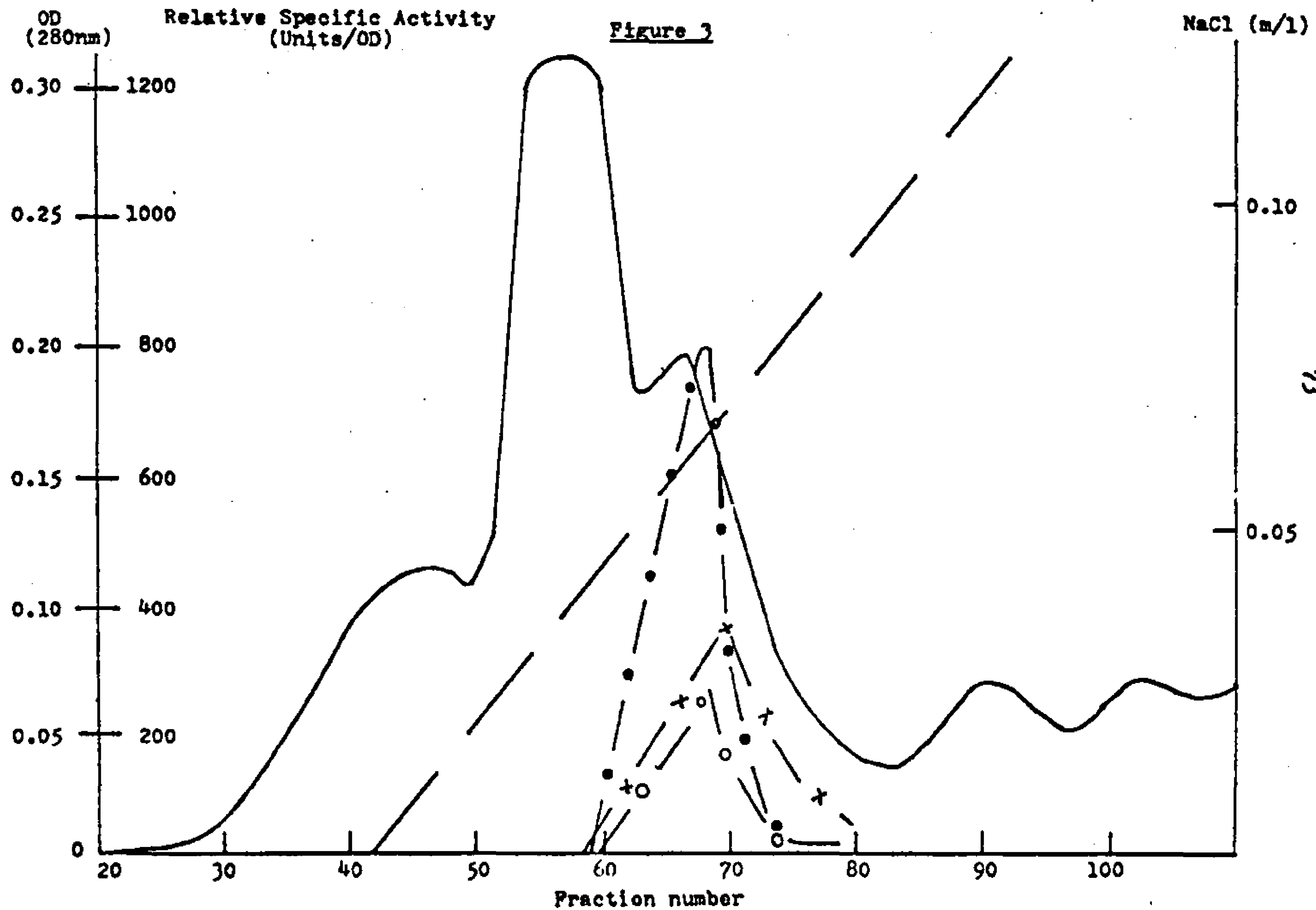


Figure 3

Figure 4

Chromatography on Hydroxylapatite

15.5 ml of dialyzed protein solution (4mM potassium phosphate buffer, pH 7.0) was applied to a 0.9 x 14.5 cm column of spheroidal hydroxylapatite. Experimental details are given in the Materials and Methods section. Dipeptidase activity was eluted (1.5ml fractions) with 10mM postassium phosphate buffer, pH 7.0. The following were assayed for; protein (solid line) Met-Met (•—•—), Leu-Gly (o—o—) and Met-Met-Met (x— x—).

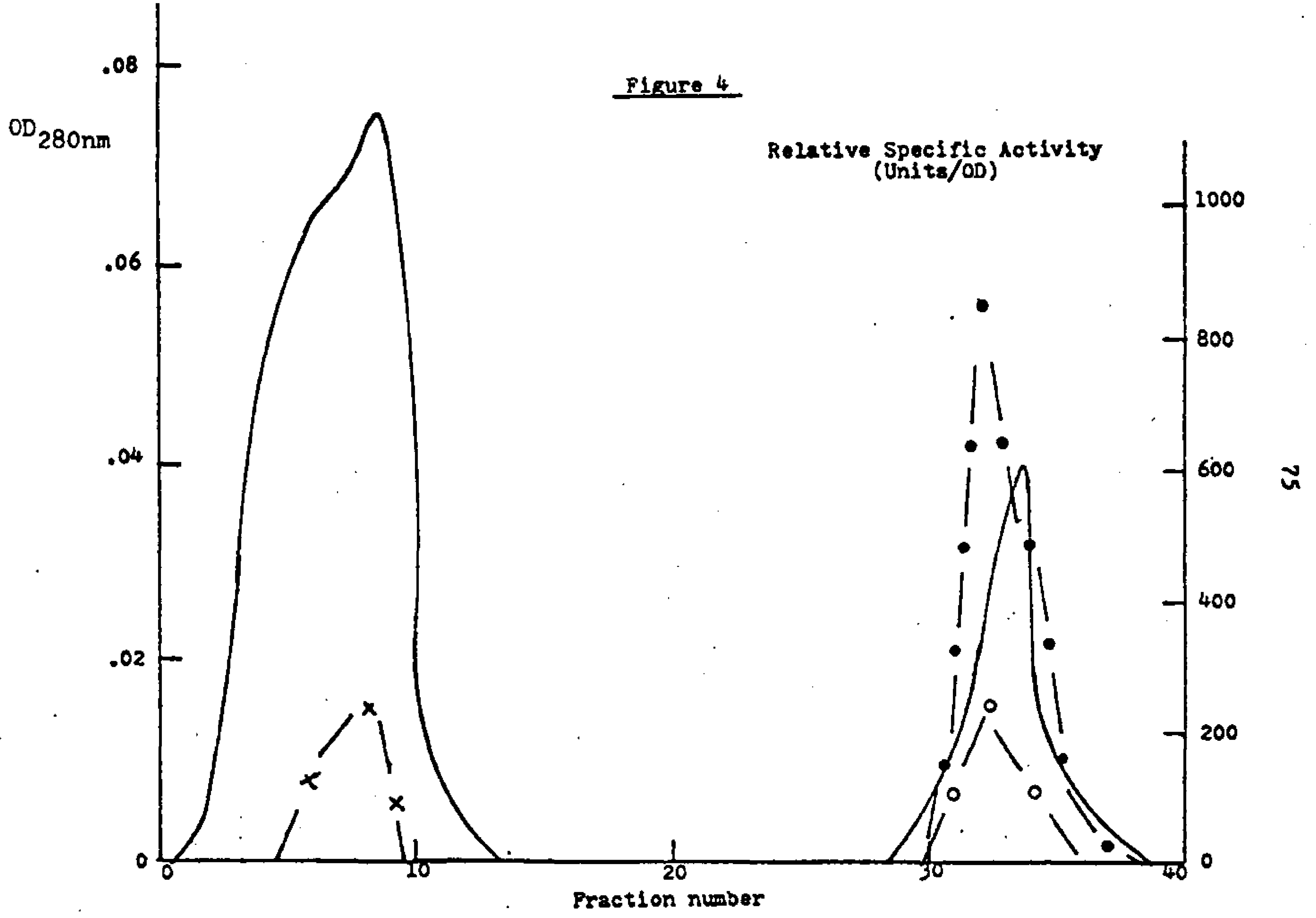
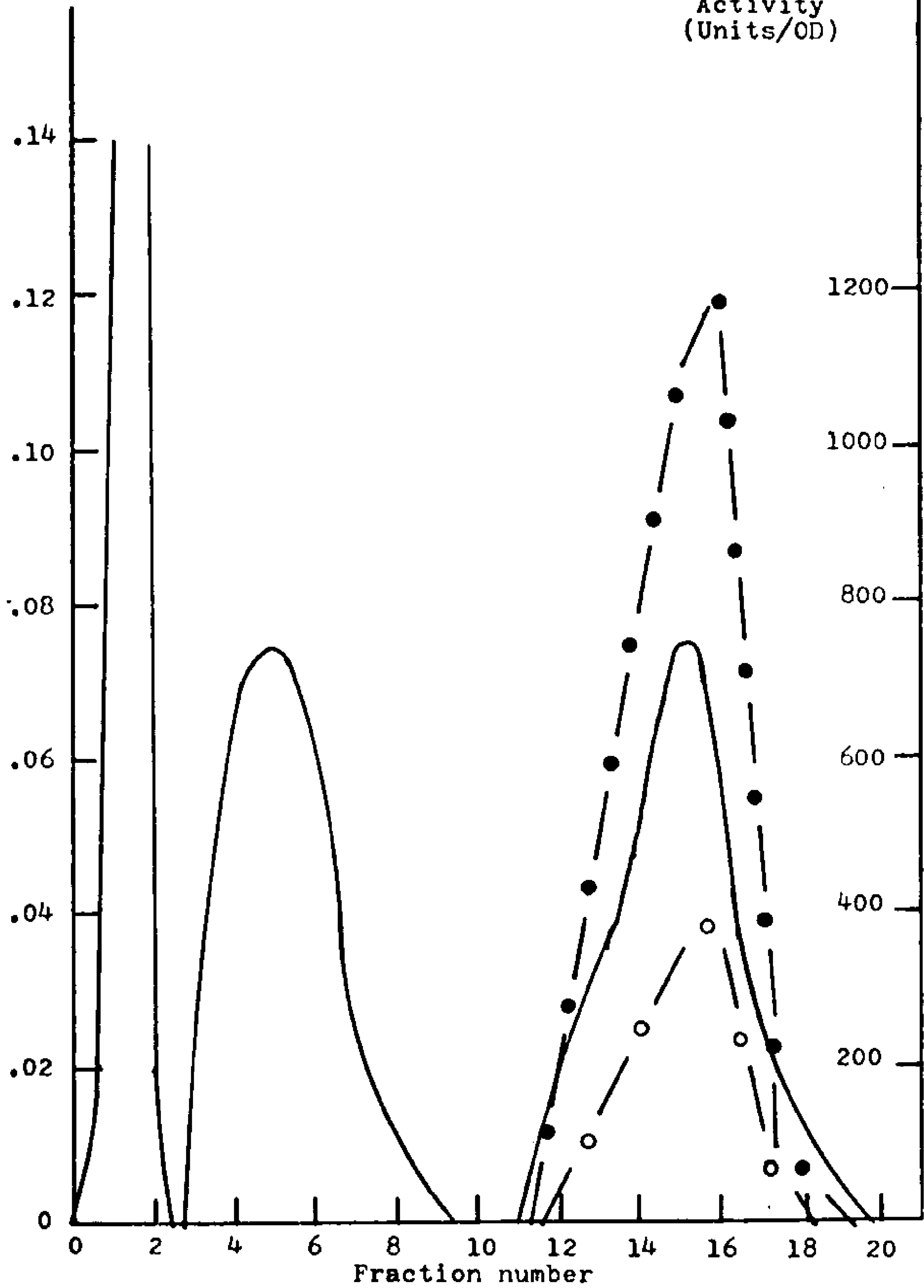


Figure 4
OD280nm
.08
.06
.04
.02
0
Relative Specific Activity
(Units/OD)
1000
800
600
400
200
0
0
10
20
30
40
Fraction number

Figure 5

Preparative Polyacrylamide Gel Electrophoresis

Dieptidase activity toward Met-Met (• —— • ——) and Leu-Gly (• —— • ——) as well as the OD_{280nm} are plotted vs. fraction number. None of the fractions collected had oligopeptidase activity (not cleaved; Met-Met-Met, Gly-Gly-Gly, Leu-Leu-Leu). The large peak appearing for fractions 1 and 2 correspond to the bromophenol blue dye marker. The protein solution applied to the preparative polyacrylamide gel was from the hydroxylapatite step and had been dialyzed against Tris buffer and concentrated prior to use. (See Materials and Methods for experimental details).



OD280nm
Figure 5
Relative Specific Activity (Units/OD)
.14
.12
.10
.08
.06
.04
.02
0
1200
1000
800
600
400
200
0
2
4
6
8
10
12
14
16
18
20
Fraction number

hydroxylapatite step was necessary in order to separate the dipeptidase from the tripeptidase activity prior to final purification of the dipeptidase by preparative polyacrylamide gel electrophoresis.

As seen in Table 4, fraction 16 from the preparative gel electrophoresis step had the highest specific activity but accounted for only 26% of the activity applied. Another 45% of the total activity units could be accounted for by other fractions. Two of these fractions had lower specific activities than fraction 16 but were also homogeneous by gel electrophoresis. The reason for the apparent difference between the various fractions is not clear although it may be due in part to errors in protein measurements at the low protein levels present or to subtle differences between these fractions.

Purity of the dipeptidase was indicated by the presence of a single band in analytical polyacrylamide gel electrophoresis (Fig. 6). Further evidence for purity is the presence of a single band in isoelectric focusing of the dipeptidase on polyacrylamide gels and the agreement between molecular weight studies conducted on the intact dipeptidase and those conducted in the presence of SDS.

C. Isoelectric Focusing

Isoelectric focusing of the purified dipeptidase was accomplished using 7% polyacrylamide gels containing ampholine (LKB Instruments Inc.), pH 5-8. A single band was observed having an isoelectric point of 6.5. An

Figure 6

Polyacrylamide Gel Electrophoresis of Crude Semi-purified, and Purified Yeast Dipeptidase

Proteins were separated on polyacrylamide gels (0.5x6.5cm, 7% acrylamide) by a method similar to that of Davis[110] (see Materials and Methods for experimental details). Electrophoresis was performed at 1 milliamp per tube with migration toward the anode. Protein staining was performed by the method of Chrombach et al[111] using Coomassie blue and the gels were photographed using a red filter.

A. Dialyzed crude cell extract (250ug)

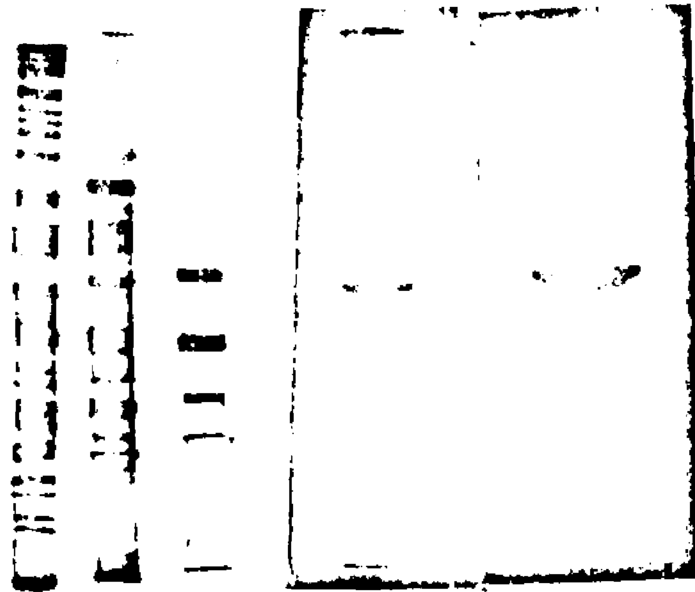
B. Pooled Sephacryl S-200 fractions (100ug)

C. Pooled DEAE-Sephadex fractions (40ug)

D. Fraction 16 from the preparative polyacrylamide gel electrophoresis step (10ug)

E. Close-up of D

Figure 6



interesting point regarding isoelectric focusing of the dipeptidase resides in the fact that dipeptidase activity could not be detected on the gels using the gel activity staining method described earlier. This may be due to interference by the carrier ampholytes with the peptidase assay. However, isoelectric focusing in a granular gel (Multiphor System, LKB Instruments Inc.) of some semi-purified dipeptidase from the ion exchange step resulted in approx. a 90% loss of specific activity under standard assay conditions (ampholine and granular gel were removed prior to assay). Therefore, the loss in activity seems to be due to the isoelectric focusing procedure but the exact cause is not known. Similar behavior was also noted by Rohm[68] for a purified yeast dipeptidase.

D. Molecular Weight and Sub-Unit Molecular Weight

The molecular weight of the dipeptidase was determined by its gel filtration behavior on Sephadex G-150 using several marker proteins. The results are given in Fig. 7 as a plot of K_{av} ($K_{av} = \frac{Ve-Vo}{Vt-Vo}$; Ve = elution volume of the protein, Vo = elution volume for Blue Dextran 2000, and V_t = total bed volume of the Sephadex G-150) vs the log of the proteins molecular weight. The dipeptidase had a K_{av} value of 0.306 yielding a molecular weight of 141,000.

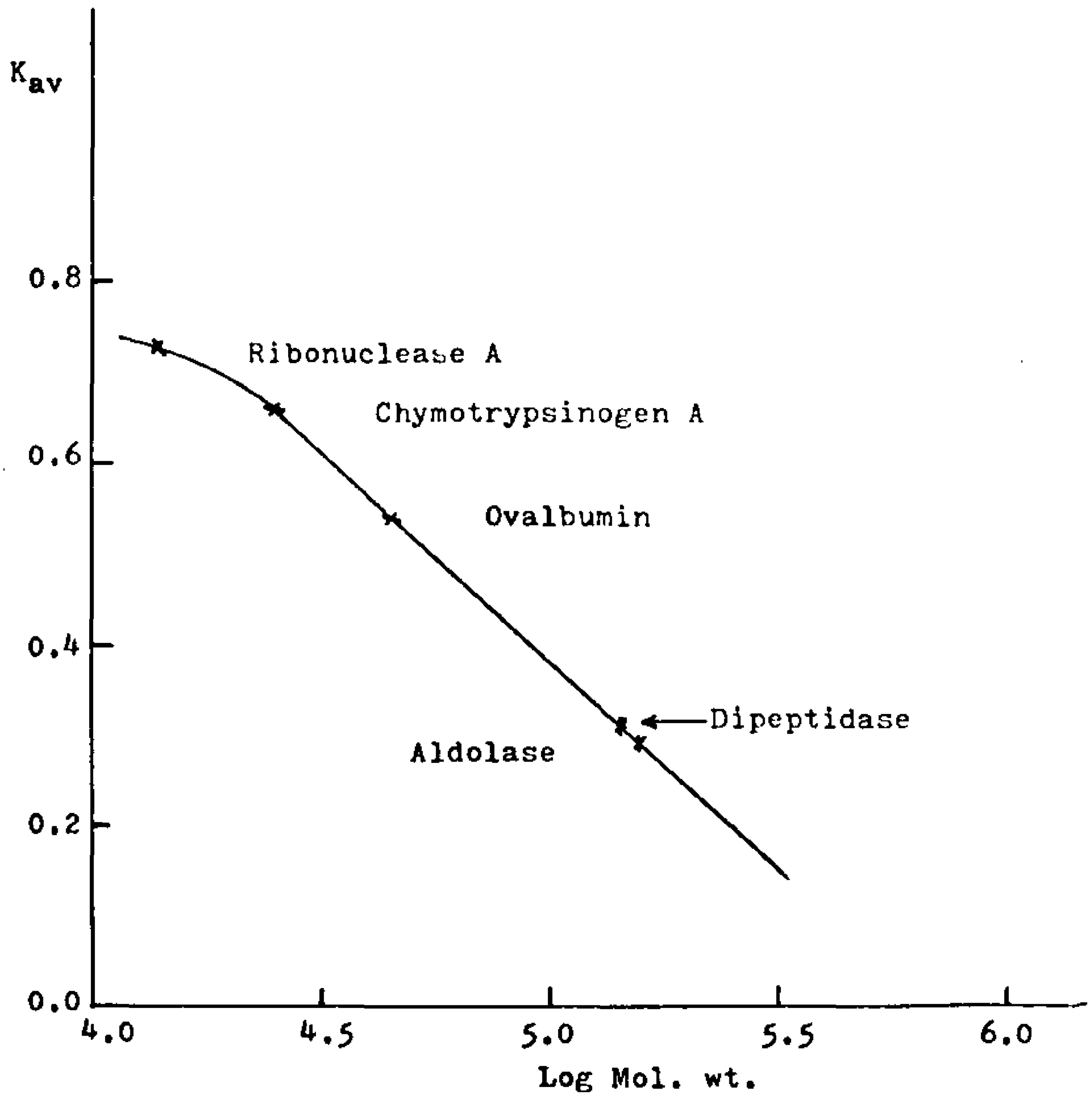
Due to the molecular weight found above and the fact that most large proteins (M.W.> 100,000) are not monomers the possibility of the presence of subunits was investigated. This was accomplished by subjecting purified dipeptidase to

Figure 7

Molecular Weight Determination of Yeast Dipeptidase by Gel Filtration on Sephadex G-150

The elution volumes for dipeptidase activity (DEAE-Sephadex fraction) and for several reference proteins were determined as described under Materials and Methods. A plot of their K_{av} ($K_{av} = \frac{Ve-Vo}{Vt-Vo}$, Ve = protein elution volume, Vo = elution volume of a totally excluded molecule, Blue Dextran 2000, and Vt = total bed volume of Sephadex G-150) values vs. the logarithms of their molecular weights are given in the figure. The marker proteins had the following molecular weights; Ribonuclease A, 13,700; Chymotrypsinogen A, 25,000; Ovalbumin, 45,000; Aldolase, 158,000.

Figure 7



SDS gel electrophoresis. The results are shown in Fig. 8 as a plot of log molecular weight vs % mobility (% mobility = $\frac{\text{distance moved by the protein}}{\text{distance moved by the tracking dye}}$ x 100). A single band was found whose molecular weight was approx. 35,000. Thus, the dipeptidase is composed of 4 subunits having equal molecular weights.

E. pH Profiles

Two types of activity - pH profiles were determined and their results shown in Fig. 9. In both cases Met-Met was the substrate and activities were measured by the standard assay procedure. For curve A the activity was tested at the pH values given whereas for curve B the enzymes was pre-incubated for 2 minutes at the pH value shown, allowed to recover for 2 minutes at pH 7.0, and the activity determined at pH 7.0. The dipeptidase is active between pH values of 4.5-10.5 with an apparent pH optimum at 8.0 (Curve A). The decrease in activity observed for the acidic branch of Curve A is probably due to the irreversible inactivation of the dipeptidase as evidenced by the acidic portion of Curve B. However, comparison of the basic portions of these two curves shows that the decrease in the observed activity for Curve A is not due to the destruction of the enzyme. It may be ascribed to a pH effect on the ionizable groups of the substrate and/or of the active site of the enzyme.

The effect of preincubation of the dipeptidase at different pH values, Curve B, is somewhat unusual. In going

Figure 8

Sub-unit Molecular Weight Determination of Yeast Dipeptidase by SDS-Polyacrylamide Gel Electrophoresis

SDS-polyacrylamide gels (0.5x9 cm, 10% acrylamide) were prepared as described under Materials and Methods and electrophoresis carried out at 8 milliamps per tube. Protein loads were prepared from 2-7.5 ug of the dipeptidase, which was previously dissociated into its sub-units by treatment with 1% SDS and 0.1% 2-mercaptoethanol in sodium phosphate buffer (10mM, pH 7.0) for 3 minutes at 100°C. The molecular weight markers (cross-linked proteins) were purchased from Gallard-Schlesinger. These 5 markers had molecular weights covering the range from 14,300 to 71,500. A plot of the logarithms of their molecular weights vs % mobility

$$\left(\frac{\text{Distance moved by the protein}}{\text{Distance moved by the dye marker}} \times 100 \right)$$

was constructed and the sub-unit molecular weight of the dipeptidase determined by the comparison of its % mobility with that of the markers.

Figure 8

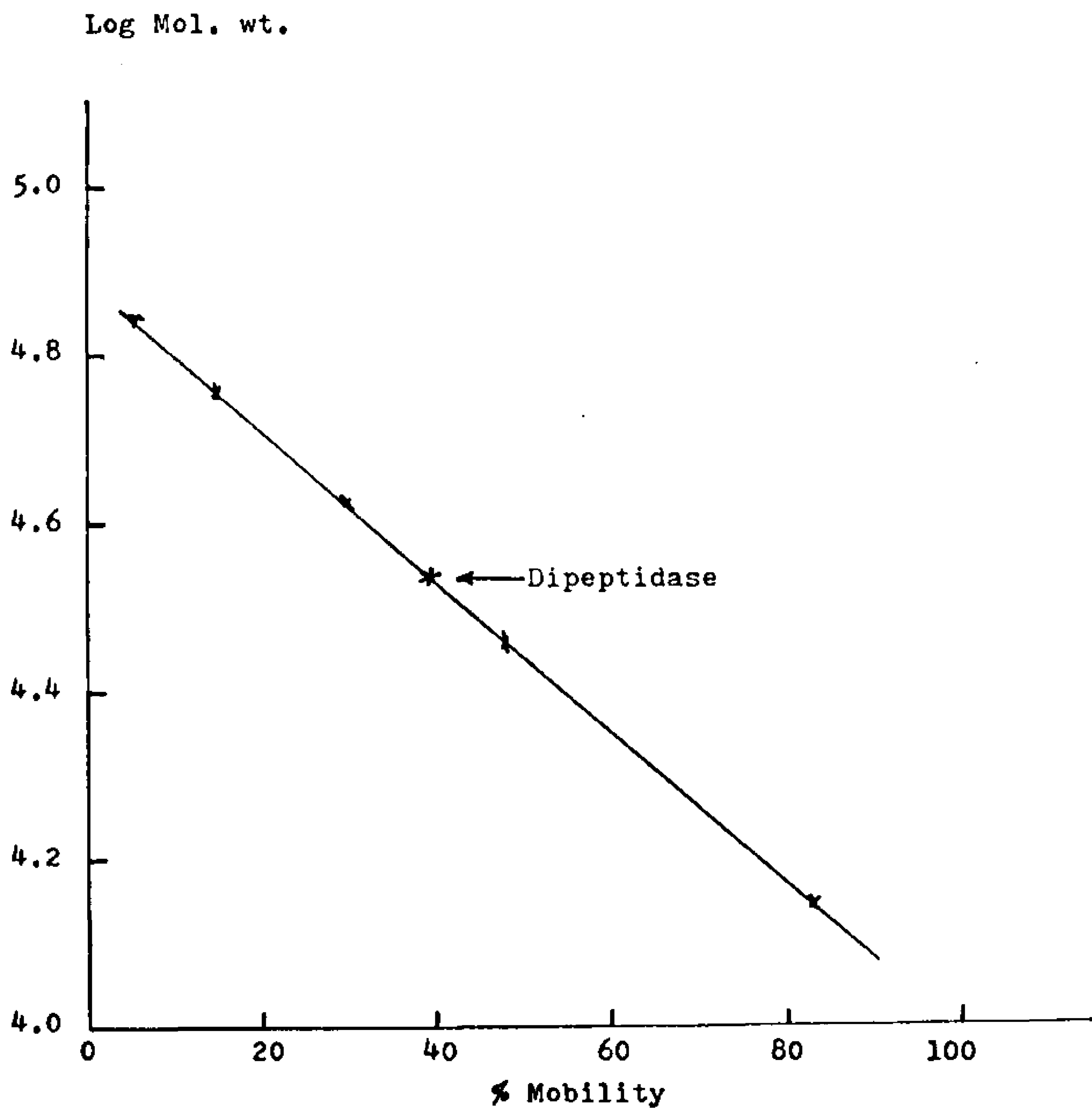


Figure 9

pH Activity Profiles of Yeast Dipeptidase

pH profiles for the dipeptidase were determined under different conditions. Curve A was obtained from data in which the activity was measured at the pH given. Curve B represents data obtained by pre-incubating the dipeptidase at the pH given and then conducting the assay at pH 7.0. Activities were measured using Met-Met as the substrate. The experimental details are given in the Results section.

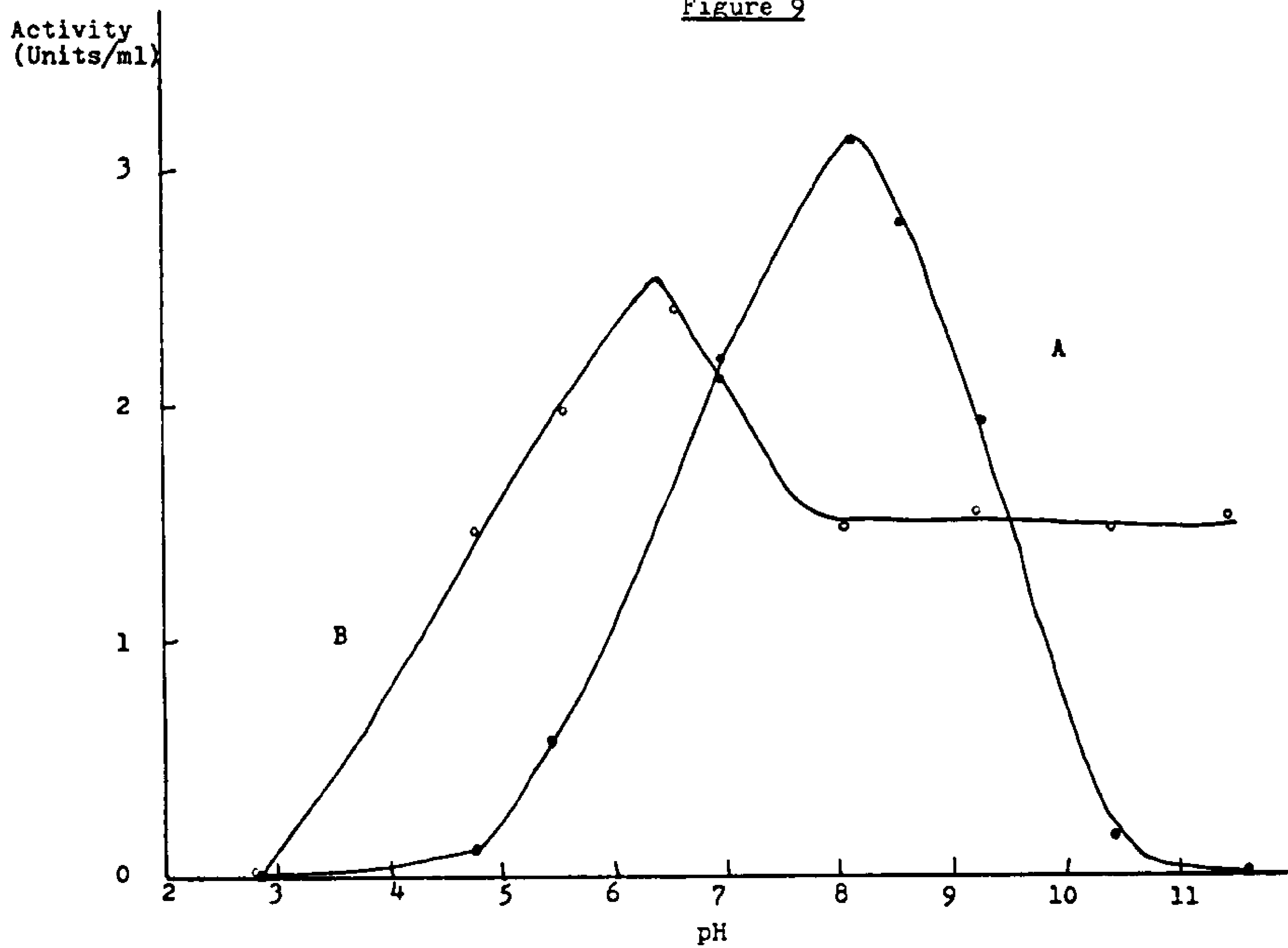


Figure 9

from low to higher pH values the curve first rises, peaks at approx. pH 6.3, then decreases reaching a minimum at approx. pH 8 and finally levels off. The decrease in activity between pH 6.3 and 8.0 may be due to the existence of different conformational forms of the dipeptidase. This point will be discussed further in the next section.

F. Effect of Metal Ions

Since most dipeptidases have been shown to be metalloenzymes the effects of several divalent metal ions on the purified dipeptidase were determined. The results given in Table 5 show a complex pattern of metal ion activation and inhibition depending upon both the divalent metal ion and the dipeptide present in the assay mixture. It is found that the hydrolysis of Met-X dipeptides is accelerated by Zn^{+2} and retarded by Co^{+2}. Mn^{+2} and Mg^{+2} are either deactivating or slightly activating toward these substrates. A very different situation is found for Gly-X and Ala-X dipeptides. The cleavage of Gly-X dipeptides is inhibited by Zn^{+2}, Mn^{+2}, and Mg^{+2}. The degree of inhibition by Zn^{+2} and Mg^{+2} are similar whereas Mn^{+2} has little or no effect. Co^{+2}, however, is strongly activating toward the cleavage of Gly-X dipeptides. For Ala-X dipeptides it is found that Zn^{+2}, Co^{+2}, and Mn^{+2} are deactivating with $Co^{+2}$and Mn^{+2} being better inhibitors than Zn^{+2}. Mg^{+2} has a small activating effect upon the hydrolysis of Ala-Met. From these observations alone it is difficult to ascertain the role that metal ions play in the activation or inhibition of the dipeptidases

TABLE 5. METAL ION EFFECTS

The dipeptidase(5ul) was incubated for 1 minute in 0.9ml of 40mM potassium phosphate buffer, 30°C, pH 7.0, containing either
a) no added metal, b)0.1mM Zn $(CH_3CO_2)_2$, c) .1mM $CoCl_2$
d)0.1mM $MnCl_2$ e)0.1mM $MgCl_2$
The reaction was begun by the addition of 0.1ml of pre-warmed Met-Met, 10mM. The final concentrations of Met-Met and metal ion are 1mM and0.09mM respectfully. Velocities ($U/_{ml}$) are expressed as % increase (+) or decrease (-) of those velocities determined without added metal ion.

Substrate	Zn^{+2}	Co^{+2}	Mn^{+2}	Mg^{+2}
Met-Gly	+4	-38	-59	-41
Met-Ala	+77	-7	2	-
Met-Met	+86	-70	+6	+15
Met-Leu	+47	-40	+10	-
Gly-Gly	-34	+400	-13	-47
Gly-Met	-34	+477	0	-34
Ala-Ala	-38	-59	-	-
Ala-Met	-47	-65	-65	+18

activity. However, it is fair to conclude that metal ions markedly affect the enzymes ability to cleave various dipeptides.

G. Storage and Thermal Stabilities

Both the storage and thermal stabilities of the dipeptidase were tested under various conditions. Storage stability was determined at 4° and - 30° C for the purified dipeptidase ($40\frac{ug}{ml}$). These studies were performed with a portion of the purified dipeptidase which had not been previously frozen. The dipeptidase exhibited excellent stability at 4° C in the presence of glycerin (20% $^{v}/v$). Activity was measured under standard assay conditions toward Met-Met as described in Materials and Methods. During the first twenty days there was little measurable change in the specific activity. From the twentieth to the forty-sixth day the specific activity continuously decreased in small amounts but the dipeptidase still retained approx. 85% of its original activity at this time. Quite opposite to this was the dipeptidases stability under identical conditions without the addition of glycerin. The dipeptidase lost 25% of its specific activity after the first two days and retained less than 5% of its activity by the fourth day. Thus glycerin exerts a large stabilizing effect upon the enzyme. A similar study was carried out in the absence of glycerin for a less highly purified dipeptidase preparation (approx. 175 fold purified). The results obtained showed no activity loss during the first seven days. By the twelfth day a 25% loss

of activity was recorded and after twenty-eight days less than 1% of its original activity remained and a precipitate was observed.

The dipeptidase may be frozen at - 30°C in the presence of glycerin (20% $^{v}/v$) and stored for several months without any apparent loss of activity. Even repeated freezing and thawing results in only small losses of activity, approx. 15%. However, freezing the dipeptidase in the absence of glycerin for even a few hours followed by thawing results in a 65% loss of activity. Here again glycerin is seen to have a pronounced stabilizing effect on the dipeptidase.

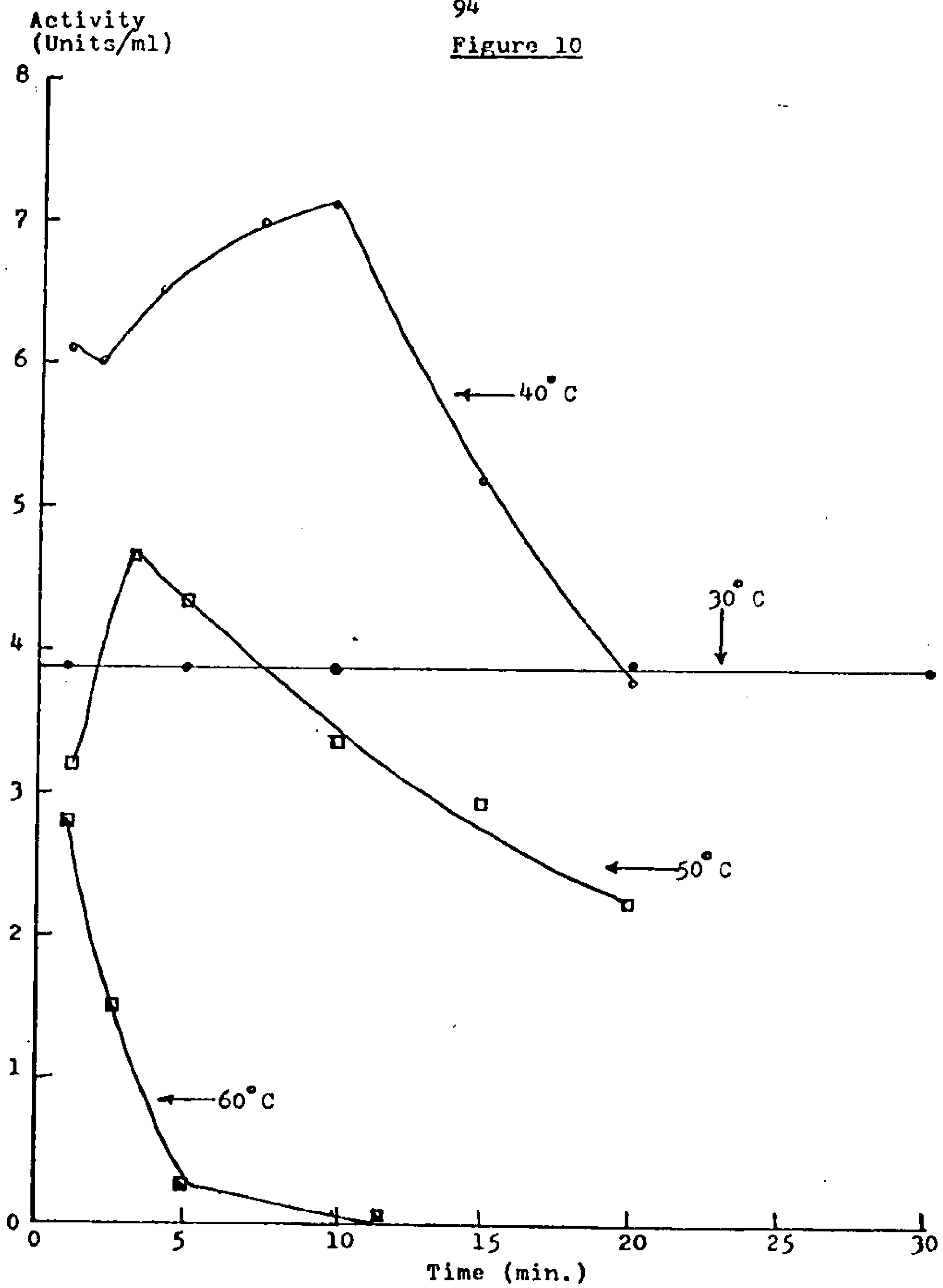
Heat stability of the dipeptidase was measured as a function of time for samples held at 30°, 40°, 50°, and 60°C and the results shown in Fig. 10. The activities were measured under standard assay conditions (see Materials and Methods). Dipeptidase activity remains constant for 30 minutes at 30°C and although not shown in Fig. 10 this activity remains unchanged for up to 4 hours. At 40° and 50°C the activity increases for a short period of time reaching a maximum which is followed by a sharp decrease in activity. This behavior is not observed at 60°C where the activity continually decreases and approaches zero after 12 minutes. The initial activations observed at 40° and 50°C may be due to conformational changes which enhance the activity of the enzyme. However, following this period the rapid loss of activity is probably due to several factors which may include denaturation, loss of metal ion, or irreversible destruction of

Figure 10

Thermal Stability of Yeast Dipeptidase

Thermal stability of the dipeptidase was measured at 30°, 40°, 50° and 60° C for the times shown in the figure under standard assay conditions using Met-Met as the substrate (see Results for details).

Figure 10



the enzyme.

The above studies were carried out in the presence of glycerin (20% $^{v}/v$) and therefore it was of interest to see what effect the absence of this stabilizer would have. However, because of the extremely low protein levels ($40\frac{ug}{ml}$) dialysis was ruled out as a method for removing the glycerin. Instead, the dipeptidase (5ul) was diluted with 0.9 ml of either potassium phosphate buffer, 40 mM, pH 7.0 or with 20% $^{v}/v$ glycerin in the same buffer and the activities measured at 50° and 60° C. After incubation at 50° C for 20 minutes the dipeptidase diluted with glycerin retained approx. 80% of the activity of the undiluted dipeptidase in glycerin. In contrast to this the dipeptidase diluted in buffer alone lost approx. 60% of its activity. Thus, glycerin stabilizes the dipeptidase against thermal effects under these conditions. However, when the same experiment was performed at 60° C for one minute, regardless of the presence or absence of glycerin, the activity of the dipeptidase was reduced by approx. 90%. Apparently at low protein concentrations glycerin cannot stabilize the dipeptidase against significantly elevated temperatures.

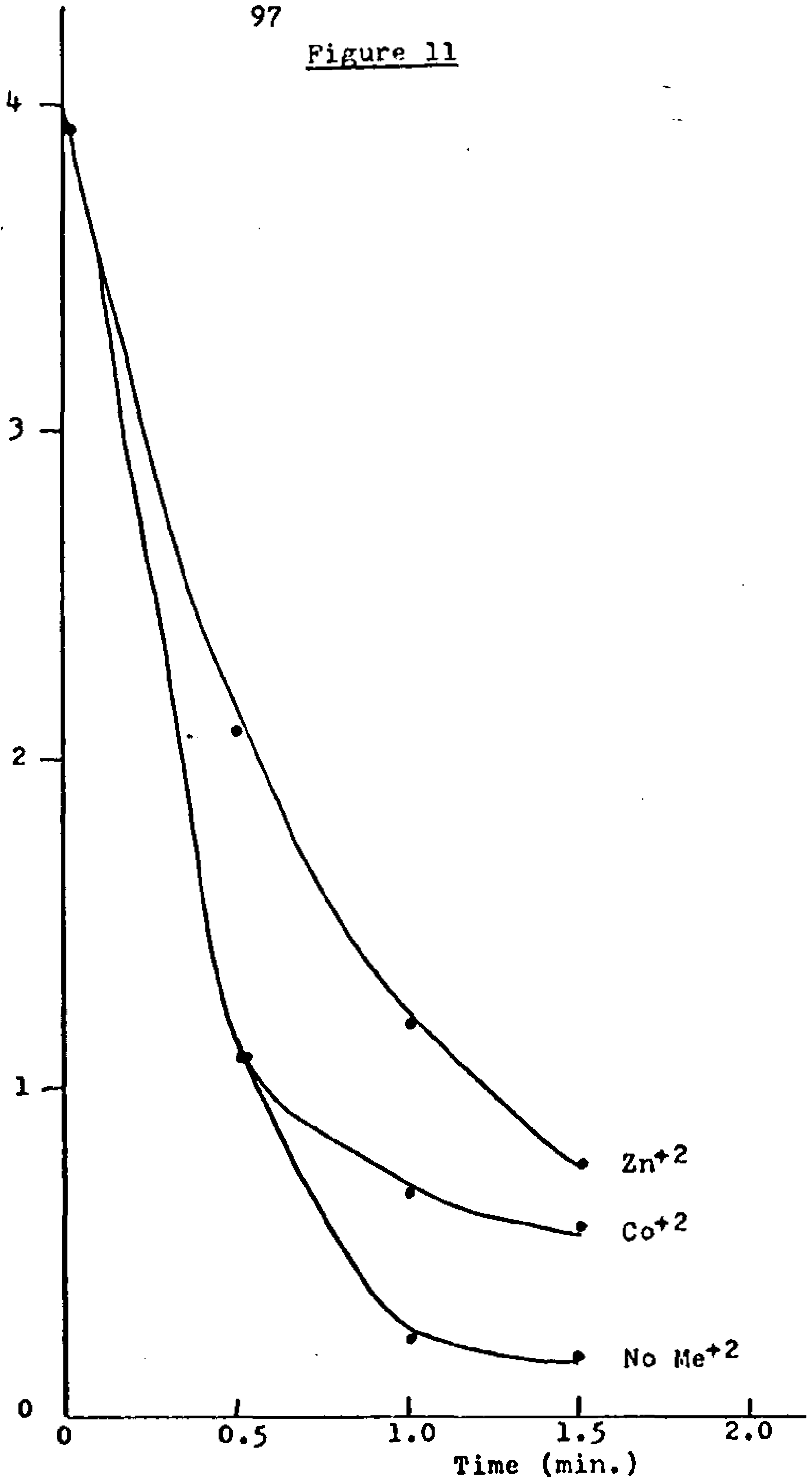
Since metal ions have a pronounced effect on dipeptidase activity, they may also affect the thermal stability of the enzyme. A study was performed as described above for the diluted dipeptidase in potassium phosphate buffer at 60° C with or without added metal ions. The results are presented in Fig. 11 using Met-Met as the substrate and Zn^{+2} and Co^{+2}

Figure 11

Effects of Metal Ions on the Thermal Stability of Yeast Dipeptidase

The effect of Zn^{+2} and Co^{+2} on the thermal stability of the dipeptidase was measured at 60°C for the time intervals given using Met-Met as the substrate (see Results for details).

Figure 11



as the metal ions. Zn^{+2} stabilizes the dipeptidase. This was expected considering its activation of the dipeptidase toward Met-Met, and the belief that at least some of the activity loss upon heating was due to the loss of a metal ion. In contrast, the result obtained for Co^{+2} was unexpected. It is seen that Co^{+2} also protects against heating at 60° C although to a lesser extent than Zn^{+2}. Since it was shown earlier that Co^{+2} deactivated the dipeptidase toward the cleavage of Met-Met it was assumed that the presence of this metal ion would speed up deactivation. This apparent discrepancy may be explained as follows. The curve in Fig. 11 for Co^{+2} is initially similar to that without metal ion. After a 30 second incubation the sample without added metal ion continued its rapid loss of activity whereas the curve for Co^{+2} starts to level off and approach the curve obtained in the presence of Zn^{+2}. It is believed that in addition to enhancing the activity of the enzyme toward Met-Met hydrolysis, Zn^{+2} is capable of stabilizing the active conformation against temperature effects. In a similar manner Co^{+2}, while exerting no enhancement of activity toward Met-Met, may also stabilize the conformation of the enzyme against temperature effects. The fact that the curves for Zn^{+2} and Co^{+2} approach each other suggests that after a 2 minute incubation at 60° C the primary influence of both metal ions may be conformational and that specific effects upon hydrolysis rates are minimal or non-operative.

H. Effects of Reagents on Dipeptidase Activity

The susceptibility of the dipeptidase to several reagents was tested and the results given in Table 6. Metal ion chelators are extremely effective inhibitors of dipeptidase activity. This result in conjunction with the effects of metal ions strongly supports the concept that the dipeptidase is a metalloenzyme. Furthermore, the addition of Zn^{+2} (final concentration $5x10^{-4}$M) to the EDTA inactivated dipeptidase brought about an activation of the dipeptidase equivalent to 80% of its original activity. This process was time dependent and the 80% activation was only achieved after an incubation time of 30 minutes at 30°C. Increasing the incubation time beyond 30 minutes did not result in any further activation.

The dipeptidase was inhibited by dithiothreitol, PCMB and iodoacetic acid but PMSF had virtually no effect. These results suggest that the dipeptidase does not contain an active serine residue as part of its active center but may contain essential disulfide bonds and sulfhydryl groups.

I. Comparison of Crude and Purified Yeast Dipeptidase

Many researchers have studied the action of dipeptidases in crude extracts and have reported on their substrate specificities. This type of study may lead to erroneous conclusions due to the presence of other peptidases in the crude extracts. A comparison of the rates of hydrolysis of several dipeptides by both a crude cell extract and the purified dipeptidase of *S. cerevisiae* Z1-2D is given in Table 7. The rates of

TABLE 6. EFFECTS OF REAGENTS ON THE DIPEPTIDASE.

The dipeptidase was incubated for 5 minutes at 30° C in the presence of the reagents (10^{-4}M) given below and the reaction begun by the addition of substrate (10^{-3}M). The assay was performed under standard assay conditions with the exception that the iodoacetic acid test was performed in the dark.

Reagent	Activity (U/ml)	Substrate
None	12.9	Leu-Leu
None	23.8	Met-Met
EDTA	0	Met-Met
o-phenanthroline	.22	Met-Met
Dithiothreitol	1.68	Met-Met
PMSF*	22.75	Met-Met
PCMB	5.6	Leu-Leu
Iodoacetic Acid	7.8	Leu-Leu

* The incubation of the dipeptidase with PMSF was extended to 15, 20, and 40 minutes. The activities observed were 21.7, 21.5, 21.5 respectively.

TABLE 7. COMPARISON OF DIPEPTIDASE ACTIVITIES OBTAINED FOR A DIALYZED CRUDE CELL-EXTRACT AND THE PURIFIED DIPEPTIDASE.

Activities toward the substrates tested were measured under standard assay conditions (see Materials and Methods). The concentration of substrate was 1.0 mM and the amount of protein added was 6.5 ug of crude cell-extract or 0.16 ug of purified dipeptidase. Specific activities are expressed relative to that of Met-Met which has been assigned a value of 100%

	Relative Specific Activities	
Substrate	Cell-Extract	Purified Dipeptidase
Met-Met	100	100
Ala-Met	175	71
Met-Ala	135	149
Leu-Met	56	59
Met-Leu	152	97
Leu-Leu	54	55
D-Leu-L-Leu	0	0
L-Leu-D-Leu	0	0
Lys-Leu	1.5	0
Gly-Gly	10.5	0.65
Pro-Met	9.5	2.5
Met-Pro	.5	0
Glu-Glu	4	1.5
Ac-Gly-Leu	0	0
Ac-Leu-Gly	0	0

hydrolysis are expressed as a percentage of the specific activities relative to Met-Met with Met-Met being assigned the value of 100%. The conditions of assay were identical except for the amounts of protein present. There are several similarities between the results obtained for the crude cell extract and those obtained for the purified dipeptidase. Neither dipeptidase preparation exhibited activity toward D-Leu-L-Leu, L-Leu-D-Leu, Ac-Gly-Leu, or Ac-Leu-Gly. The relative % hydrolysis of several of the dipeptides which are cleaved by both preparations are similar (Leu-Met, Met-Ala, Leu-Leu,) whereas for other dipeptides the relative rates are quite different (Ala-Met, Pro-Met, Met-Leu, Gly-Gly, Glu-Glu). This may reflect the fact that the purified dipeptidase was not saturated with substrate whereas the crude extract was probably saturated with substrate. As will be shown shortly this reasoning could be applied to all but one (Met-Leu) of the latter group of the dipeptides tested since they have K_{app}'s greater than the concentration of substrate used (1mM). Perhaps the most important difference lies in the activity toward Met-Pro and Lys-Leu. Both of these substrates are cleaved by the crude cell extract although they are poor substrates. However, neither is cleaved by the purified dipeptidase illustrating that in the crude cell extract these two dipeptides were cleaved by other peptidases. It is possible, therefore, that the studies of other investigators using either crude cell-extracts or semi-purified preparations may

contain errors regarding substrate specificities of their dipeptidase(s).

J. Specificity of the Dipeptidase

The response of the purified dipeptidase toward various peptides, peptide derivatives and amino acid derivatives was tested and the results given in Table 8. It is clear from the results shown that the purified dipeptidase is active toward a large number of dipeptides provided that the dipeptide is composed of only L-amino acids, (not cleaved, L, D and D, L Leu-Leu) possesses a true amide bond (not cleaved Gly-Sar, Met-Pro, Leu-Pro and Gly-Pro) and contains both free amino and carboxy termini (not cleaved, Ac-Met-Met, Ac-Leu-Gly, Cbz-Phe-Leu, Cbz-Leu-Leu, Gly-Met-OMe, Leu-Leu-OBzl, Leu-Gly-NH_2,). The dipeptidase is also inactive toward free tripeptides, blocked tripeptides, tetrapeptides, and several types of amino acid derivatives. Thus, the purified dipeptidase possesses a substrate specificity profile characteristic of a true dipeptidase. It is also seen that the dipeptidase is inactive toward dipeptides containing N-terminal lysine or histidine. This point will be discussed later.

K. Kinetic Parameters

More detailed studies on the substrate specificity of the dipeptidase were carried out by examining the kinetic constants for hydrolysis of several dipeptides. As stated earlier it became obvious from Lineweaver-Burk plots that while most substrates adhered to Michaelis-Menten kinetics

TABLE 8. RESPONSE OF THE DIPEPTIDASE TOWARD VARIOUS SUBSTRATES

The response of the dipeptidase toward various substrates was measured under standard assay conditions. The substrates were present at a concentration of 1mM. Cleavage of a good substrate is denoted by (+++), an average substrate by (++), and a poor substrate by (+). A (-) designates that the substrate was not cleaved. Good, average, and poor refer to the rates of cleavage of substrates relative to that obtained for Met-Met which is a good substrate.

Substrate	Response	Substrate	Response
Met-Gly	++	Glu-Glu	+
Met-Ala	+++	Glu-Met	+
Met-Met	+++	His-Met	-
Met-Leu	+++	Lys-Leu	-
Met-Ser	+++	Lys-Lys	-
Met-Glu	+++	Ac-Met-Met	-
Met-His	+	Ac-Leu-Gly	-
Met-Pro	-	Ac-Gly-Leu	-
Leu-Gly	++	Cbz-Phe-Leu	-
Leu-Met	++	Cbz-Leu-Leu	-
Leu-Leu	+++	Leu-Gly-NH_2	-
L-Leu-D-Leu	-	Met-Met-OMe	-
D-Leu-L-Leu	-	Gly-Met-OMe	-
Leu-Pro	-	Ac-Phe	-
Gly-Gly	+	*Leu-NH_2	-
Gly-Met	+	*Leu-p-NA	-
Gly-Leu	+	*Leu-β-NA	-
Gly-Pro	-	Met-Met-Met	-
Gly-Sarc	-	Leu-Leu-Leu	-
Ala-Ala	++	Met-Gly-Met	-
Ala-Met	+++	Gly-Met-Gly	-
Ala-Gly	+	Gly-Gly-Gly	-
Ser-Ser	++	Ac-Met-Met-Met	-
Ser-Met	+++	Met-Met-Met-OMe	-
Pro-Met	+	Gly-Leu-Leu-Gly	-
Pro-Leu	+	Gly-Leu-Gly-Leu	-

*These substrates were not assayed for by the standard method. See Materials and Methods for the procedures.

(Figure 12) some did not (Figure 13). Therefore, the kinetic data was fit directly to saturation curves. The results for a computer best fit analysis of saturation curves determined by either the Hill equation $V = \frac{Vmax\ S^{n_H}}{Kapp + S^{n_H}}$ or the Michaelis-Menten equation $V = \frac{Vmax\ S}{Km + S}$ are given in Table 9, and actual plots of both kinetic expressions for Leu-Met are shown in Figure 14. The substrates which do not adhere closely to Michaelis-Menten kinetics (i.e. $n_H \neq 1$) contain either glycine, N-terminal leucine, or C-terminal glutamic acid. The data in Table 9 reflect the role of the side chains of the dipeptides in binding and catalysis. For instance, in going from Met-Met to Met-Leu Vmax is increased by 21% but Kapp increases almost 240%. The large change in Kapp is unexpected if one considers that leucine and methionine are similar in their size and hydrophobicities. Furthermore, defining the catalytic efficiency as $Vmax/_{Kapp}$, it is seen that the values obtained for the parameter vary by more than 300 fold among the dipeptides tested. The kinetic parameters (Vmax, Kapp, n_H, and $Vmax/_{Kapp}$) of the dipeptidase toward several dipeptides will be discussed in the following section.

Figure 12

Lineweaver-Burk Plot for Met-Met

Initial rate data for the hydrolysis of Met-Met by the dipeptidase was obtained under standard assay conditions. The plot of $\frac{1}{v}$ vs $\frac{1}{[S]}$ is linear and therefore obeys Michaelis-Menten kinetics.

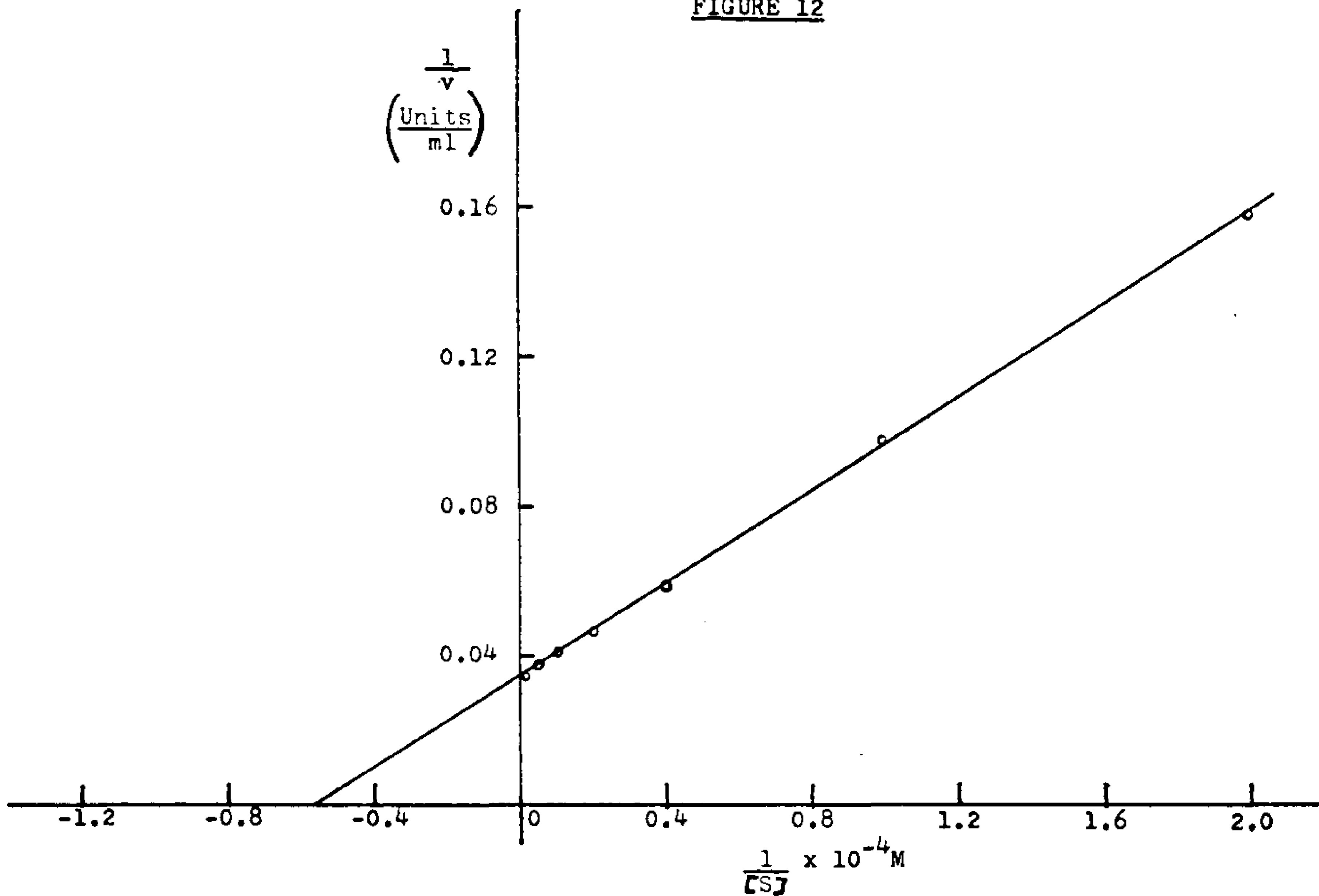
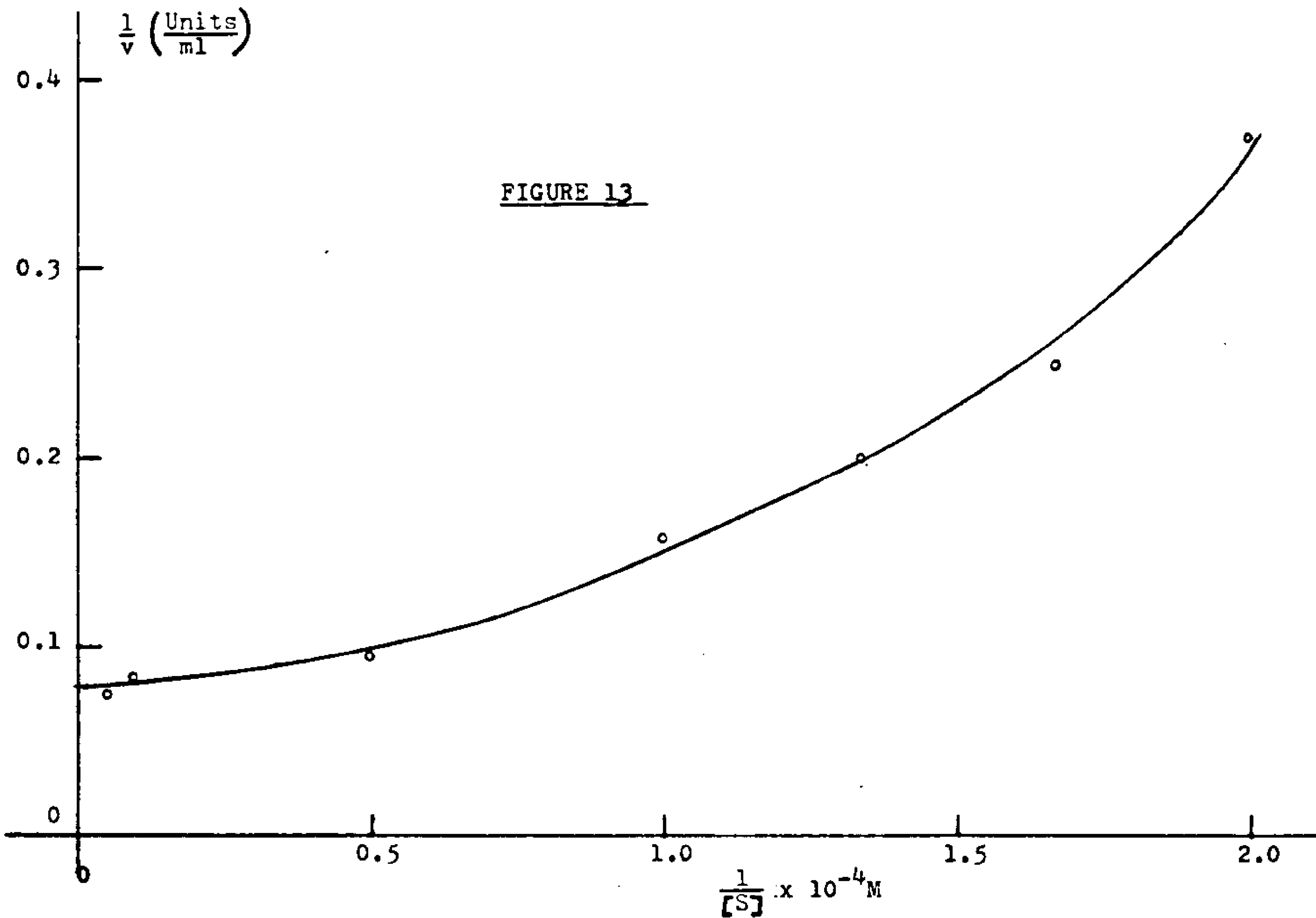


FIGURE 12
1/v (Units/ml)
0.16
0.12
0.08
0.04
-1.2
-0.8
-0.4
0
0.4
0.8
1.2
1.6
2.0
1/[S] x 10^-4 M

Figure 13

Lineweaver-Burk Plot for Met-Gly

Initial rate data for the hydrolysis of Met-Gly by the dipeptidase was obtained under standard assay conditions. The plot of $\frac{1}{v}$ vs $\frac{1}{[S]}$ is non-linear and therefore deviates from Michaelis-Menten kinetics.



$\frac{1}{v}\left(\frac{Units}{ml}\right)$
0.4
0.3
0.2
0.1
0
FIGURE 13
0
0.5
1.0
1.5
2.0
$\frac{1}{[S]}$ x 10^{-4}M

TABLE 9. KINETIC CONSTANTS FOR YEAST DIPEPTIDASE

Experiments were performed at 30°C in potassium phosphate buffer, pH 7.0. Maximal Velocities are expressed relative to that obtained for the hydrolysis of Met-Met, taken as 1.0

Dipeptide	Vmax	Kapp[a] (mmol/l)	n_H	Vmax/Kapp
Met-Met	-1.0-	0.18	MM[b]	5.6
Leu-Leu	1.19	0.72	0.65	1.65
Ala-Ala	0.62	0.86	MM	0.72
Ser-Ser	0.87	1.07	MM	0.81
Met-Gly	0.44	0.09	2	4.9
Met-Ala	1.77	0.28	MM	6.3
Met-Leu	1.21	0.43	MM	2.8
Met-Ser	1.20	0.35	MM	3.4
Met-Glu	0.99	0.80	0.89	1.24
Met-His	0.18	0.07	MM	2.57
Gly-Met	0.035	1.97	1.4	0.018
Ala-Met	1.36	1.36	MM	1.0
Leu-Met	0.67	0.23	0.80	2.9
Ser-Met	0.89	1.97	MM	0.56

[a] Asymptotic Standard Deviations for K_{app} values were less than ± 15% of the values shown

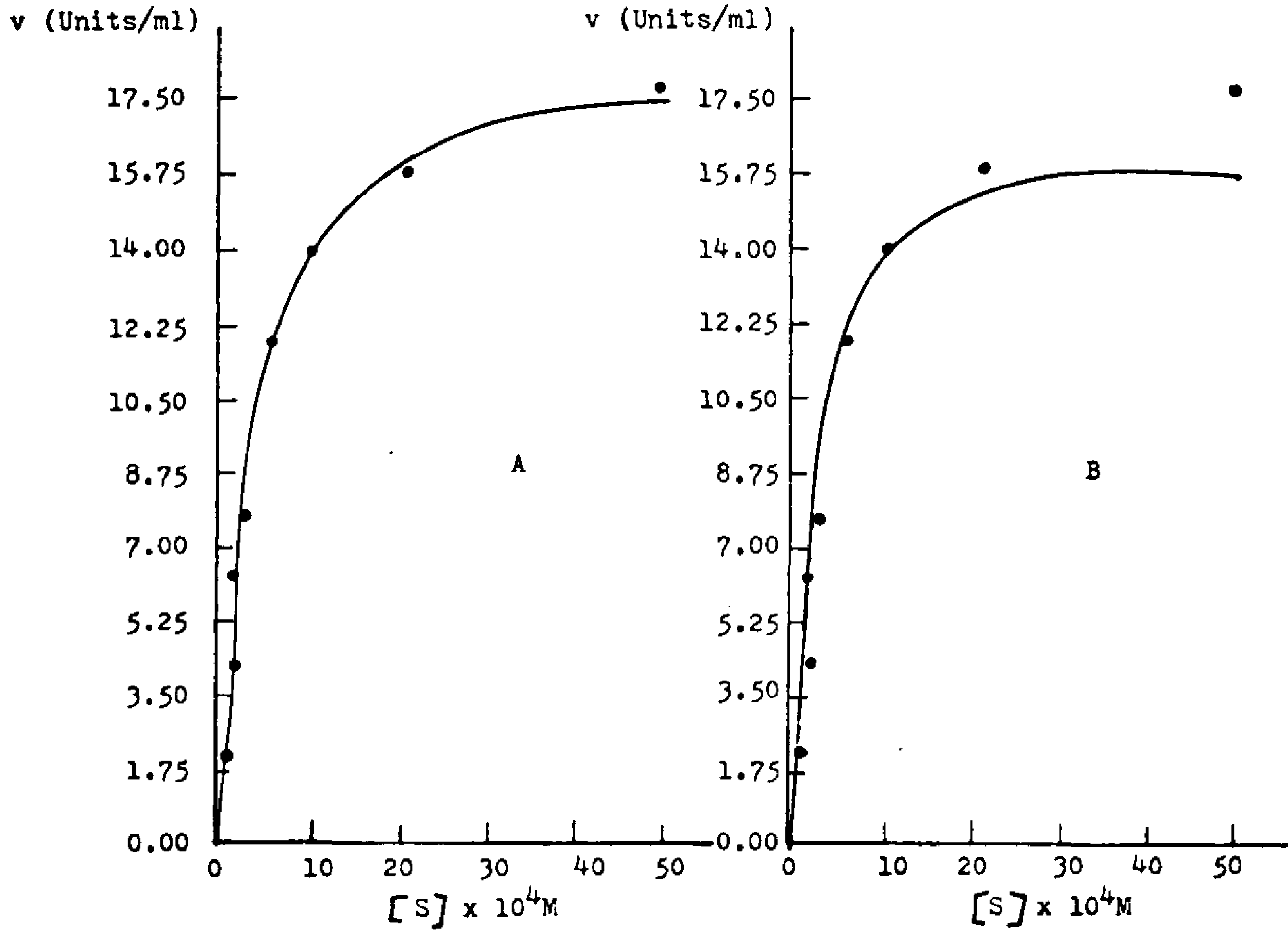
[b] MM = computer best fit was based on Michaelis-Menten equation

Figure 14

Computer Evaluation of Kinetic Data for Leu-Met

Initial rate data for the hydrolysis of Leu-Met by the dipeptidase was obtained under standard assay conditions. The concentration of Leu-Met was varied between 0.25×10^{-4}M and 50×10^{-4}M. The velocity data was fit directly to either the Hill equation $\left(v = \frac{V_{max}\ S^{nH}}{K_{app} + S^{n_H}} \right)$ with a constant Hill coefficient (n_H) or to the Michaelis-Menten equation $\left(v = \frac{V_{max}\ S}{K_{app} + S} \right)$. A computer generated best fit is shown in the figure for both the Hill equation (Curve A) and the Michaelis-Menten equation (Curve B). Goodness of fit to each of the kinetic equations was determined by comparison of their correlation coefficients (r). For Leu-Met a better fit was obtained by use of the Hill equation. (See Materials and Methods for details).

Figure 14



IV. DISCUSSION

It has been demonstrated that S. cerevisiae Z1-2D contains at least four aminopeptidases, one dipeptidase, and carboxypeptidase activity. At least two of the aminopeptidase activities must be different from those reported by others[9,21,55] due to the fact that they lack esterase activity toward amino acid derivatives. Under the conditions employed for polyacrylamide gel electrophoresis the main fractionating power of the gel resides in its ability to act as a molecular sieve. Thus, proteins will be separated mainly according to their molecular size, which assuming a spherical shape, is proportional to their molecular weights. Using this as a guide the following observations may be made. The aminopeptidase activity associated with band 1 seems to have the broadest specificity and the highest molecular weight (Rf=.18) of the aminopeptidases found. Furthermore, this aminopeptidase was one of two of the peptidases capable of cleaving leucyl-p-nitroanilide. In these respects the aminopeptidase activity of band 1 resembles aminopeptidase I (mol. wt. 640,000) which has been purified and characterized in detail by Metz and Rohm.[58] The aminopeptidase activity found in band 3 should have a molecular weight in the the area of 140,000 since it migrates with the dipeptidase activity (the dipeptidase having a molecular weight of 141,000). To date, no yeast aminopeptidase having this molecular weight has been reported. The aminopeptidase activities associated with bands 2 and 4 represent peptidases

which should have molecular weights higher and lower than 140,000 respectively. If one constructs a plot of R_f vs log Mol. Wt. for the peptidases found using a molecular weight for activity band 1 of 640,000 and for activity band 3 of 141,000 then activity bands 2 and 4 have molecular weights in the range of 218,000 and 51,000 respectively. Masuda et al[9] found two aminopeptidases in the acidic fraction of S. cerevisiae possessing molecular weights of 200,000 and 34,000 based upon gel filtration. The high molecular weight peptidase was able to cleave leucyl-p-nitroanilide whereas the other one could not. The fact that activity band 2 has an estimated molecular weight near 200,000 and that it has activity toward leucyl-p-nitroanilide suggests that it is similar to the high molecular weight aminopeptidase described by Masuda et al.[9] It is not clear whether the low molecular weight aminopeptidase (34,000) of Masuda et al[9] and a similar aminopeptidase (mol. wt. less than 50,000) described by Frey and Rohm[55] are similar to the aminopeptidase activity found in band 4.

The utility of the PAGE-activity stain assay is manifested in its ability to rapidly screen cell extracts for peptidase activity, yielding information regarding the number and types of peptidases present. It may also supply information regarding substrate specificity and relative molecular weights. The substrate specificity profile obtained can be used to differentiate between several peptidases during their purification. However, caution must be observed in

interpreting the results of the gel-activity assay since it is a qualitative rather than quantitative procedure.

Discussion of the number of dipeptidases in S. cerevisiae dates back to some of the early work in the late 1920's and early 1930's by Grassman and coworkers.[56,59-62] In 1966, Cordonnier[64] characterized the dipeptidase activity(ies) of a semi-purified fraction from S. cerevisiae var. ellipsoideus and concluded that more than one dipeptidase might be present. His view was based on the observations that the dipeptidase's behavior toward Gly-Gly and other substrates differed in the presence of metal ions and other effectors. Also, the ratio of dipeptidase activity toward Gly-Gly and Ala-Gly varied during the purification. Although these findings may be explained (see Introduction) in terms of a single dipeptidase the question as to the number of dipeptidase activities remained open. In 1974 Rohm[68] reported the first homogeneous preparation and characterization of a dipeptidase from brewers yeast. He concluded that only one dipeptidase existed and attributed Cordonniers observations to the fact that the dipeptidase is greatly influenced by metal ions.

The results of the polyacrylamide gel electrophoresis-coupled peptidase staining method showed the existence of a single broad specificity dipeptidase. Purification of the dipeptidase was monitored by following its activity toward Met-Met and Leu-Gly. The ratio of activities for these substrates remained essentially constant during purification and their activity profiles coincided with one another. No other

dipeptidase activity was found. Thus this study supports the conclusion that S. cerevisiae contains one dipeptidase.

The purified dipeptidase is very similar in some of its physical and chemical properties to other yeast dipeptidase preparations as given in Table 10. The dipeptidase appears to be a metalloenzyme having a molecular weight of 141,000 and is composed of four equal subunits. It has a pH optimum of 8.0, an isoelectric point of 6.5, and is inhibited by PCMB, EDTA, o-phenanthroline and amino acids. Furthermore from Table 6 it is seen that the dipeptidase is also inhibited by iodoacetic acid and dithiothreitol but not by PMSF. The failure of PMSF to inhibit the dipeptidase establishes that the dipeptidase is not a "serine hydrolase". The fact that dithiothreitol severely inhibits dipeptidase action is probably due to the reagents ability to reduce disulfide bonds which are essential to maintaining the dipeptidases conformational integrity (i.e. the dipeptidase is composed of subunits which may be held together by disulfide bridges). Although PCMB and iodoacetic acid, which are both sulfhydryl reagents, inhibit the enzyme it is not clear whether this effect is caused by inhibition of an active center sulfhydryl group or to a conformational change of the dipeptidase.

The properties described for the yeast dipeptidase(s) are similar to those found for other dipeptidases (see Table 2). The most outstanding similarities are that they are metalloenzymes and they are usually inhibited by sulfhydryl reagents and amino acids. The fact that sulfhydryl reagents are

TABLE 10. PHYSICAL AND CHEMICAL PROPERTIES OF YEAST DIPEPTIDASE(S)

	Present Study	Rohm[a]	Cordonnier[b]
Molecular Weight	141,000	130,000	100,000[c]
No. of Subunits	4	4	n.d.[d]
Subunit Molecular Weight	35,000	35,000	n.d.
pH optimum	8.0	7.8-8.3	7.5-8
Isoelectric Point	6.5	6.3	n.d.
Metal Ion	Yes	Yes, Zn^{+2}	Yes
EDTA	I[e]	I	I
o-phenanthroline	I	I	I
PCMB	I	n.d.	I
Amino Acids	I	I	n.d.

[a] Ref. 68, for a purified yeast dipeptidase

[b] Ref. 64, for a partially purified yeast dipeptidase essentially lacking other peptidase activity

[c] Determined by comparison of elution volumes for the dipeptidase on Sephadex G-100 and G-200

[d] n.d. = not determined

[e] I = inhibitory

usually found to inhibit the action of dipeptidases suggests that these enzymes possess active center sulfhydryl groups. The active center sulfhydryl group may participate in binding and/or catalysis directly or may be one of the ligands involved in the binding of essential metal ion cofactors.

The effects of metal ions on the dipeptidases substrate specificity and stability are given in Table 5 and Fig. 11 respectively. In Table 5 it is seen that Zn^{+2} is activating toward Met-X dipeptides and deactivating toward Gly-X and Ala-X dipeptides. Although the native metal ion in this study has not been determined it is likely to be Zn^{+2} as was found by Rohm[68] for his yeast dipeptidase which is very similar if not identical to my dipeptidase (Table 10). This may explain why the dipeptidase in the absence of added metal ions cleaves Met-X dipeptides faster than the other dipeptides (see Table 7 and 8). It appears that the native dipeptidase shows a greater specificity (i.e. efficiency) for Met-X dipeptides than for other dipeptides. The effects of other metal ions on the dipeptidase are also given in Table 5. These effects vary from that of a 70% decrease in activity for Met-Met in the presence of Co^{+2} to an increase of 477% in the cleavage of Gly-Met in the presence of Co^{+2}. The amounts of activation and inhibition vary with respect to the combination of both metal ion and dipeptide. The tremendous increase in the hydrolysis of Gly-X dipeptides in the presence of Co^{+2} has been noted by several investigators[64,68,87,88,103,104] for their dipeptidase preparations.

However, the reason for this phenomena is not well understood. It must be noted that although the dipeptidase is a metalloenzyme (i.e. the metal ion is retained by the dipeptidase during purification) the exact sequence of binding of metal ion, enzyme, and substrate has not been elucidated. The presence of metal ions at the concentration used in this study (.09mM) as well as those of other studies[64,68,103,104] (usually .01mM to 1mM) is probably higher than would be required to saturate the dipeptidase alone. The addition of metal ions may bring about changes in the substrate specificity of the enzyme due to conformational perturbations, and/or direct complexation with the substrate thus making it more susceptible to the dipeptidase. The conformational argument is supported by the observation that both Zn^{+2} and Co^{+2} are able to stabilize the dipeptidase against heating effects toward Met-Met although Co^{+2} was found to inhibit the hydrolysis of this dipeptide under standard assay conditions (see Results and Fig. 11). Furthermore, the pH profiles for the dipeptidase (Fig. 9) show the possible existence of the active dipeptidase in more than one conformational form. Finally, lag phases were noted in the kinetics for several of the dipeptides tested. This observation may indicate that the dipeptidase must alter its conformation in order to accomodate a given substrate. All of these findings lead to the conclusion that the dipeptidase is extremely susceptible to external effects which manifest themselves by altering the conformation of the dipeptidase. However, it is

not clear as to whether or not these conformational changes affect the gross conformation of the enzyme or only the conformation of the active center. Rohm and coworkers[68,91,92] have observed a similar pH profile to that described in this work and different values of kinetic parameters below pH 6.0 and above pH 7.0. In order to elucidate the type of conformational change occurring for the dipeptidase they studied its sedimentation behavior in sucrose density gradients and its far ultraviolet circular dichroism spectra at different pH values. They found that the dipeptidase does not alter its sedimentation behavior as a function of pH and therefore its subunit composition remains intact. Also, there was no change in the CD spectra of the dipeptidase between pH values of 5.5 and 7.2. Since the gross conformation of the dipeptidase does not change it is reasonable that changes in pH, substrate, and metal ion may affect the conformation of the active center.

Kinetic parameters of the dipeptidase toward several dipeptides are given in Table 9. For the sake of comparison the sizes of amino acid residues are those determined from C.P.K. space filling models and their relative hydrophobicities based upon determinations made by Tanford and coworkers.[113,114] The efficiency of dipeptidase action will be defined as the ratio of Vmax to Kapp ($\frac{Vmax}{Kapp}$). For the homodipeptides tested it is seen that the efficiency increases as the size of the dipeptide increases (Met > Leu > Ser $\geq$ Ala). Although not given in Table 9

estimates of the kinetic paramters for Glu-Glu and Gly-Gly were attempted by computer analysis but due to insufficient data complete saturation curves could not be obtained. However, the data clearly indicated, regardless of the kinetic equation used to fit the data, that both Gly-Gly and Glu-Glu would have efficiencies lower than that of Gly-Met with Gly-Gly having the lowest efficiency. In addition it has already been shown in Table 8 that Lys-Lys is not cleaved at all. Based upon size Gly-Gly fits the pattern established for the homodipeptides tested (Table 9) but Lys-Lys and Glu-Glu do not. Both of these dipeptides contain charged side chains. Lys-Lys is positively charged and is not cleaved at all although it is the largest dipeptide. Glu-Glu, which is similar in size to Met-Met, is negatively charged and exhibits a greatly reduced efficiency. Thus the catalytic ability of the dipeptidase is greatly affected by the presence of charged amino acid residues. It is not clear from the above observations as to which terminal amino acid residue if any is mainly responsible for the observed results. To investigate this point further several heterodipeptides were tested and the results given in Table 9. A comparison of the efficiencies for Met-X and X-Met dipeptides, where X= Ala, Gly, Ser or His clearly shows that Met-X dipeptides have higher efficiencies than their X-Met counterparts. However, there is no efficiency trend for either Met-X or X-Met dipeptides as X is varied with respect to either the size or hydrophobicity of X.

Furthermore a comparison of Met-Leu and Leu-Met shows that although methionine is greater in size than leucine these dipeptides have almost identical efficiencies. The reason for this is not clear but may reflect the shape of the active site of the dipeptidase since methionine has a linear side chain whereas leucine has a branched side chain. Also, it is seen (Tables 8 and 9) that the dipeptidase cannot cleave dipeptides possessing a positively charged N-terminal amino acid i.e. Lys-Leu, Lys-Lys and His-Met. The dipeptidase can however act upon dipeptides having a positively charged C-terminal residue e.g. Met-His. It can also accomodate a negative charge at either terminus but its action is greatly reduced when the negative charge is at the N-terminus (Table 8 and 9, i.e. Glu-Met vs Met-Glu). These results suggest that the N-terminal amino acid rather than the C-terminal amino acid is mainly responsible for controlling the efficiency of the dipeptidase. Furthermore, it is seen in Table 9 that of the dipeptides tested, Gly-Met has the lowest Vmax and highest Kapp making it the poorest substrate. Its efficiency is approximately 30 times lower than that of the next poorest substrate Ser-Met and 350 times lower than the best substrate Met-Ala. This result illustrates the importance of the interaction between side chains of the dipeptide and the active center of the dipeptidase since glycine has no side chain (i.e. R=H).

The only substrates having Hill coefficients (n_H) significantly different from unity (i.e. when n_H=1, Michaelis-Menten kinetics are present) are Met-Gly, Gly-Met, Leu-Leu, Leu-Met and Met-Glu. This was judged based on goodness of fit by examining the saturation curves for both the Hill and Michaelis-Menten equations and determining their correlation coefficients (r). These dipeptides do not have any single feature in common which differs from the other dipeptides tested. The allosteric effect may be due to the peptide itself, to the products of the cleaved peptide, or to a combination of both. Since the dipeptides containing glycine had the largest values of n_H it was of interest to see if the addition of glycine alone could produce such an effect for other dipeptides.

An experiment was performed under standard assay conditions with Met-Met (a substrate following Michaelis-Menten kinetics) as the substrate in the presence of glycine, $1x10^{-6}$M. The results clearly demonstrated that the hydrolysis of Met-Met in the presence of glycine did not adhere to Michaelis-Menten kinetics and yielded a value for n_H of 1.20. Also, Kapp was essentially unchanged whereas Vmax decreased by approximately 15%. Therefore, glycine may act as an allosteric effector interacting with a site causing a change in the conformation of the active site of the dipeptidase. However, the possibility of non-competitive inhibition cannot be ruled out. Furthermore, it may explain why Rohm and coworkers[91] found that all dipeptides containing N-terminal leucine did not follow Michaelis-Menten

kinetics. However, to complicate matters further Rohm and coworkers[91] also found that leucine actually acts as an activator at low concentrations but as an inhibitor at higher concentrations and that the type of inhibition depends upon the composition of the dipeptide.

In summation the present study has demonstrated the existence in S. cerevisiae Z1-2D of four aminopeptidases, one dipeptidase and at least one carboxypeptidase. The dipeptidase was purified to homogeneity and characterized. It is a metalloenzyme which is extremely susceptible to modifier effects. These effects are most probably the result of changes in the conformation of the active site which are caused by binding of the substrate and/or metal ion and by changes in pH.

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