

PROLINASE AND PROLIDASE IN HUMAN TISSUES

David Andrew Priestman (BSc)

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DECLARATION

I declare that, apart from from the assistance acknowledged, the research described was performed by myself and this thesis is of my own composition.

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ABSTRACT

Peptide bonds containing proline residues are often resistant to the action of peptidases because of the unique pyrrolidine-ring structure of proline. Therefore, in order to hydrolyse proline peptide bonds there are a number of proline-specific peptidases. At the final stage of hydrolysis, dipeptides with N-terminal proline are hydrolysed by prolinase and those with C-terminal proline by prolidase. These two enzymes have been little studied in human tissues and the assay methods used up until now are relatively insensitive.

Initially, prolinase was studied. A new sensitive fluorimetric assay was developed for estimating prolinase activity and a study was made of its properties. Using ion-exchange chromatography, gel filtration and isoelectrofocusing, two distinct forms of prolinase were found to be present in cultured skin fibroblasts. Human kidney, however, had only a single form which also seemed to have non-specific dipeptidase activity. To study this further, the enzyme was purified from human kidney using a wide variety of separative techniques and a number of properties studied. The results confirmed that prolinase substrates are hydrolysed by a non-specific dipeptidase in human kidney.

Two new methods for assaying prolidase were also developed after which, detailed studies of the enzyme's properties could be made. Prolidase deficiency is an autosomal recessive disease associated with chronic ulcerative dermatitis, mental retardation and imidodipeptiduria. Using cultured skin fibroblasts from two unrelated cases of prolidase deficiency, the residual activity was determined with a variety of substrates. The results showed a marked change in substrate specificity rather than a complete deficiency of prolidase in the cases. Later, using ion-exchange chromatography, it was found that there are also two forms of prolidase. These were designated peaks I and II. The two forms differed in substrate specificity, heat stability and inhibition with p-hydroxymercuribenzoate. In the two cases of prolidase deficiency, fibroblast peak I was markedly reduced although still detectable, whereas peak II was active against all substrates except for a 90% reduction against glycyl-proline. These results imply that the two forms of prolidase are structurally related.

Finally, the first peak of prolidase was purified from human kidney and injected into rabbits to raise specific antisera to the enzyme. Using immunoblotting after polyacrylamide gel electrophoresis, it was then possible to show that the first peak of prolidase is present, though inactive, in fibroblasts from the two cases.

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ABBREVIATIONS

DEAE	Diethylaminoethyl
FCS	Foetal calf serum
K _m	Michaelis constant
K _i	Inhibitor constant
M _r	Molecular mass
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PHMB	p-Hydroxymercuribenzoate
pI	Isoelectric point
SDS	Sodium dodecyl sulphate
TBS	Tris-buffered saline
TCA	Trichloroacetic acid

1. GENERAL INTRODUCTION

1.1. PROLINE AND PROLINE-SPECIFIC PEPTIDASES

The pyrrolidine-ring structure of proline makes it unique among the amino acids, it also lacks the amino group common to all the other amino acids. The nitrogen atom is part of the rigid pyrrolidine ring not allowing any rotation of the N-C_α bond. Because of this, whenever proline (or hydroxyproline) occurs in a peptide chain, it interrupts the α-helix and creates a kink or bend. In this way proline residues have a large effect upon the structure and conformation of peptides and proteins in solution. Proline and hydroxyproline, which comprise about one quarter of all the amino residues in collagen, are regularly spaced forcing the chain to assume a peculiar kinked type of helix because of the rigidity of the pyrrolidine ring. In collagen this gives rise to the triple helix.

Because of this unusual structure, proline-containing peptides are often resistant to the action of peptidases (Walter et al., 1980). Thus to complete the hydrolysis of proteins and peptides to their constituent amino acids, there are a number of proline-specific peptidases. At the final stage of hydrolysis dipeptides of the form Proline-X (where X = C-terminal amino acid with one amino group) are hydrolysed by the specific dipeptidase prolinase [E.C.

3.4.13.8] (see fig. 1a). Alternatively, dipeptides of the form X-Proline (X = N-terminal amino acid with one amino group) are hydrolysed by the specific dipeptidase prolidase [E.C. 3.4.13.9] (see fig. 1b). Other dipeptides not containing proline are hydrolysed by one or more non-specific enzymes.

Prolidase deficiency (McKusick 26413) is an autosomal recessive disease with characteristic clinical features and is discussed in section 1.4. No such deficiency of prolinase has been observed.

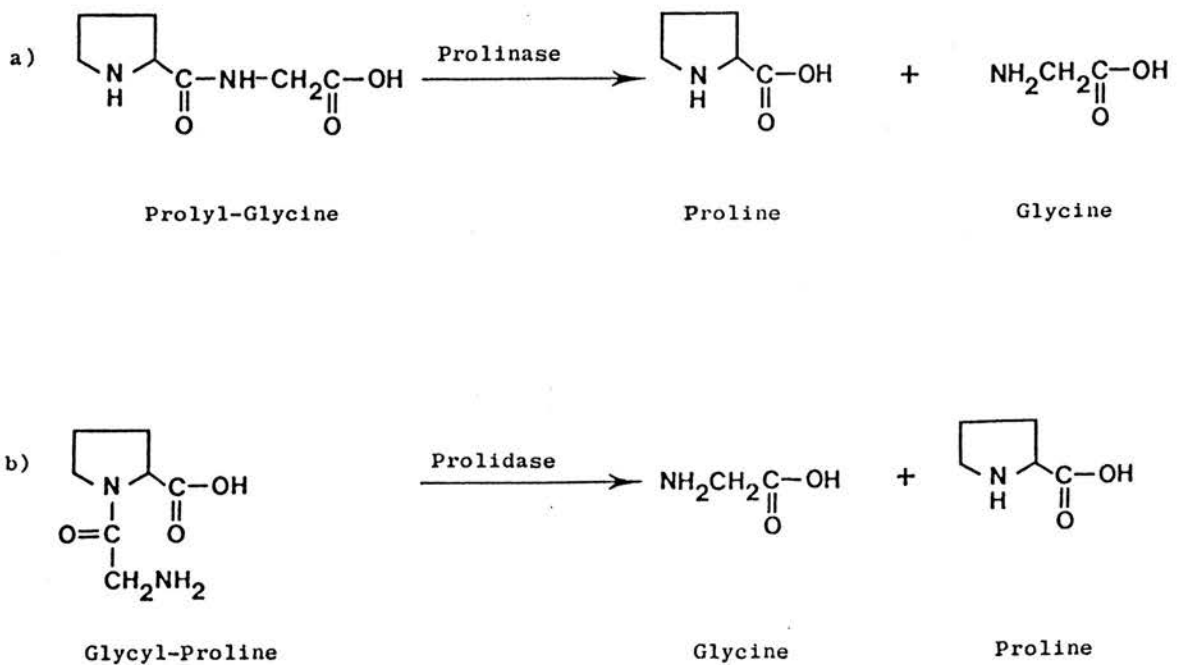


Fig. 1 The actions of a) Prolinase and b) Prolidase.

1.1.1. The physiological roles of prolinase and prolidase

The precise physiological roles of proline-specific enzymes are unknown. It is assumed that they aid in the general catabolism of the peptides and proteins to their constituent amino acids for eventual reutilisation by the cell. Some of these enzymes have widely different distributions in tissues suggesting that they may be intimately related to the specialised functions of particular tissues (Walter et al., 1980; Imai et al., 1982). The high content of proline in collagen probably signifies that these enzymes must be important in collagen turnover with prolinase and prolidase acting at the final step.

A large number of neuroactive peptides and hormones contain proline (see Table 1), so these enzymes are likely to be of importance in the termination of hormone functions. Also, proline is a putative neurotransmitter (Felix and Kunzle, 1976) so it is quite possible that prolinase and prolidase play a key role in its availability. Inhibition or activation of prolinase and prolidase may also have an important influence on the distribution and levels of proline and proline dipeptides (Hui and Lajtha, 1980).

Table 1. Peptide hormones containing proline

Adrenocorticotrophic hormone (ACTH)
Angiotensins I, II and III
Bradykinin
Calcitonin
Cholecystokinin
Corticotropin releasing factor
C-peptide
Dynorphin
Endorphins
Gastrin
Gastrin releasing peptide
Insulin
Luteinising hormone releasing hormone (LH-RH)
 β -Lipotropin
Melanocyte stimulating hormone (MSH)
Motilin
Neuropeptide Y
Neurotensin
Oxytocin
Parathyroid hormone
Substance P
Thyrotropin releasing hormone (TRH)
Vasopressin (ADH)

1.2. PROLINASE

The action of prolinase was first described by Grassman et al. (1932) and has subsequently been purified to varying degrees from porcine (Davis and Smith, 1953; Mayer and Nordwig, 1973) and bovine (Akrawi and Bailey, 1976; 1977) kidney. The enzyme has no activity against either prolinamide, tripeptides or dipeptides in which the imino group is acylated (Smith, 1955) and may therefore be considered to be a strict dipeptidase. Various preparations have had low activities against prolyl-leucinamide and towards dipeptides without N-terminal proline (Sarid et al., 1962; Nordwig and Mayer, 1973; Akrawi and Bailey, 1976) but it is not clear whether these activities were due to contaminating enzymes. However, it has been proposed that the prolinase and non-specific dipeptidase activities may be due to a single enzyme (Hayman and Patterson, 1971).

1.3. PROLIDASE

Prolidase activity was first detected by Bergmann and Fruton (1937) and is widely distributed in man and animals (Smith, 1951; Hui and Lajtha, 1978). The enzyme has been purified from porcine kidney (Davis and Smith, 1957) and intestine (Sjöström et al., 1973).

More recently human erythrocyte prolidase has been purified for the first time (Endo et al., 1982).

Like prolinase, prolidase is a strict dipeptidase requiring both a free α -amino and α -carboxyl group. Tripeptides or N-acyl derivatives of dipeptides are not cleaved. The enzyme is highly specific for proline at the C-terminus although cleavage of dipeptides with C-terminal hydroxyproline and sarcosine does occur. Prolidase therefore probably recognises the imide bond rather than the pyrrolidine ring. It shows little specificity for amino acids at the N-terminus (Walter et al., 1980), and has been shown to be specific for the trans isomers of X-Pro dipeptides (Lin and Brandts, 1979; King et al., 1986). The enzyme is reported to be composed of two identical non-covalently bound subunits with a molecular weight of 55,000 giving a molecular weight of 110,000 for the native enzyme (Endo et al., 1982).

Manganese ions have been shown to activate prolidase (Smith, 1948a; Walter et al., 1980), whereas EDTA, fluoride, citrate (Adams and Smith, 1952) and iodoacetamide and p-chloromercuribenzoate (Davis and Smith, 1957) exhibit an inhibitory effect. If manganese is added prior to iodoacetamide no inhibition is observed, indicating that manganese binds to an SH group.

Recently a hypothetical active-site model has been proposed for prolidase (King et al., 1986)

modifying an earlier model proposed by Hui and Lajtha (1978). This new model is shown in Fig. 2. Its main characteristics are: i) cationic and anionic binding sites for the C- and N-termini respectively; ii) a pocket to bind the pyrrolidine ring; iii) a binding site for Mn^{2+} oriented such that the peptide bond carbonyl group can only be polarised if the substrate is in the trans conformation; and iv) no specific binding site for the N-terminal amino acid.

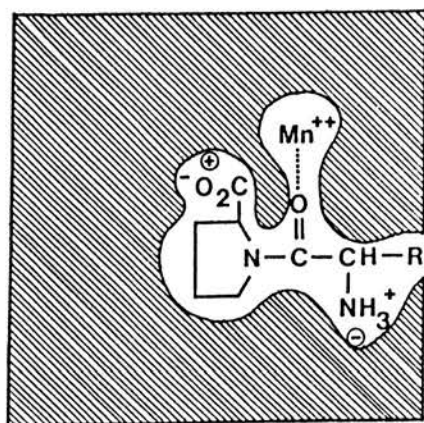


Fig. 2 Hypothetical active site of prolidase as proposed by King et al. (1986); the hatched area represents the enzyme.

1.4. PROLIDASE DEFICIENCY

1.4.1. History

Nearly 20 years ago, Goodman et al. (1968) described a patient with massive urinary excretion of glycyl-proline and skin and visceral symptoms resembling lathyrism. Buist et al. (1972) reinvestigated this patient and found that purified porcine prolidase hydrolysed the imidodipeptides in the urine and hypothesised a prolidase deficiency. In 1974 Powell et al. were the first to directly demonstrate a deficiency of prolidase activity in both the erythrocytes and leucocytes from a 7-year old boy with imidodipeptiduria and similar clinical features to Goodman's patient. Prolinase activity was normal. Prolidase deficiency was then confirmed in the patients of Jackson et al. (1975). Up until now about 20 cases of prolidase deficiency have been reported (Myara et al., 1984a).

1.4.2. Clinical findings

Recently Der Kaloustian et al. (1982) made an exhaustive clinical review of prolidase deficiency. The first symptoms of the disease can appear as early as six days of life and are usually manifested in the first years of life. Symptoms related to collagen

disturbance are the most important: skin ulceration or fragility with scar formation, purpuric lesions, telangiectasia, poliosis, photosensitivity and thickening of the skin with lymphoedema. There may also be musculoskeletal abnormalities including joint laxity, osteoporosis and structural anomalies. The predominant dermatological feature is chronic recalcitrant ulceration on the lower parts of the legs. Among prominent non-dermatological findings associated with prolidase deficiency are: mental retardation, unusual facial appearance, splenomegaly, optical findings and deafness. Recurrent infections including otitis media, sinusitis and cellulitis are also common and could be due to a disturbance of the complement fraction Clq which contains a high level of iminoacids (Porter and Reid, 1978; Scriver et al., 1983).

Although the lymphoedema might possibly be explained by osmotic disturbances resulting from the accumulation of non-degraded dipeptides, the manner in which deficiency of prolidase is responsible for the clinical manifestations of the disease is unclear, particularly since there are reports of asymptomatic cases (Umemura et al., 1978; Isemura et al., 1979). Therefore it appears that other factors as yet unknown may be involved.

1.4.3. Imidodipeptiduria

Massive imidodipeptiduria is the major laboratory finding and patients may excrete as much as 3g proline/day (Scriver et al., 1983). Characterisation of the urinary dipeptides reveals a number of proline-containing dipeptides the major ones of which are shown in Table 2. Glycyl-Proline comprises one fifth of the total imidodipeptides excreted (Scriver et al., 1983).

Since proline and hydroxyproline together account for 22% of the amino acid residues in collagen, it would be expected that collagen metabolism would be most affected by an enzyme defect such as proliidase deficiency. Originally, it was proposed that the imidodipeptides were excreted as the result of a massive increase in collagen turnover with increased collagen metabolism (Powell et al., 1974). However, more recent data indicate the imidodipeptides to be derived to a considerable extent from sources other than collagen, as proposed by Sheffield et al. (1977). In collagen there are approximately equivalent concentrations of proline and hydroxyproline residues. In proliidase deficiency the imidodipeptides are mostly of the form X-proline with only a small amount of X-hydroxyproline excreted (Powell et al., 1977; Sheffield et al., 1977). Moreover, Glu-Pro and Asp-Pro

Dipeptide	μmol excreted/24hr
Gly-Pro	2232
Pro-Pro	1084
Leu-Pro	599
Ile-Pro	561
Glx-Pro	504
Val-Pro	427
Phe-Pro	389
Asx-Pro	383
Thr-Pro	361
Ser-Pro	313
Ala-Pro	207

Table 2. The major dipeptides excreted in the urine of a patient with prolidase deficiency
(after Powell et al., 1974)

are in relatively high concentrations whereas glutamate and aspartate have very low levels in collagen.

Royce and Danks (1982) showed a normal proline in collagen secreted by skin fibroblasts from hydroxylation of \wedge a prolidase deficient patient supporting the hypothesis that proteins other than collagen contribute to the constitution of the imidodipeptiduria. This imidodipeptiduria is not symptomatic of prolidase deficiency and has been reported in rickets (Scriver et al., 1964; Scriver, 1964; Saville and Alderman, 1970), hyperparathyroidism (Thomson et al., 1969; Cahill et al., 1970) and unusual bone diseases (Marshall, 1962; Seakins, 1963; Alderman et al., 1969; Isaacs et al., 1971). Thus prolidase activity should be determined to confirm an enzymatic nature to the imidodipeptiduria.

1.4.4. Collagen investigations

A number of investigations have been undertaken to study the effect of prolidase deficiency on collagen. Amino acid composition, the hydroxylation rate of proline and lysine residues and glycosylation of hydroxylysine were similar to that in normal collagen (Myara et al., 1984a). Also, Isemura et al. (1981) examined the skin collagen of a patient with prolidase deficiency for the distribution of borohydride-reducible cross-links and the ratio of type III to type I collagen. The patient's skin contained

more dihydroxylysinoxidation and a larger proportion of type III collagen. This modification was also noted by Myara et al. (1983) studying prolyl-4-hydroxylase-deficient fibroblasts in which they found: i) collagen is present in normal amounts; ii) a modified ratio of type I to type III collagen; iii) collagen hydroxylation is normal; iv) the proline pool is significantly decreased because proline is trapped for imidodipeptide synthesis and thus cannot be reused.

Jackson et al. (1975) proposed a blocking of the normal re-cycling of proline, slowing the normal rate of production of collagen or leading to the formation of an abnormal collagen. The collagen-related clinical manifestations of prolyl-4-hydroxylase deficiency may therefore be related to this proline deficit. However, patients do not seem to benefit from proline-supplemented diets.

1.4.5. Genetics

The gene locus of prolyl-4-hydroxylase has been assigned to chromosome 19 in man (McAlpine et al., 1976; Brown et al., 1978). Prolyl-4-hydroxylase deficiency is a genetic disease inherited as an autosomal recessive trait (Powell et al., 1974; Gejyo et al., 1980; Isemura et al., 1981). Heterozygotes present without any clinical or biochemical symptoms and their erythrocyte prolyl-4-hydroxylase activity was about 50% of that of controls (Ogata et al., 1981). As with other genetic diseases there is a

fair deal of heterogeneity and some asymptomatic cases have been reported (Isemura et al., 1981; Arata et al., 1979).

Lewis and Harris (1969) studied prolydase in erythrocytes from a number of different individuals after starch gel electrophoresis. This showed the existence of various prolydase phenotypes. The usual phenotype is referred to as Pep D1 and the variants Pep D2, Pep D2-2 and Pep D3-1.

1.4.6. Treatment

Because of the massive loss of proline through imidodipeptiduria, Powell et al. (1974) suggested treatment with a dietary supplement of proline. This was attempted by Sheffield et al. (1977) and Isemura et al. (1979) but without any clinical improvement. Sheffield et al. (1977) then proposed therapy with drugs that may act on collagen synthesis or inhibit collagenase such as diphenylhydantoin or oxaceprol. Manganese and ascorbic acid, cofactors for prolydase activity and collagen synthesis, were also suggested for treatment. Diphenylhydantoin and oxaceprol failed to improve the clinical condition of one patient but manganese and ascorbic acid led to a disappearance of skin rashes and a dramatic reduction in glycyL-proline excretion, but without any improvement in the leg ulcerations (Charpentier et al., 1981). In an earlier

study high doses of ascorbic acid in intravenous infusions had been ineffective (Lapiere and Nusgens, 1969). Pedersen et al. (1983) treated their four-year old patient with manganese, ascorbic acid and proline daily and saw a definite clinical improvement of the skin manifestations. Discrepancies between the results of various authors may be due to the different ages of the patients, the severity of the skin lesions or the combination of drugs used.

Enzyme replacement therapy has been attempted in two patients (Isemura et al., 1981; Endo et al., 1982). No significant changes in the imidodipeptiduria were observed and the clinical symptoms were unchanged though Endo et al. (1982) found prolidase activity in the patient's erythrocytes to be raised to about 35% of control values after erythrocyte infusion.

Ogata et al. (1981) treated the leg ulcerations with wet dressings containing antibiotics or proline and also attempted split thickness skin grafting. This had a temporary beneficial effect for about a month followed by rejection in one patient whereas another patient kept the grafts for two years before they were rejected.

The anti-leprosy drug, Dapsone (diamino 4,4' diphenyl sulphone), has been administered to several patients and did cause accelerated epithelialisation of the ulcers. Dapsone may therefore be considered for

use in the treatment of prolidase deficiency, though its mechanism of action is unknown.

The most recent proposal for the treatment of the leg ulcerations seems to be the most promising. Arata et al. (1986) applied a topical ointment containing both glycine and proline to the leg ulcers of an adult patient and observed a dramatic improvement. These ulcers still remain healed after six months.

It seems to be most important that prolidase deficiency is diagnosed in early childhood in order to begin therapy at as early an age as possible. Then the leg ulceration and maybe even the mental retardation might be prevented by drug therapy.

1.5. AIMS OF THIS PROJECT

Although there are a number of published methods for measuring prolinase and prolidase, they all suffer drawbacks in the analysis of human cultured cells used in the diagnosis of enzyme deficiencies. For example, the method of Troll and Lindsley (1955) as modified by Powell et al. (1974) is time-consuming, uses benzene and insensitive, requiring high levels of protein in the assay. The method of Arata et al. (1979) used an amino acid analyser to separate the products for the substrate, followed by ninhydrin detection and also needed high levels of protein in the assay. Thus there

was a need to develop simple sensitive assays for both enzymes.

Initially a new fluorimetric assay was developed for prolinase adapting the method of Roth (1971) and using o-phthalaldehyde to detect the amino acid liberated. Using this method it was then possible to derive optimal assay conditions for prolinase and make a study of its properties. Previous work on prolinase has been performed on animal tissues and few studies have been undertaken with the human enzyme (Smith, 1960; Delange and Smith, 1971; Walter et al., 1980). A variety of chromatographic procedures were undertaken to study the possibility of more than one form of prolinase being present. Ion-exchange chromatography and gel filtration showed two distinct components of prolinase to be present in human cultured skin fibroblasts but only one in human kidney. As it has been proposed that a single enzyme can hydrolyse both prolinase and non-specific dipeptidase substrates (Binkley et al., 1968; Hayman and Patterson, 1971), prolinase was isolated from human kidney to study further the relationship between the two enzymes.

Two methods were developed for prolidase which detect the C-terminal amino acid liberated from the hydrolysed substrate. The methods were adapted from the assays of Shoaf et al. (1974) and Matsuomoto et al. (1982) and used to determine the conditions of assay and to investigate the properties of prolidase.

Prolidase-deficient fibroblasts from two unrelated cases of prolidase deficiency were used to study the properties of the residual prolidase activity. The major form of prolidase was then purified from human kidney using a variety of chromatographic procedures and injected into rabbits to raise specific antibodies to prolidase. These were then used to investigate the presence of cross-reacting material in the prolidase-deficient fibroblasts.

2. DEVELOPMENT OF A FLUORIMETRIC ASSAY FOR PROLINASE

2.1 INTRODUCTION

Existing methods for the estimation of prolinase activity (Josefsson and Lindberg, 1965; Powell et al., 1974; Sheffield et al., 1977; Arata et al., 1979) are relatively insensitive and are therefore unsuitable for the rapid assay and characterisation of prolinase in human cultured cells. Also, the described conditions of assay have been derived from animal tissues, as human prolinase has not been characterised (Smith, 1960; Delange and Smith, 1971; Walter et al., 1980). The action of prolinase on a proline dipeptide produces an equimolar amount of proline and amino acid so a highly sensitive method detecting the primary amino-group on the amino acid would be ideally suited for the assay of prolinase. The fluorogenic reaction between o-phthalaldehyde and amines in the presence of a sulphhydryl reagent as described by Roth (1971), is one of the most sensitive methods for the determination of primary amines. A modification of the Roth reaction was therefore developed for the estimation of prolinase activity.

Conditions of assay were assessed for human cultured skin fibroblasts and prolinase activity determined in cultured amniotic fluid cells,

erythrocytes, white blood cells and a range of tissues
obtained at autopsy.

2.2. METHODS

2.2.1. Enzyme source and preparation of extract

Human skin fibroblasts and amniotic fluid cells were cultured as described in the appendix (section 11.1.). Tissue samples were obtained at autopsy 24hr after death and red and white blood cells were prepared from venous blood from children by Dextran sedimentation (Snyder and Brady, 1969). All samples were stored at -70°C prior to assay.

Extracts were prepared by the addition of ice-cold distilled water, sonication at 4-6 μm (MSE Ultrasonic Disintegrator Mk11) for 30 sec for cells and 60 sec for tissues followed by centrifugation at 1000 x g for 10 min.

2.2.2. Fluorimetric enzyme assay

The basic assay system developed consisted of 50 μl extract (0.2 mg protein/ml), 50 μl 50 mM barbital / HCl buffer (pH range 6.8-9.6) and 100 μl 30 mM prolyl-glycine (Pro-Gly, ^{Sigma Ltd}_^). Each assay tube was incubated for up to 120 min at 37°C after which 200 μl 7.2 g/l perchloric acid was added to stop the reaction. After keeping at 5°C for 15 min, the assay tubes were spun at 2000 x g for 10 min to remove precipitated protein, and 50 μl portions taken for estimation of liberated

glycine. Substrate and extract blanks and a glycine standard were run simultaneously.

Glycine was estimated by a modification of the method of Roth (1971) based on that used for tyrosine (Guy and Butterworth, 1978). The reagent (100 ml) consisted of 3 ml o-phthalaldehyde (12 g/l methanol), 3 ml 2-mercaptoethanol (0.6% v/v in ethanol) and 94 ml 150 mM Na_2CO_3 / NaHCO_3 buffer pH 9.5 and was freshly prepared. 850 μl of this reagent was added to 50 μl incubation mixture or amino acid standard. The reaction was timed from addition of reagent and the development and subsequent decay of fluorescence was followed in a Perkin-Elmer 1000 fluorimeter (excitation 365 nm, emission 455 nm). Before use the fluorimeter was standardised against the stable fluorochrome (excitation 365nm, emission 455nm) quinine_A to give 250 fluorescence units at 1 mg/l. Standard curves for each amino acid were then constructed using a range of concentrations up to 0.25 mM. pH profiles (pH 6.8-9.6) were also constructed with 0, 0.025 and 1 mM MnCl_2 final concentration in the assay. The effect of Manganese ions on prolinase activity was also studied at pH 7.4 with a range of MnCl_2 concentrations 0-2 mM and at pH 9.2 with a range 0-0.2 mM.

2.2.3. Spectrophotometric enzyme assay

For comparison with the fluorimetric method, the spectrophotometric method for prolinase as given by Powell et al. (1974) was used. The assay consisted of 50 μ l cell extract (5 mg/ml), 100 μ l 200 mM borate buffer pH 8.0 and 50 μ l 24 mM Pro-Gly. The assay tubes were incubated for 30 or 60 min at 37°C after which 1.3 ml ethanol ^{was} added, allowed to stand for 10 min, spun at 2000 x g for 10 min and a 1 ml sample read at 220 nm on a Perkin-Elmer dual beam spectrophotometer. The assay was also stopped and analysed as for the fluorimetric assay.

2.2.4. Time and protein linearities for prolinase

The linearity of the hydrolysis of Pro-Gly was determined for skin fibroblasts with incubations up to 3 hr and for levels of protein up to 1.6 mg/ml. Protein was measured (Lowry et al., 1951) using bovine serum albumin as standard as described in the appendix (section 11.3.).

2.2.5. Prolinase activities in cells and tissues

Prolinase activity was also determined at pH 7.4 and 9.2 for a range of human skin fibroblast cultures (n = 15), amniotic fluid cells (passage 2 + 3, n = 19),

erythrocytes (n = 3), white cells (n = 3) and a number of tissues. The specific activities of Cathepsin B and β -hexosaminidase were simultaneously determined for the tissue extracts as described by Butterworth and Duncan (1979, 1980) and Butterworth and Guy (1979).

2.3. RESULTS

2.3.1. Reaction of amino acid standards with o-phthalaldehyde-mercaptoethanol reagent

After addition of o-phthalaldehyde-mercaptoethanol reagent, the time taken to reach the maximum fluorescence and the rate of decay of this fluorescence depended upon the particular amino acid. Glycine and alanine were the most reactive (Fig.3) of the amino acids studied and reached a maximum fluorescence after one minute, however this fluorescence then decayed and 25% of this reading was lost after 8 minutes. The reaction for valine, leucine, phenylalanine and glutamic acid was maximal after 1½ minutes and stayed stable for at least 5 minutes. Because of the decay of fluorescence it was important to take a reading on the fluorimeter 1 minute after addition of reagent for glycine and alanine and at any time between 1½ and 5 minutes for the other amino acids. In this way standard curves for the amino acids were constructed (Fig.4) and were found to be linear within the limits of the capability of the fluorimeter. The method was highly sensitive and 10 nmoles glycine, alanine, valine, leucine, phenylalanine and glutamic acid gave 950, 830, 1050, 990, 1050, and 1060 fluorescence units respectively. The blank due to the reagent was less than 20 units and increased by only a few units after

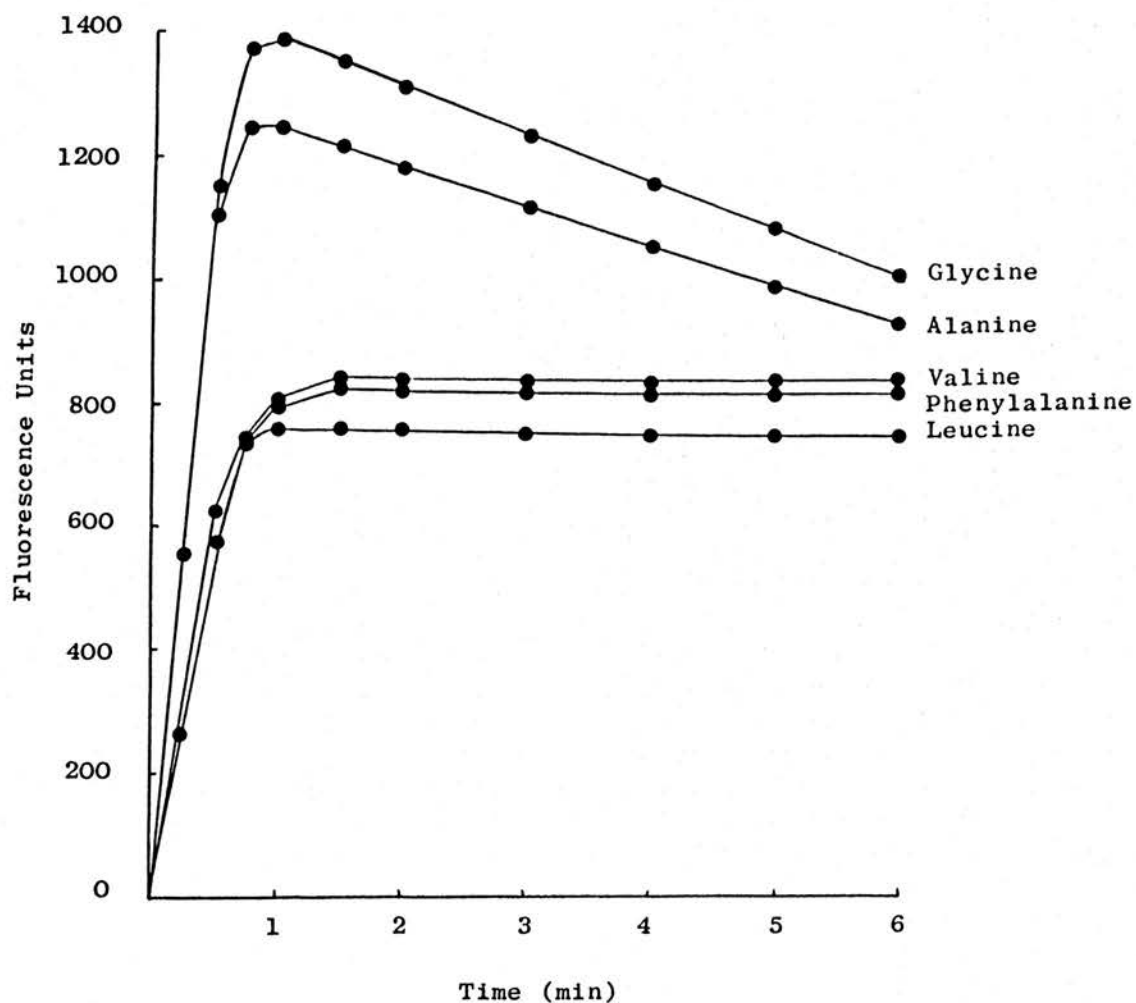


Fig. 3 Time course of the fluorescent reaction of o-phthalaldehyde - mercaptoethanol reagent with amino acid standards. 850 μ l reagent was reacted with 50 μ l assay mixture containing 15 nmol glycine or alanine and 7.5 nmol valine, phenylalanine or leucine.

Proline did not react with the reagent.

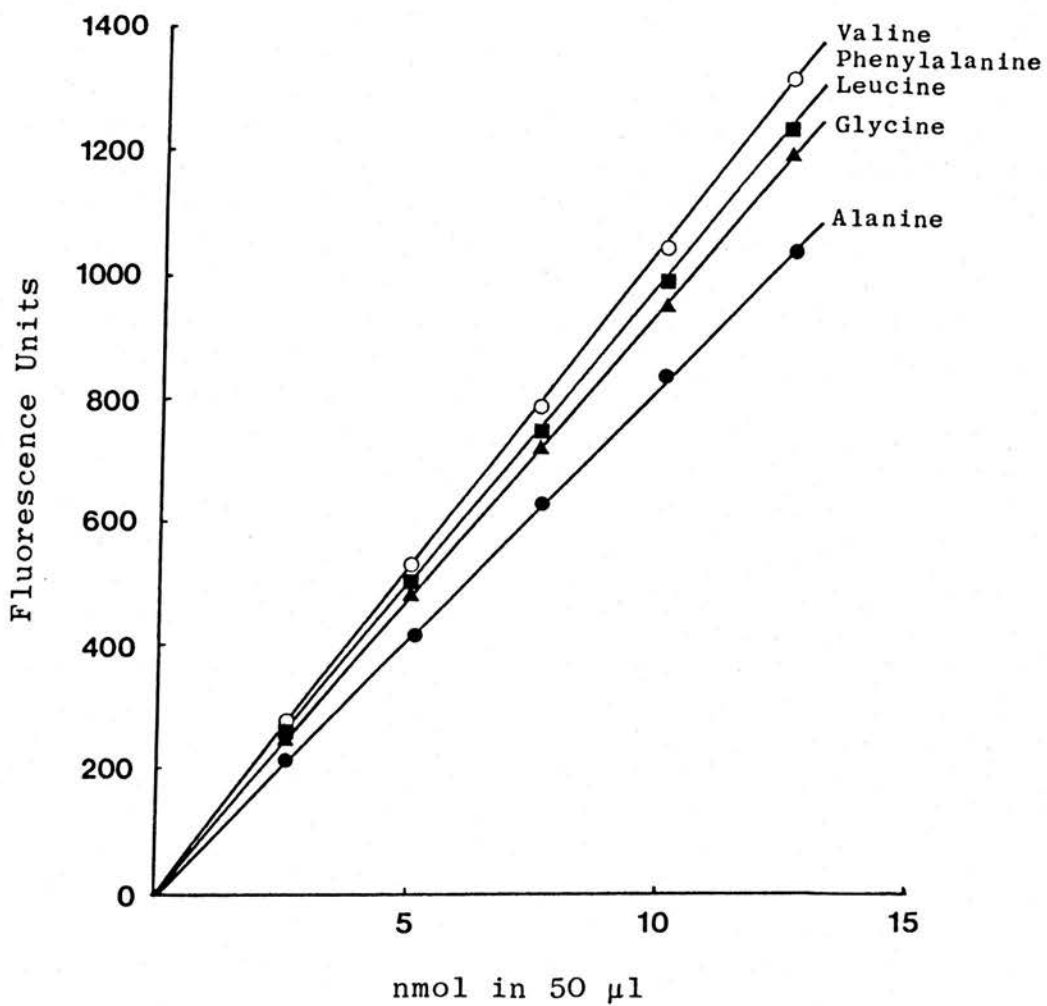


Fig. 4 Standard curves for amino acid standards (50 μ l) reacting with 850 μ l o-phthalaldehyde - mercaptoethanol reagent.

several hours. By comparison the spectrophotometric method of Powell et al. (1974) relies on the fall in absorption at 220 nm as peptide bonds are hydrolysed and resulted in a drop in optical density of 0.025 for 100 nm of substrate hydrolysed.

The pH of the reaction mixture has a large effect on the rate of the fluorescence-producing reaction and is optimal close to pH 9.5. It is therefore important to ensure that the reagent is sufficiently buffered to counteract the low pH of the final assay mixture resulting from the addition of perchloric acid as protein precipitant. The amount of assay mixture taken to estimate the liberated amino acid can be increased but the reagent buffer molarity must be increased accordingly. Although 100 mM will suffice for 50 μ l assay volume, 150 mM was routinely used to permit either 50 or 100 μ l to be taken. 1000 mM buffer was required for 400 μ l of assay volume, the maximum tested.

2.3.2. Determination of pH optimum for prolinase

in 25 mM barbital / HCl buffer

The pH profile ^A for the hydrolysis of Pro-Gly by prolinase in skin fibroblasts was highly dependent upon the level of manganese ions present (Fig.5). Without added manganese a broad optimum at pH 8.4 to 9.4 was found, whilst the optimum with 1 mM $MnCl_2$ was 7.4 - 7.8 and with 0.025 mM $MnCl_2$, pH 9.2, this last condition

giving the highest activity. The optimal concentration of MnCl_2 at pH 7.4 was 1 mM and at pH 9.2, 0.025 mM with inhibition above these respective concentrations (Fig.6).

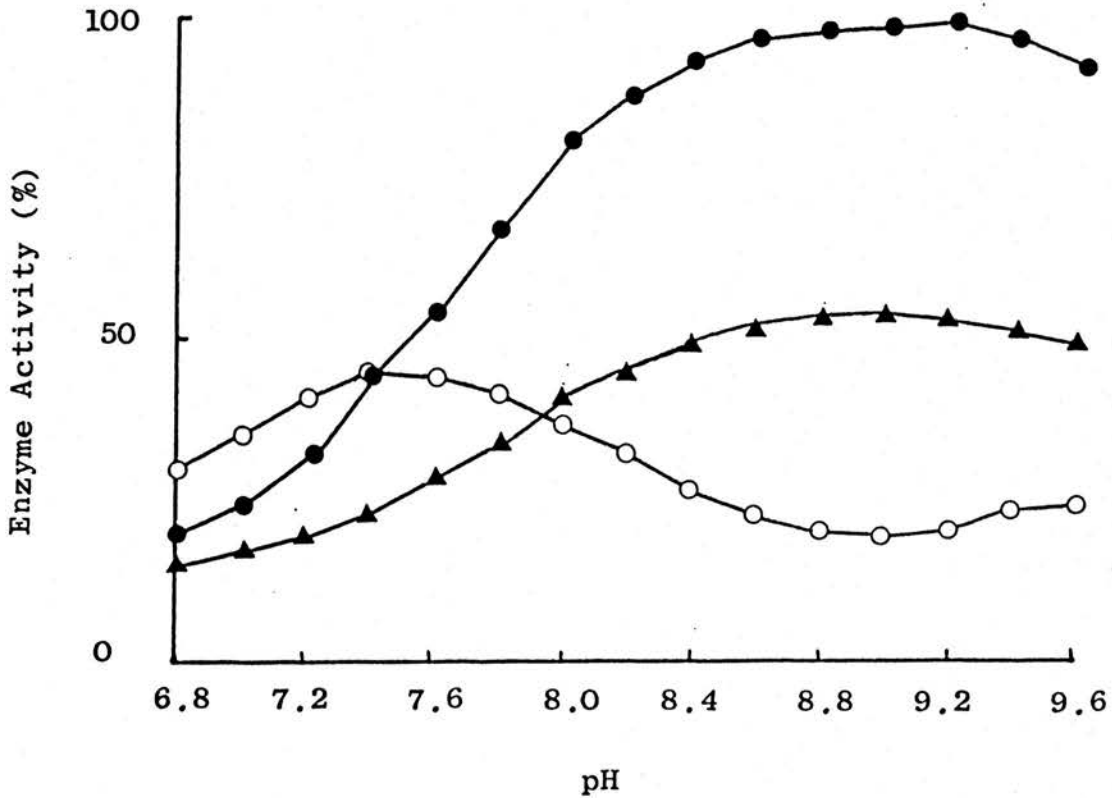


Fig. 5 pH profiles for human skin fibroblast prolinase assayed with Pro-Gly in the presence of (\blacktriangle) 0; (\bullet) 0.025 mM; and (\circ) 1 mM MnCl_2 .

100% = maximum activity with 0.025 mM MnCl_2

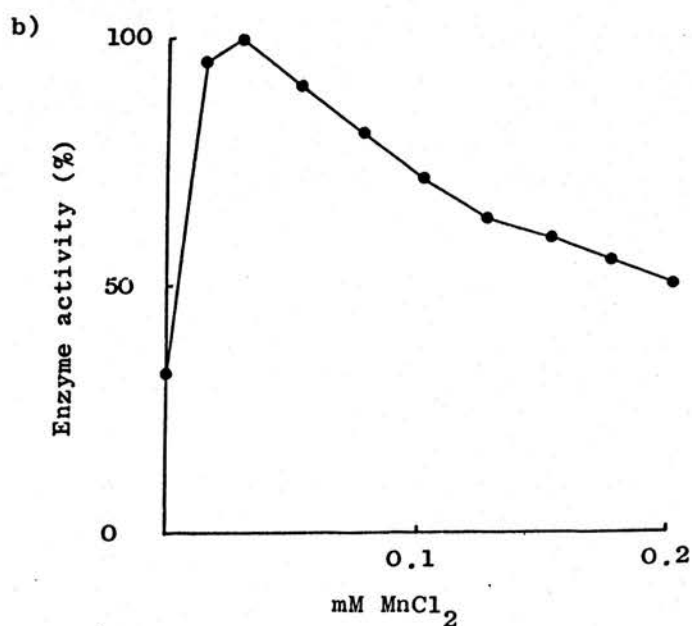
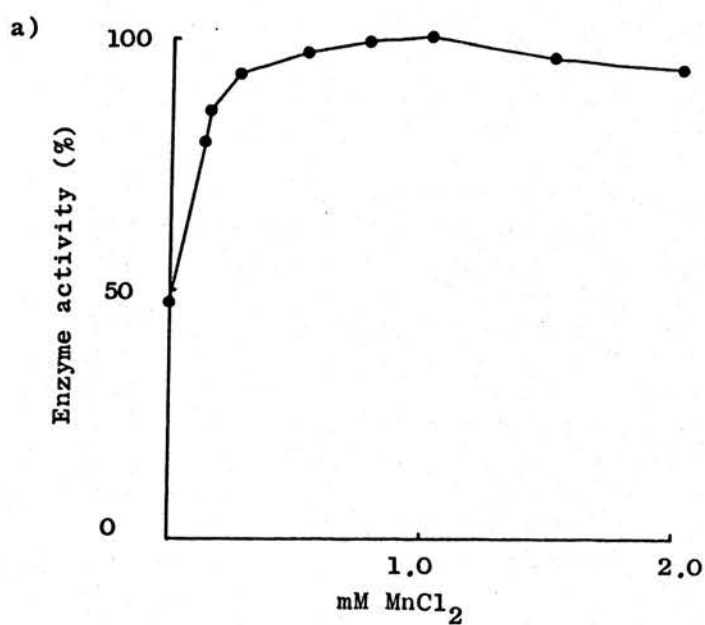


Fig. 6 Effect of different levels of manganese on human skin fibroblast prolinase activity against Pro-Gly assayed at a) pH 7.4 and b) pH 9.2.

a) 100% = 45 nmol/min/mg

b) 100% = 78 nmol/min/mg

2.3.3. Time and protein linearities

The prolinase assay was linear with time for at least 3 hr at pH 7.4 and 9.2 and with protein concentrations up to a level hydrolysing as much as 20% of substrate (Fig.7). A typical assay containing 10 ug fibroblast protein hydrolysed less than 4% of the substrate.

2.3.4. Prolinase activities

Using Pro-Gly, cultured skin fibroblasts (n = 15) had a prolinase mean specific activity (nmol/min/mg protein \pm SD) of 42.4 ± 12.2 (range 26-72) at pH 7.4 and 80.1 ± 24.2 (range 47-134) at pH 9.2, whilst amniotic fluid cells (n = 19) were 56.9 ± 30.3 (range 21-124) and 108.5 ± 58.5 (range 41-237) respectively (Fig.8). The omission of four epithelioid amniotic fluid cell cultures tested, which had high prolinase values, resulted in mean values very similar to those for fibroblasts. Three white blood cell samples (polymorphonuclear cells) had specific activities of 34, 55 and 82 at pH 7.4 and 51, 77 and 122 at pH 9.2 respectively. Three red blood cell samples had rather low specific activities of 7.3, 8.7 and 8.8 at pH 7.4 and 10.5, 11.8 and 12.9 at pH 9.2 respectively. Prolinase was also estimated in a number of tissues obtained at autopsy (Table 3). Apart from heart

muscle, all the tissues had moderate activities, with kidney being particularly active.

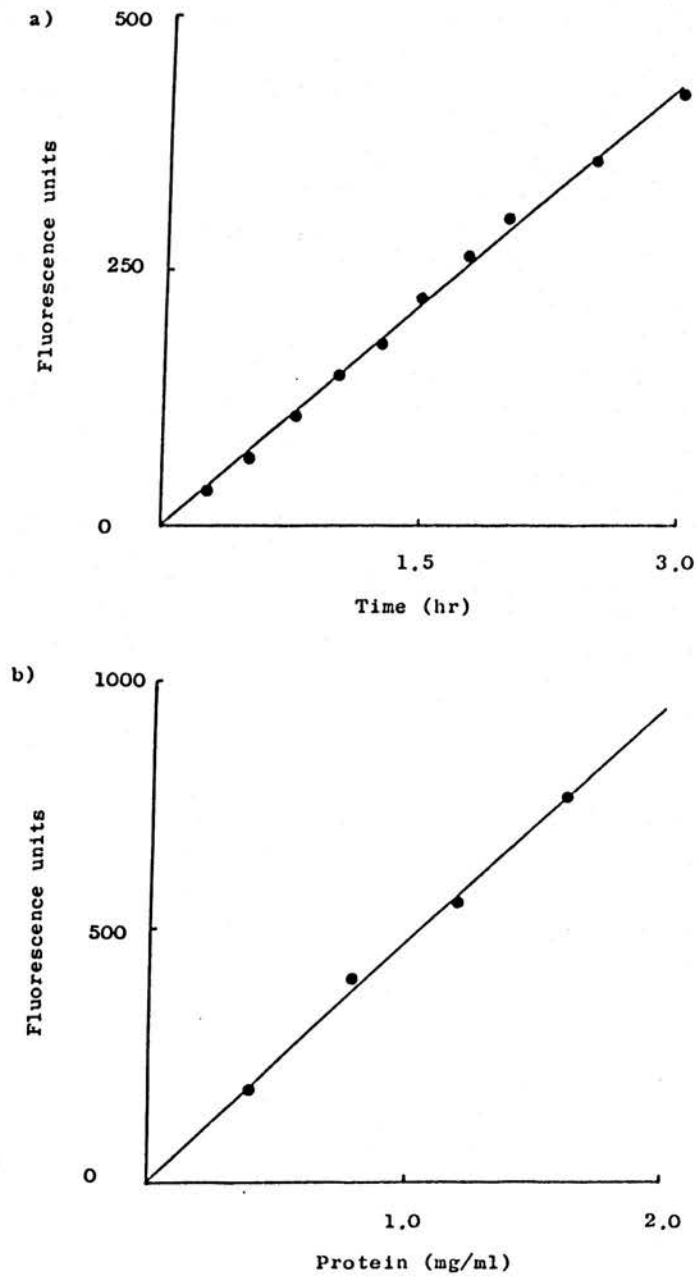


Fig. 7 a) Time and b) protein linearities for human skin fibroblast prolinase assayed with Pro-Gly at pH 7.4 with 1 mM $MnCl_2$.

(950 Fluorescent Units = 10 nmol glycine in 50 μ l assay mixture)

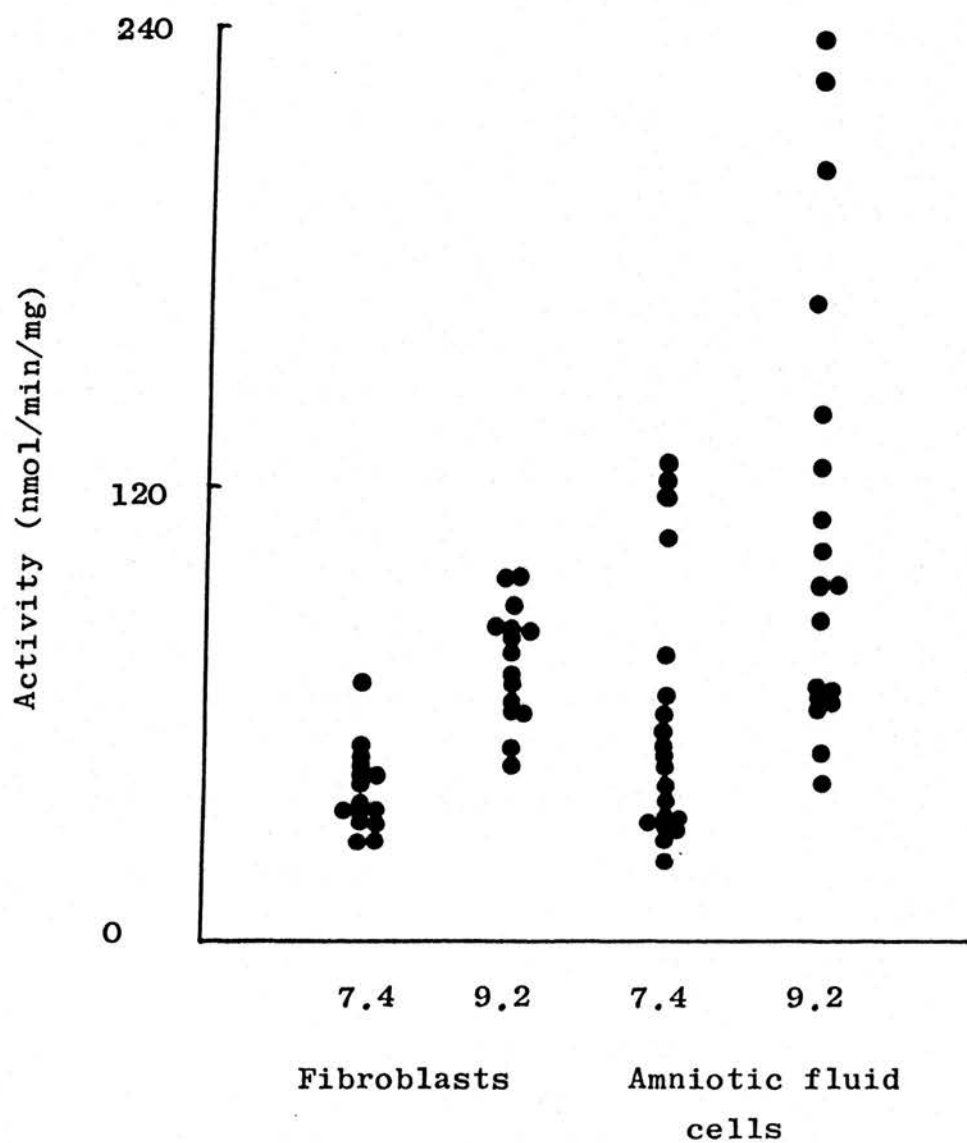


Fig. 8 Prolinase activities at pH 7.4 with 1 mM $MnCl_2$ and pH 9.2 with 0.025 mM $MnCl_2$ for cultured skin fibroblasts and amniotic fluid cells assayed with Pro-Gly.

Tissue	Prolinase		Cathepsin B	β -Hex
	pH 7.4	pH 9.2		
Kidney	451	974	8.2	49.5
Thyroid	338	554	94.2	41.9
Thymus	200	324	28.6	82.6
Pancreas	160	317	5.3	29.1
Adrenal	153	273	7.9	52.4
Pituitary	150	270	2.9	26.2
Liver	115	237	6.9	68.4
Choroid Plexus	116	234	5.2	31.1
Cerebral Cortex	123	231	0.8	5.8
Small Bowel	107	211	3.8	69.1
Lung	101	183	2.2	14.4
Skeletal Muscle	87	155	1.1	6.5
Skin	80	125	2.6	7.2
Spleen	64	117	2.4	15.9
Cartilage	54	108	4.0	23.4
Placenta	55	89	1.8	9.7
Heart	19	29	2.4	13.8

Table 3. Specific activity (nmol/min/mg protein) of Prolinase, Cathepsin B and β -Hexosaminidase in human tissues.

2.4. DISCUSSION

2.4.1. Fluorimetric assay

The o-phthalaldehyde-mercaptoethanol method provides a simple and sensitive assay for amino groups. It has an advantage over other methods in that it does not react with proline, hence substrates of the type proline-amino acid which have no N-terminal amino groups, will not produce a fluorescence. In alternative assays, for example the ninhydrin method (Mayer and Nordwig, 1973), there is a reaction with proline requiring the separation of substrate and products. Furthermore, the estimation of amino acids using ninhydrin is about ten times less sensitive than with o-phthalaldehyde. The method given for prolinase by Powell et al. (1974), based on the detection of proline using the ninhydrin method of Troll and Lindsley (1955), is time-consuming, uses benzene and needs at least twenty times as much enzyme protein in the assay as in the present method. Also this technique doesn't lend itself to large numbers of samples as generated by kinetic and chromatographic studies. Comparison was therefore made with the method of Josefsson and Lindberg (1965) as modified by Powell et al. (1974), which depends on the fall in absorbance at 220 nm as the substrate peptide bond is hydrolysed. A 10% hydrolysis resulted in a drop in optical density of

about 0.03 representing 120 nm glycine. The present method would give a reading of about 1400 for a blank of less than 20. The small drop in absorbance, read against the initial high value resulted in low sensitivity and required a very accurate technique. Also the spectrophotometric method was unsuitable in the presence of haemoglobin, whereas it had no effect in the present fluorimetric method. By using the complete assay volume of 400 μ l instead of 50 μ l for amino acid estimation, the amount of enzyme needed may be as little as 1 μ g per assay, making this method as much as 200 times as sensitive as previous methods.

Prolyl-Glycine was used as the preferred substrate, despite glycine's short-lived fluorescence, because this was the substrate most frequently used in other studies on prolinase (Mayer and Nordwig, 1973; Powell et al., 1974) and allowed comparison of the present with previous findings.

2.4.2. pH profile

The broad pH optimum, pH 8.4-9.4, in the absence of manganese ions compares with pig kidney prolinase which showed optimal activity at pH 8.75 (Mayer and Nordwig, 1973). The optimum pH of 9.2 in the presence of 0.025 mM MnCl_2 was similar to that observed in human plasma (Myara et al., 1985a).

2.4.3. Prolinase activities

Prolinase activity in human skin fibroblasts was similar to the mean value of 67 nmol/min/mg recently reported by Myara et al.(1984b). However, the mean value of 16 nmol/min/mg observed by Arata et al. (1979) was probably much lower because of sub-optimal assay conditions without added manganese. Although erythrocyte prolinase activity was relatively low compared with fibroblasts, it was markedly higher than previously reported values of 0.20 (Powell et al., 1974) and 0.14 (Isemura et al., 1981). White blood cell prolinase was also higher than the activity of 24 nmol/min/mg reported by Powell et al. (1974). These large discrepancies are probably the result of sub-optimal assay conditions.

3. STUDIES ON PROLINASE IN HUMAN TISSUES

3.1. INTRODUCTION

Having developed a rapid and sensitive method for the estimation of prolinase activity, it was possible to characterise the human enzyme and elucidate some of its properties. Very little research into human prolinase has been undertaken so a study was made to determine i) intracellular localisation, ii) effect of heat, iii) effect of pH, iv) activities in a variety of human tissues, v) substrate specificity and vi) kinetics. Also a variety of separative procedures were performed including ion-exchange chromatography, gel filtration and isoelectrofocusing.

3.2. METHODS

3.2.1. Subcellular fractionation

The intracellular localisation of prolinase was determined in fibroblasts by subcellular fractionation as described by Guy and Butterworth (1978). A cell extract (1 mg/ml) was prepared in 0.25 M sucrose by homogenising in an all glass Potter-Elvehjem homogeniser (10 up-down strokes). The homogenate was centrifuged at 800 x g for 5 min to bring down non-disrupted cells and then re-homogenised. The resulting homogenate was then used for differential centrifugation. The total enzyme activity was then determined in a portion of this homogenate. The remaining homogenate was then centrifuged at 3,500 x g for 10 min to yield a pellet which was resuspended to obtain the heavy fraction. The supernatant was then centrifuged at 22,500 x g for 20 min for the light fraction and then 100,000 x g for 30 min for the microsomal fraction, the final supernatant being the soluble fraction. Prolinase (section 2.2.2.), lactate dehydrogenase (Bergmeyer, 1965), fumarase (Whittaker and Barker, 1972), α -L-fucosidase and β -hexosaminidase (Butterworth and Guy, 1979) activities were determined in all fractions, the latter two enzymes were also assayed in the presence of 0.1% Triton-X-100 (Sigma Ltd). Protein was also determined by the method of

Lowry et al. (1951) as described in the appendix (section 11.3.).

3.2.2. Effect of heat

For heat stability, extracts in 10 mM barbital / HCl buffer pH 7.4 were treated at 45-60°C for 5 min or at 52°C for up to 60 min prior to assay. 100 mM NaCl and 0.2 mg/ml BSA were added to see if they might stabilise prolinase to heat.

3.2.3. pH stability

pH stability was determined by adjusting the pH of an extract (0.5 ml) with 0.01 mM HCl or NaOH, leaving for 30 min and neutralising prior to assay.

3.2.4. Substrate Specificity

Substrate specificity was studied in fibroblasts and amniotic fluid cells with a number of dipeptide substrates. Amino acids coupled to proline (Sigma Ltd) included glycine, alanine, leucine, phenylalanine, valine and glutamic acid (Pro-Gly, Pro-Ala, Pro-Leu, Pro-Phe, Pro-Val, Pro-Glu). These substrates were assayed at pH 7.6 with 1 mM $MnCl_2$ and pH 9.2 with 0.025 mM $MnCl_2$. Hydroxyprolyl-glycine (Hypro-Gly) was assayed at pH 8.8 with 1mM $MnCl_2$. Also, the hydrolysis

of two non-specific dipeptidase substrates, Glycyl-Leucine (Gly-Leu) and Phenylalanyl-Alanine (Phe-Ala) was determined using a micromethod based upon the assay of Shoaf et al. (1974).

3.2.5. Substrate kinetics

Substrate Kms were obtained for prolinase using Pro-Gly assayed at pH 7.6 with or without 1 mM $MnCl_2$ at concentrations up to 10 mM substrate or at pH 9.2 with or without 0.025 mM $MnCl_2$ at concentrations up to 20 mM substrate.

3.2.6. Dialysis

The effect of dialysis on prolinase was also studied by dialysing fibroblast extracts for 60 min against distilled water, 10 mM barbital / HCl buffer pH 7.4 or 100 mM NaCl.

3.2.7. Ion-exchange chromatography

Ion-exchange chromatography was performed using DEAE-Cellulose 52 (Whatman Ltd). 25g resin was dispersed in 150 ml 10 mM barbital / HCl and then titrated back to pH 7.4 with 0.5M HCl. The slurry was allowed to settle then the supernatant decanted. The remaining slurry was made up to 150 ml again with

buffer, the pH checked, then poured into a 1 x 25 cm column. The extract (2 ml) was dialysed for 60 min against the elution buffer, centrifuged at 100,000 x g for 30 min, then applied to the column. Elution was performed with a 0-250 mM NaCl linear gradient in 10 mM barbital / HCl buffer pH 7.4 and 1.25 ml fractions collected.

3.2.8. Gel filtration

Gel filtration was carried out with Sephadex G 150-120 (Sigma Ltd). The gel was swollen and equilibrated according to the manufacturers directions and poured into a column (2.5 x 55 cm). The extract (2 ml) was dialysed for 60 min against the elution buffer, centrifuged at 100,000 x g and applied to the column. Elution was performed with 10 mM barbital / HCl pH 7.4 containing 100 mM NaCl with an ascending flow-rate of 4 ml/hr and 1.25 ml fractions collected.

3.2.9. Sucrose density isoelectrofocusing

Isoelectrofocusing was performed in a sucrose gradient using a modification of the method of Godson (1970) employing " J-tubes " as described in the appendix (section 11.4.).

3.2.10. Hydrophobic chromatography

For hydrophobic chromatography, butyl-agarose (Miles Ltd) in a 0.8 x 10 cm column was equilibrated with 20 mM maleate buffer pH 6.5. A 2 ml sample, dialysed against this buffer, was applied to the column and eluted with a 0-300 mM NaCl linear gradient in the same buffer.

3.2.11. Affinity chromatography

Affinity chromatography was performed by applying a 2ml sample, dialysed against elution buffer, to a 0.8 x 9 cm column of Concanavalin-A Sepharose (Pharmacia Ltd) equilibrated with 10 mM barbital / HCl pH 7.4 containing 0.1 mM $MnCl_2$, 100 mM NaCl and 0.1% (v/v) Triton-X-100 and eluted with a 0-1 M α -methyl-D-glucoside (Sigma Ltd) linear gradient in the same buffer. Wheat-germ lectin-Sepharose (Sigma Ltd) affinity chromatography was also performed in a 0.8 x 9 cm column equilibrated with 10 mM barbital / HCl pH 7.4 containing 100 mM NaCl and 0.1% (v/v) Triton-X-100. Elution was carried out with a 0-1 M N-acetyl-D-glucosamine (Sigma Ltd) linear gradient.

3.3. RESULTS

3.3.1. Intracellular localisation

Prolinase was found to be almost entirely in the final supernatant on subcellular fractionation (Table 4). Lactate dehydrogenase and fumarase showed their typical localisation as did the lysosomal enzymes, α -L-fucosidase and β -hexosaminidase, which also showed their latency effect with Triton-X-100.

3.3.2. Heat stability

The enzyme was thermolabile with a log-linear fall-off in activity at 52°C (Fig.9a), but with a marked transition point at about 55°C and a rapid loss of activity at higher temperatures (Fig.9b). The presence of NaCl or BSA during the heat treatment increased the thermolability.

Enzyme	Enzyme Activity (%)				
	Total	Heavy	Light	Microsomal	Soluble
Prolinase	100	2	0	0	98
Lactate Dehydrogenase	100	10	0	10	80
Fumarase	100	54	0	0	46
α -L-Fucosidase					
a) + Sucrose	100	36	4	1	52
b) + 0.1% Triton-X-100	400	304	30	3	54
β -Hexosaminidase					
a) + Sucrose	100	37	10	10	52
b) + 0.1% Triton-X-100	748	510	57	15	49
Protein	100	24	15	13	48

Table 4. Subcellular distribution of prolinase in cultured skin fibroblasts.

Total refers to the activity in the original extract.

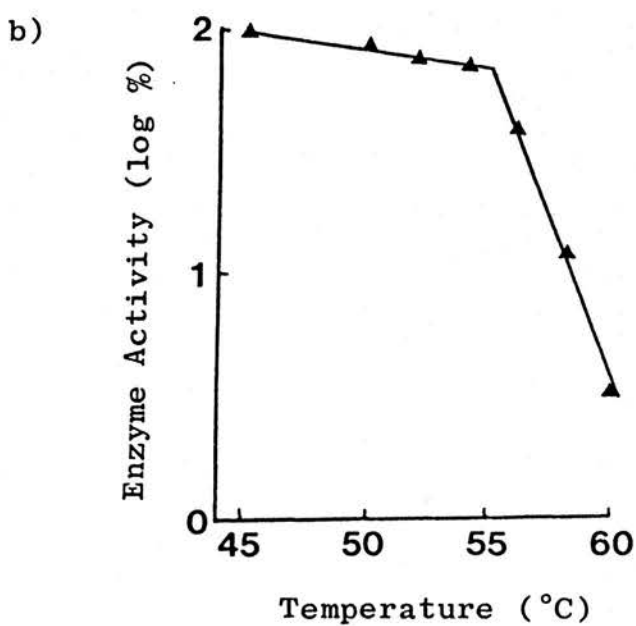
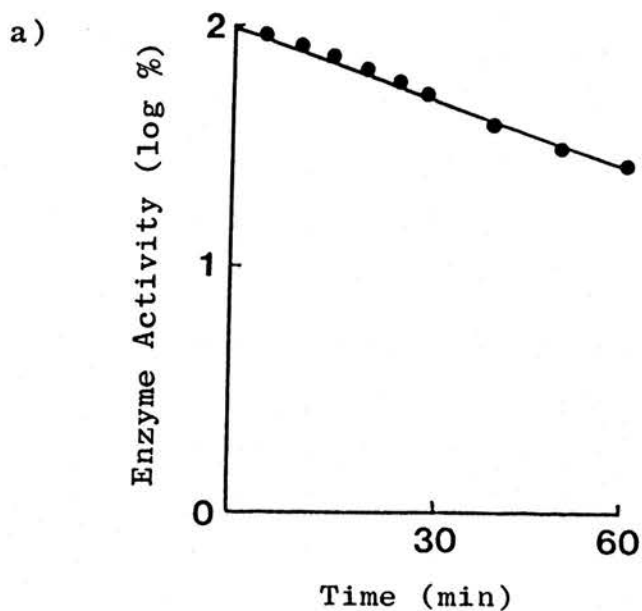


Fig. 9 Effect on fibroblast prolinase activity of
 a) heating at 52°C for up to 60 min and
 b) heating at various temperatures for 5 min .
 Prolinase was assayed with Pro-Gly at pH 7.4
 in the presence of 1 mM MnCl₂.

3.3.3. pH stability

Prolinase was stable between pH 6.5 and 9.5 but the activity was markedly reduced by up to 70% at pH 6 or below (Fig.10).

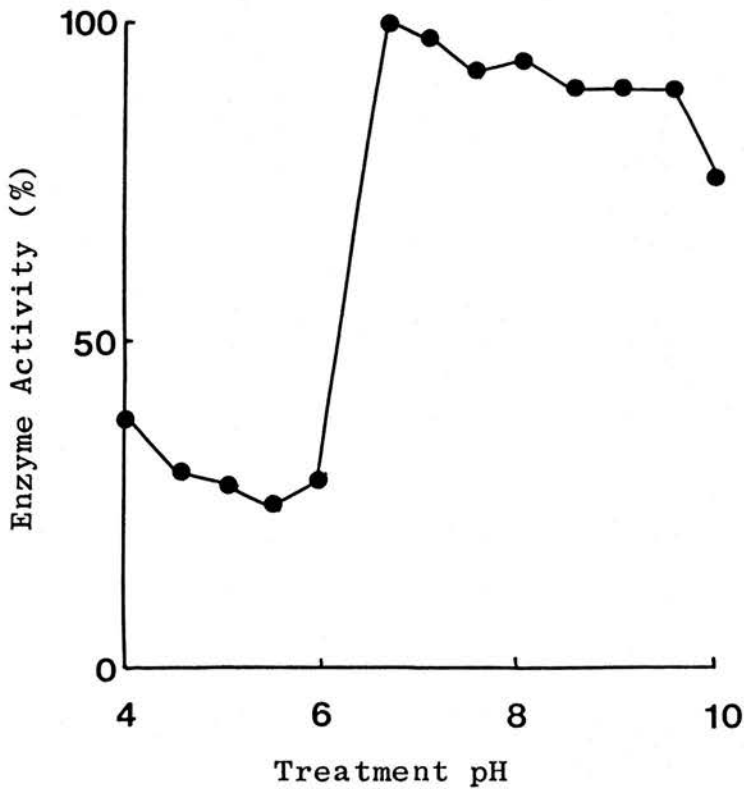


Fig. 10 pH stability of fibroblast prolinase activity assayed with Pro-Gly. The pH of an extract (0.05 ml) was adjusted with 0.01 mM HCl or NaOH, left for 30 min and neutralised prior to assay at pH 7.4 with 1 mM $MnCl_2$.

3.3.4. Substrate specificity

The hydrolysis of a number of proline dipeptides, hydroxyprolyl-glycine and two non-specific dipeptidase substrates Gly-Leu and Phe-Ala by skin and amniotic fluid cell cultures are given in Table 5. Pro-Leu, Pro-Phe and Pro-Val gave almost twice the activities of Pro-Gly and Pro-Ala at both pHs and for both cell types. Hypro-Gly gave much lower activities than all the proline dipeptides having 20% or less of the activity of Pro-Gly. Pro-Glu was not hydrolysed by cultured cell prolinase. Gly-Leu was hydrolysed at a much faster rate than any of the prolinase substrates

This was not a result of the use of different assay buffers. in both fibroblasts and amniotic fluid cells. A high activity was also found for Phe-Ala in fibroblasts though amniotic fluid cells had a relatively low activity against this substrate.

3.3.5. Substrate kinetics

Double-reciprocal plots were constructed for Pro-Gly (Fig 11) according to Lineweaver and Burk (1934). The hydrolysis of Pro-Gly was found to obey normal Michaelis-Menten kinetics giving Kms of 6.8 mM at pH 7.4 and 2.3 mM at pH 9.2.

Substrate	pH	Fibroblasts	Amniotic fluid cells
Pro-Gly	7.6	27	53
	9.2	42	89
Pro-Ala	7.6	44	77
	9.2	66	115
Pro-Leu	7.6	63	117
	9.2	106	189
Pro-Phe	7.6	57	127
	9.2	115	211
Pro-Val	7.6	69	129
	9.2	81	175
Hyp-Gly	8.8	6	12
Gly-Leu	8.0	462	150
Phe-Ala	8.0	218	68

Table 5. Dipeptidase activity (nmol/min/mg protein) of cultured cells at indicated pH values*.

* Barbital / HCl buffer, pH 7.6 + 1mM MnCl₂, pH 9.2 + 0.025 mM MnCl₂, pH 8.8 + 1 mM MnCl₂; Tris / HCl buffer, pH 8.0.

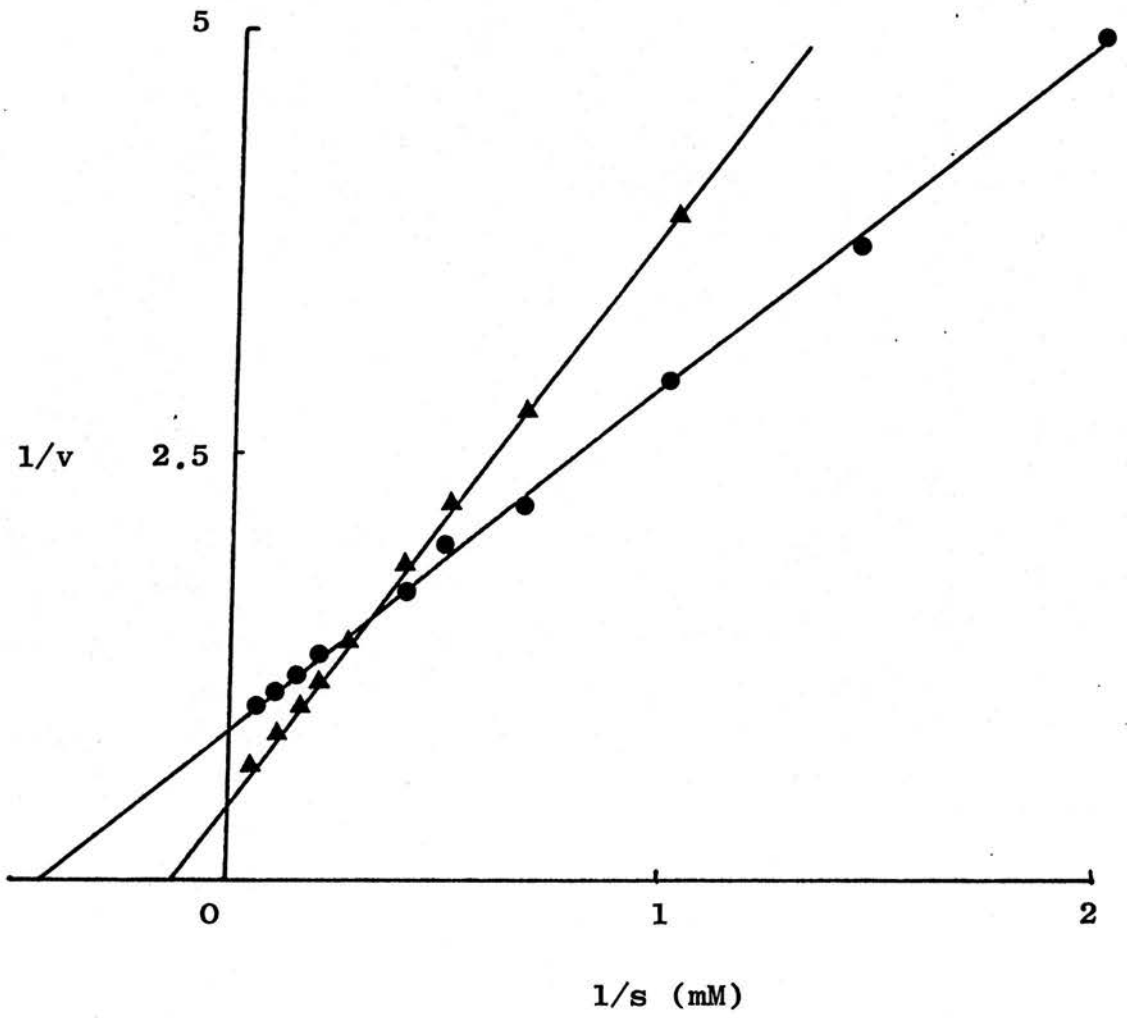


Fig. 11 Fibroblast prolinase activity at pH 7.4 (▲) and pH 9.2 (●) in relation to Pro-Gly concentration (Lineweaver-Burk plot).

3.3.6. Dialysis

Dialysis of the enzyme for 60 min against distilled water reduced prolinase activity by 75-85%, but by only 25% against 100 mM NaCl and less than 20% against 10 mM barbital / HCl pH 7.4.

3.3.7. Chromatography

For assays of prolinase by the fluorescent method after chromatographic procedures, it was not usually necessary to centrifuge following addition of perchloric acid, as the enzyme blank was very low. The chromatographic profiles obtained for DEAE-Cellulose, gel filtration, isoelectrofocusing and hydrophobic chromatography are shown in Figs. 12, 14, 15 and 16.

3.3.7.1. DEAE-Cellulose

Ion-exchange chromatography with DEAE-Cellulose (Fig.12) showed two forms of prolinase, a large peak eluting with 120 mM NaCl and a small peak eluting with 145 mM NaCl. The smaller peak was more pronounced when assayed at pH 7.4 than pH 9.2. The two peaks gave the same pH profile in the presence of 0.025 MnCl₂ with a pH optimum at about pH 9.4, but with 1mM MnCl₂ the first peak gave an optimum near pH 8.0, whilst that of the second peak was nearer pH 7.4 (Fig.13).

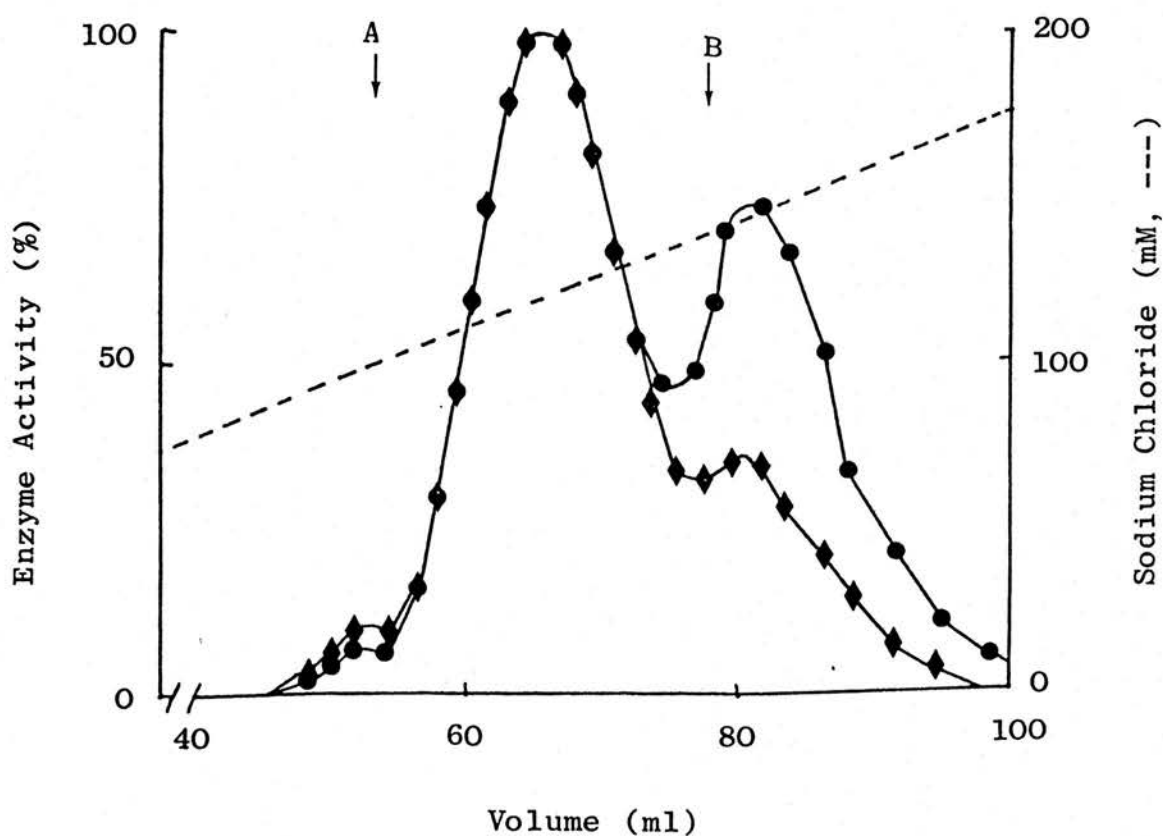


Fig. 12 DEAE-Cellulose chromatography of human skin fibroblast prolinase assayed at pH 7.4 (●) and pH 9.2 (◆) with Pro-Gly. 1 x 25 cm column eluted with 0-250 mM NaCl in 10 mM barbital / HCl buffer pH 7.4. A, β -hexosaminidase; B, α -L-fucosidase.

100% = 15 nmol/min/ml at pH 9.2 and 9 nmol/min/ml at pH 7.4

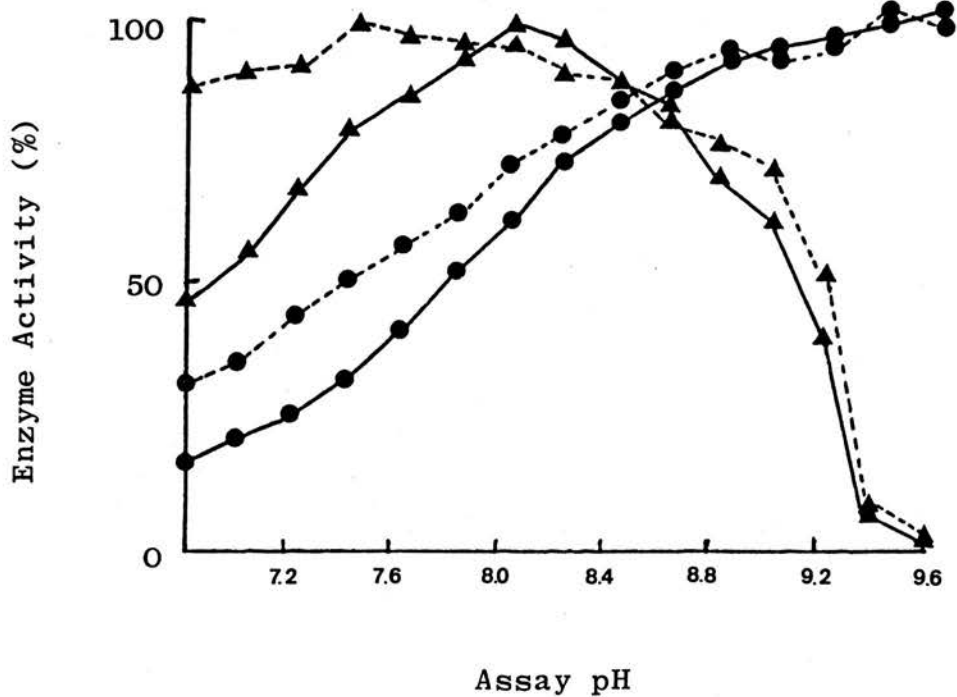


Fig. 13 pH profile of peak I (—) and peak II (----) prolinase activity separated by DEAE-Cellulose chromatography assayed with 1 mM (▲) and 0.025 mM (●) MnCl₂.

100% = 6 nmol/min/ml

3.3.7.2. Sephadex G150-120

Gel filtration (Fig.14) also separated prolinase into a large and a small peak and again the latter was more pronounced when assayed at pH 7.4.

3.3.7.3. Isoelectrofocusing

For sucrose density isoelectrofocusing it was necessary to dilute the fractions tenfold to reduce the blanks produced by the reaction between the ampholines and o-phthalaldehyde. A large peak was obtained at pI 5.75 with a smaller peak at pI 4.8 (Fig 15). The latter peak was associated with a protein band, of the original extract although it was not removed by centrifugation and showed a relatively higher activity at pH 7.4.

3.3.7.4. Butyl-agarose

Hydrophobic chromatography with butyl-agarose yielded only a single peak of prolinase activity (Fig.16) eluting at 90 mM NaCl. Cathepsin B was unbound, β -D-glucuronidase was released almost immediately on application of the chloride gradient whilst β -hexosaminidase was tightly bound.

3.3.7.5. Concanavalin-A-Sepharose and Wheat-germ lectin
Sepharose

Prolinase did not bind to Concanavalin-A-Sepharose nor to wheat-germ lectin Sepharose, although β -hexosaminidase did.

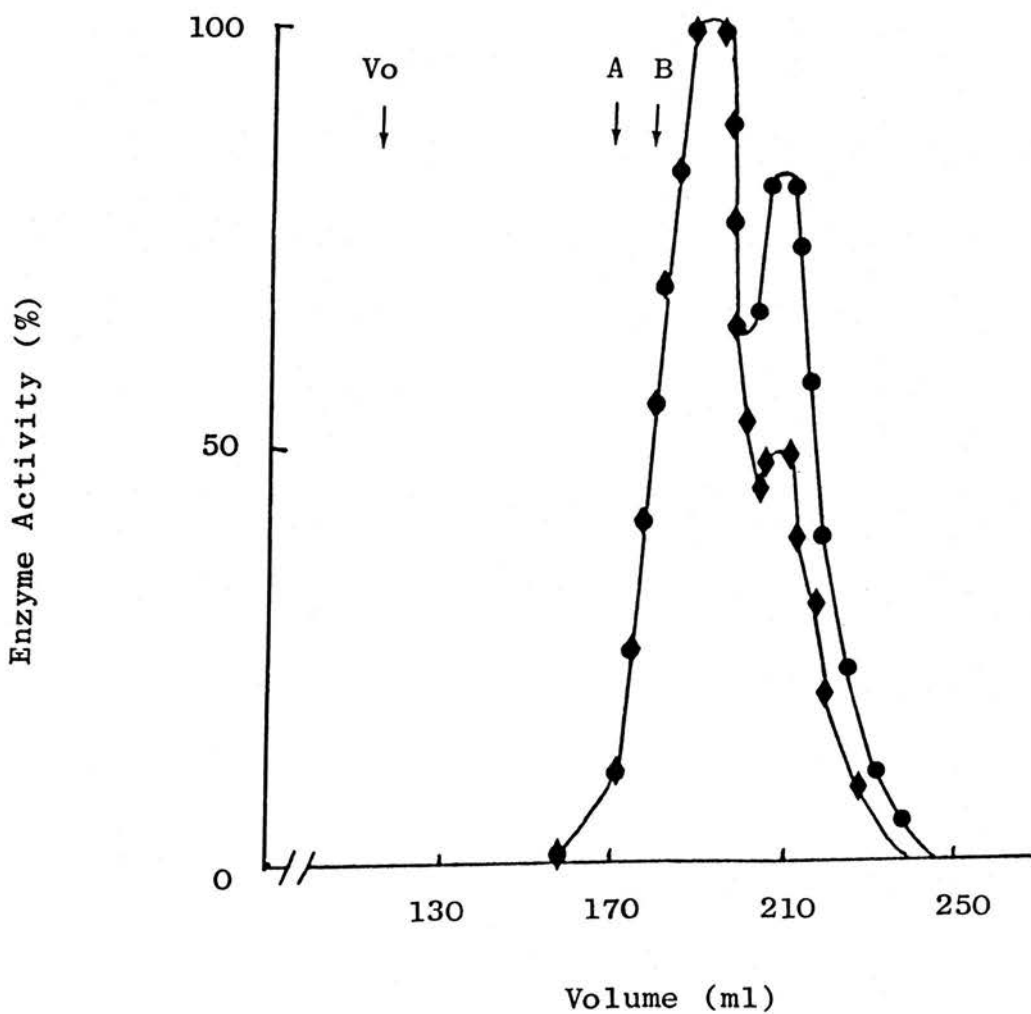


Fig. 14 Sephadex G150-120 gel filtration of fibroblast prolinase assayed with Pro-Gly at pH 7.4 (●) and pH 9.2 (◆). 2.5 x 55 cm column eluted with 10 mM barbital / HCl buffer pH 7.4, containing 100 mM NaCl. Vo, void volume; A, β -hexosaminidase; B, α -L-fucosidase.

100% = 11 nmol/min/ml at pH 9.2 and 6 nmol/min/ml at pH 7.4

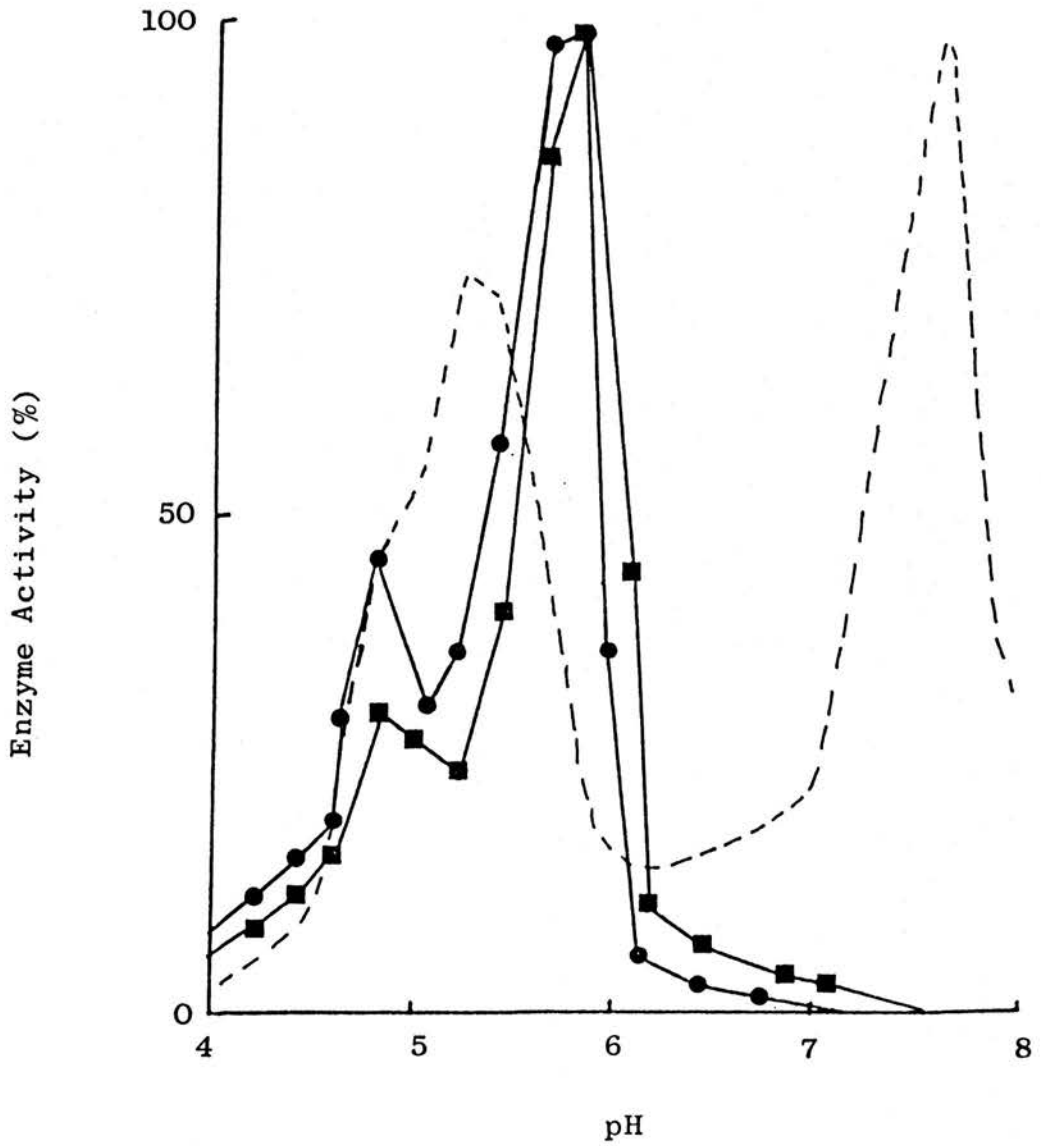


Fig. 15 Sucrose density isoelectrofocusing of human skin fibroblast prolinase assayed with Pro-Gly at pH 7.4 (●) and pH 9.2 (■). (-----) β-hexosaminidase. Isoelectrofocusing was performed as described in the appendix, section 11.4.

100% = 7 nmol/min/ml at pH 9.2 and 4 nmol/min/ml at pH 7.4

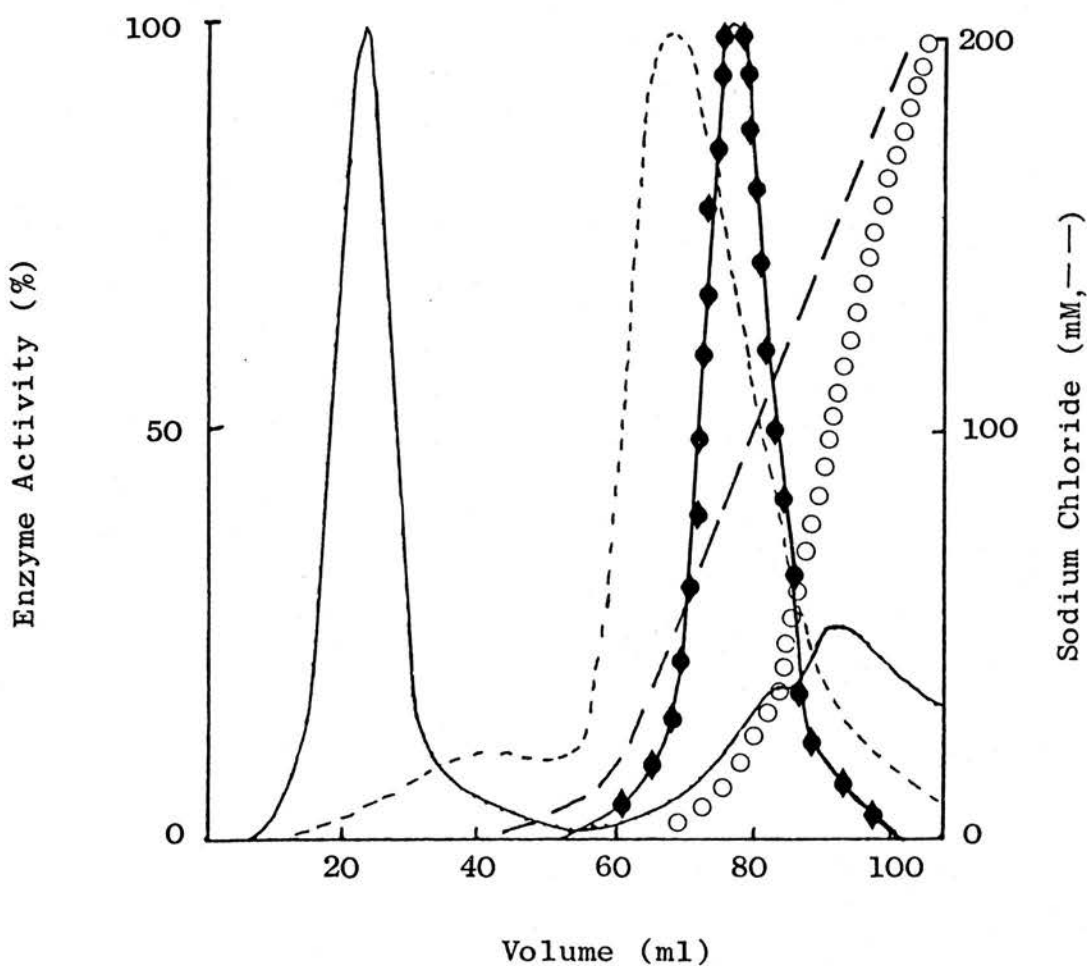


Fig. 16 Butyl-agarose hydrophobic chromatography of human skin fibroblast prolinase assayed with Pro-Gly at pH 7.4 (●) and pH 9.2 (◆).

0.8 x 10 cm column equilibrated with 20 mM maleate buffer pH 6.5, and eluted with 0-300 mM NaCl in the same buffer.

(○○○○) α -L-fucosidase; (- - - -) β -glucuronidase;

(—) acid phosphatase.

100% = 9 nmol/min/ml at pH9.2 and 5 nmol/min/ml at pH 7.4

3.4. DISCUSSION

3.4.1. Intracellular localisation

Prolinase in cultured skin fibroblasts was completely soluble, similar to pig kidney prolinase in which 87% of the activity remained in the supernatant after centrifugation of a homogenate at 100,000 x g (Mayer and Nordwig, 1973).

3.4.2. Substrate specificity

The variable ratios of activities at pH 7.6 to 9.2 for the different substrates (Table 5) indicate that there may be a single enzyme with variable responses for each substrate or possibly more than one form of prolinase.

Pig kidney prolinase was found to hydrolyse Hypro-Gly faster than Pro-Gly (Davis and Adams, 1955; Sarid et al., 1962) and was claimed to be a more specific substrate for the enzyme. However, Mayer and Nordwig (1973) found the reverse to be true with Hypro-Gly being hydrolysed at a third of the rate of Pro-Gly also in pig kidney. This probably highlights the importance of conditions of assay. In contrast, for human cultured cells, Hypro-Gly was a very poor substrate and of little use in the estimation of prolinase. Pro-Glu was not hydrolysed by prolinase in

cultured cells which agrees with the finding for the pig kidney enzyme (Davis and Smith, 1953).

The relative cleavage rates of the different substrates by cultured cell prolinase contrasted markedly with those of pig kidney prolinase in which Pro-Gly and Pro-Ala were hydrolysed at up to twenty times the rate of Pro-Leu and Pro-Phe (Mayer and Nordwig, 1973). It is noteworthy that the non-specific dipeptidase activity was higher than prolinase activity. The difference between the fibroblast and amniotic fluid cell cultures for the two non-specific substrates deserves further study.

3.4.3. Substrate kinetics

As analysed by Lineweaver-Burk plots the K_m of 2.3 mM for Pro-Gly was of the same order as the value of 6.1 reported recently for human plasma prolinase assayed at pH 9.0 with 0.02 mM $MnCl_2$ (Myara et al., 1985a).

3.4.4. Chromatography

The two chromatographic techniques, DEAE ion-exchange and gel filtration, used to characterise the human skin fibroblast enzyme confirmed the existence of two components of prolinase activity.

4. PURIFICATION OF PROLINASE FROM HUMAN KIDNEY AND ITS RELATIONSHIP WITH NON-SPECIFIC DIPEPTIDASE

4.1. INTRODUCTION

Prolinase has been purified from a number of animal tissues (Sarid et al., 1962; Mayer and Nordwig, 1973; Akrawi and Bailey, 1976) and in all cases the final preparations had activity against non-specific dipeptidase substrates, which was attributed to a contaminating enzyme. However, it has been proposed that the two activities may be due to a single enzyme (Hayman and Patterson, 1971). As kidney has been widely used in studies on dipeptidases from animals (Sarid et al., 1962; Mayer and Nordwig, 1973; Akrawi and Bailey, 1976) and had the highest prolinase activity of the human organs tested (Table 3.) prolinase was purified using human kidney. Increasing lability of dipeptidases with purification appears to preclude purification to homogeneity (Hayman and Patterson, 1971; Mayer and Nordwig, 1973). To ascertain whether human kidney prolinase and non-specific dipeptidase were the same enzyme, properties of the enzyme were studied, including substrate kinetics (Dixon and Webb, 1964, pp 84 -87), after purification using a wide range of separative techniques.

4.2. METHODS

4.2.1. Enzyme assays

Prolinase was assayed in 25 mM barbital / HCl buffer pH 9.2 using Pro-Ala, Pro-Gly, Pro-Leu, Pro-Phe and Pro-Val. The liberated amino acid was determined fluorimetrically as described earlier (Section 2.2.2.). Non-specific dipeptidase was assayed in 25 mM barbital / HCl buffer, pH 8.0, containing 10 mM Gly-Leu, alanyl-leucine (Ala-Leu), valyl-leucine (Val-Leu), seryl-leucine (Ser-Leu), phenylalanyl-glycine (Phe-Gly) or leucyl-glycyl-glycine (Leu-Gly-Gly) and the liberated leucine or phenylalanine determined spectrophotometrically by the L-amino acid oxidase / peroxidase-coupled oxidation of o-dianisidine method of Shoaf et al. (1974). Prolidase was assayed against glycyl-proline (Gly-Pro) by estimation of liberated proline (Mayer and Nordwig, 1973) and against phenylalanyl-proline (Phe-Pro) by estimation of liberated phenylalanine as above. Proline imino-peptidase (EC 3.4.11.5) activity was determined by estimation of liberated proline from prolyl-glycyl-glycine (Pro-Gly-Gly) (Butterworth and Priestman, 1984a).

Column fractions were diluted appropriately before enzyme assay with BSA (0.25 mg/ml) in 10 mM barbital buffer pH 7.4, containing 50 mM NaCl. Protein

was determined by the method of Lowry et al. (1951) as described in the appendix (section 11.3.). One unit of enzyme activity was defined as the formation of 1 μmol of product/min at 37°C.

4.2.2. Extract preparation

Human kidney was obtained at autopsy 24 hr after death and stored at -70°C. A 20% (w/v) extract was prepared by homogenising in 10 mM barbital / HCl buffer pH 7.4 containing 50 mM NaCl (buffer A). The homogenate was sonicated at 8-12 μm for 60 sec and centrifuged at 100,000 x g for 60 min.

4.2.3. Chromatographic procedures

All chromatographic procedures were carried out at 4°C unless otherwise stated.

4.2.3.1. DEAE-Cellulose

Kidney extract (50 ml) was applied to a 3.5 x 45 cm DEAE-cellulose column equilibrated with buffer A. After washing with 200 ml buffer A, the column was eluted with 500 ml of a 50-300 mM NaCl linear gradient and 10 ml fractions collected.

4.2.3.2. Ultrogel AcA44

The DEAE-cellulose peak (95 ml) was concentrated ten-fold using a PM10 ultrafiltration membrane system (Diaflo Ltd), applied to a 2.5 x 75 cm Ultrogel AcA 44 (LKB Ltd) column, eluted with buffer A with an ascending flow-rate of 20 ml/hr and 10 ml fractions collected.

4.2.3.3. Metal-ion-chelate chromatography

For metal chelate chromatography, iminodiacetic acid was coupled to epoxy-activated Sepharose 6B (Sigma Ltd) (Porath et al., 1975) and converted to a metal chelate gel by the passage of 5 ml 50 mM NiCl_2 or 50 mM MnCl_2 in water through 2.5 ml gel in a 1 cm x 3.5 cm column. The Ni^{2+} gel was equilibrated with buffer A and 40 ml of Ultrogel prolinase peak was applied to the column. After washing with 20 ml 10 mM barbital / HCl pH 7.4 containing 10 mM NaCl (buffer B), the column was eluted with 60 ml of a 0-100 mM imidazole linear gradient (in buffer B) and 2 ml fractions collected.

4.2.3.4. Hydrophobic chromatography

The prolinase peak (16 ml) from the metal chelate chromatography was applied to a 1.4 cm x 25 cm butyl-agarose column equilibrated with buffer B. The

column was washed with 75 ml buffer B, eluted with 100 ml of a 10-300 mM NaCl linear gradient and 4 ml fractions collected.

4.2.3.5. Hydroxylapatite

30 ml prolinase peak from the hydrophobic chromatography was applied to a 1.3 cm x 5 cm hydroxylapatite (BDH Ltd) column equilibrated with buffer A. The column was washed with 50 ml buffer A, eluted with 100 ml of a 0-150 mM phosphate linear gradient in buffer A_(pH 7.4) and 5 ml fractions collected.

4.2.3.6. Chromatofocusing

After hydroxylapatite chromatography the prolinase peak (15 ml) was concentrated ten-fold by ultrafiltration, diluted (1+1) with 25 mM imadazole / HCl buffer pH 7.4 and applied to a 1.3 cm x 5 cm PBE 94 (Pharmacia Ltd) column equilibrated with this buffer. Elution was carried out with 60 ml polybuffer 74 (Pharmacia Ltd) titrated with HCl to pH 4.0 and 2 ml fractions collected.

4.2.4. Molecular weight determination

For molecular weight determination a 1% (w/v) kidney extract (10 ml) in buffer A was applied to a 2.5 cm x 75 cm Ultrogel AcA 44 column, eluted with buffer A with an ascending flow-rate and 10 ml fractions collected. The elution volumes of prolinase, β -hexosaminidase and α -mannosidase were determined. A mixture (10 ml) containing 1 ml of the enzyme peak from hydrophobic chromatography and five protein standards was applied to the same column and 3 ml fractions collected. The elution volumes of the protein standards were determined following detection at 280 nm.

4.2.5 Polyacrylamide gel electrophoresis

Discontinuous polyacrylamide gel electrophoresis (4.5% stacking, 7.5% separating gel, 0.75 mm thick) was performed using a Bio-Rad Protean slab electrophoresis cell (Davis, 1964). Concentrated samples (10 μ l) of the original kidney extract and the hydroxylapatite peak were applied and electrophoresed at 14 mA for 4.5 hr. The gel lanes were cut into 0.25 cm strips and bisected, one half for prolinase and the other half for non-specific dipeptidase estimation. For prolinase each gel strip was put into 100 μ l 50 mM barbital / HCl pH 9.2 containing 0.025 mM MnCl_2 , assayed for 60 min

following the addition of 100 μ l 10 mM Pro-Phe and liberated phenylalanine estimated (section 4.2.1.). For non-specific dipeptidase each gel strip was put into 100 μ l barbital / HCl pH 8.0, assayed for 60 min after the addition of 100 μ l Gly-Leu and liberated leucine estimated as for phenylalanine above. Protein bands were detected using the silver staining method of Merril and Goldman (1984).

4.2.6. Enzyme characteristics

Studies were performed on the dipeptidase peak eluted after hydrophobic chromatography and after chromatofocusing following equilibration with buffer A by ultrafiltration.

4.2.6.1. Heat stability

For heat studies, enzyme samples were incubated at 53°C for up to 30 min prior to assay of prolinase (Pro-Ala) and non-specific dipeptidase (Gly-Leu).

4.2.6.2. p-Hydroxymercuribenzoate

The inhibition of prolinase and non-specific dipeptidase by p-hydroxymercuribenzoate (PHMB) (Sigma Ltd) was also studied. Enzyme samples were treated

with 1-50 μ M PHMB for 5 min at 37°C prior to assay with Pro-Ala and Gly-Leu.

4.2.6.3. Substrate specificity

A number of potential substrates were tested with the enzyme preparation retained after chromatofocusing to determine the substrate specificity.

4.2.6.4. Substrate kinetics

The type of inhibition and K_i values were determined (Dixon, 1953) by studying the hydrolysis of Pro-Ala (2 or 5 mM) at pH 9.2 in the presence of Gly-Leu (0.5-10 and 1-25 mM respectively) and of Gly-Leu (0.5 or 2 mM) at pH 8.0 in the presence of Pro-Ala (0.1-2 and 1-10 mM respectively). As a test of the activities being due to a single enzyme (Dixon and Webb, 1964, pp 84-87; see appendix, section 11.5.), Pro-Ala (5 mM) hydrolysis at pH 9.2 with or without Gly-Leu (5 mM), Gly-Leu (5 mM) hydrolysis at pH 9.2 with or without Pro-Ala (5 mM) and the K_i value for Pro-Ala at pH 9.2 were determined.

4.3. RESULTS

4.3.1. Enzyme purification

The purification of prolinase and non-specific dipeptidase is summarised in Table 4. To assay the two enzyme activities in the original kidney extract, it was necessary to dilute to a low protein level (< 0.01 mg/ml) at which the enzyme was unstable. Addition of BSA (0.25 mg/ml) stabilised the enzyme to dilution. During the purification procedure the protein level of the active fractions up to and including the hydroxylapatite column was sufficient to maintain stability, but addition of BSA was required for dilution of column fractions prior to assay. A preparation retained after hydrophobic chromatography was stable for 9 months at 4°C in the presence of BSA. Coelution of both of the enzyme activities as a single peak was observed for all the separative procedures, either performed singly with a crude kidney extract or during the sequential purification. The ratio of the two activities was similar following each of the separative procedures.

During the sequential purification procedure the two enzyme activities coeluted with the highest activity at 110 mM NaCl for DEAE-Cellulose column, 30 mM imidazole for the Ni²⁺ chelate column, 100 mM NaCl for the butyl-agarose column and 30 mM phosphate for

the hydroxylapatite column. A manganese chelate gel did not retain either enzyme activity. The relative elution volumes (V_e/V_o) of β -hexosaminidase and α -D-mannosidase fell on the gel filtration molecular weight calibration curve at 110,000 and 180,000 respectively. The position of the enzyme, as detected by its prolinase and non-specific dipeptidase activity, indicated a molecular weight of 100,000 (Fig.17).

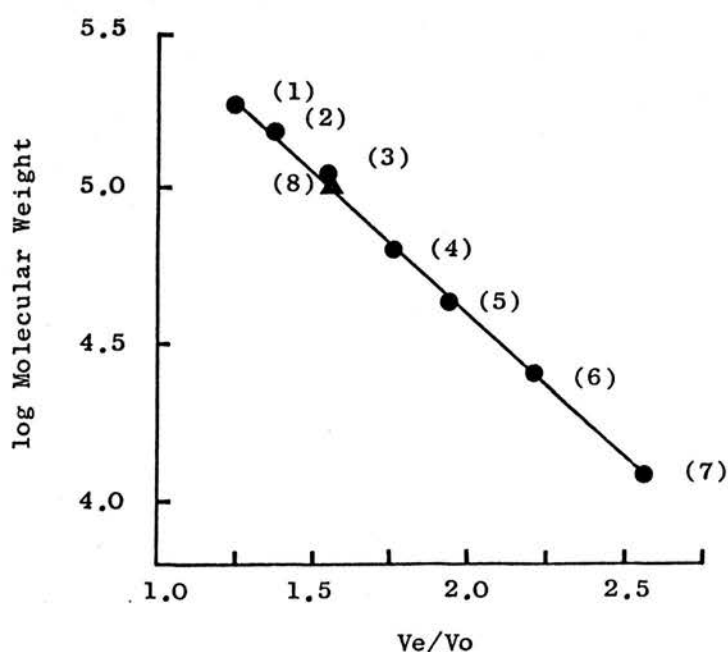


Fig. 17 Calibration of Ultragel for M_r determination.

Key: (1) α -D-mannosidase; (2) alcohol dehydrogenase; (3) β -hexosaminidase; (4) haemoglobin; (5) ovalbumin; (6) chymotrypsinogen; (7) cytochrome C; (8) prolinase and non-specific dipeptidase.

Elution from the chromatofocusing column indicated a pI of 5.3 for the enzyme. Silver staining of the polyacrylamide gel following electrophoresis of the pooled enzyme activity from the hydroxylapatite column revealed the presence of two major and three minor bands (Fig.18a). Band I was the marker bromophenol blue and band II was a staining artifact. The gel showed a coincident band of prolinase and non-specific dipeptidase (Fig.18b). A crude kidney extract gave the same position for the two enzyme activities.

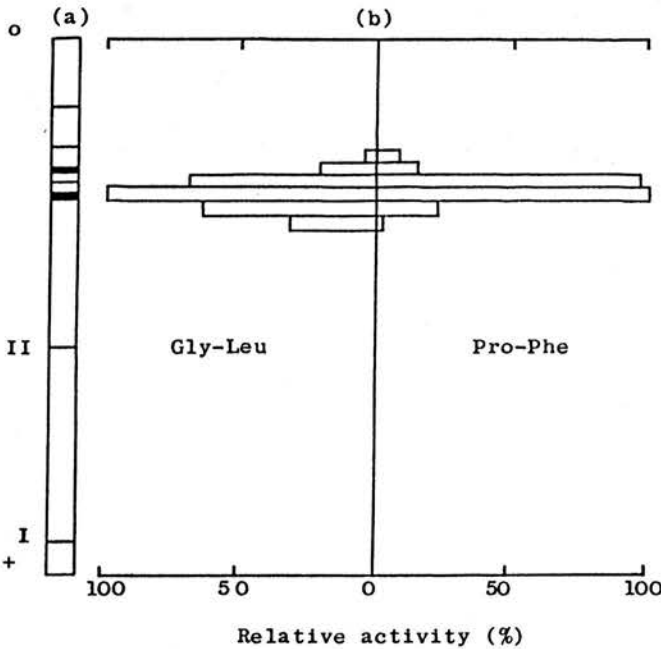


Fig. 18 Polyacrylamide gel electrophoresis of human kidney dipeptidase. (a) Silver staining for protein in the separating gel (10 cm). o, origin; +, anode end of gel. (b) Dipeptidase relative activity in gel slices (0.25 cm) against Gly-Leu and Pro-Phe assayed throughout the length of the gel.

4.3.2. Enzyme properties

As recovery of enzyme activity was poor from the chromatofocusing column and involved exposure to acidic pH, properties of the enzyme were studied following the hydrophobic as well as the chromatofocusing procedures. However the results were the same for both enzyme preparations. Heat treatment of the enzyme at 53°C resulted in a similar rate of loss of activity against Gly-Leu and Pro-Ala with a 50% loss of activity after about 12 min (Fig.19). The thiol reagent PHMB was markedly inhibitory of enzyme activity against both substrates with 50% inhibition at about 14 μ M (Fig.20).

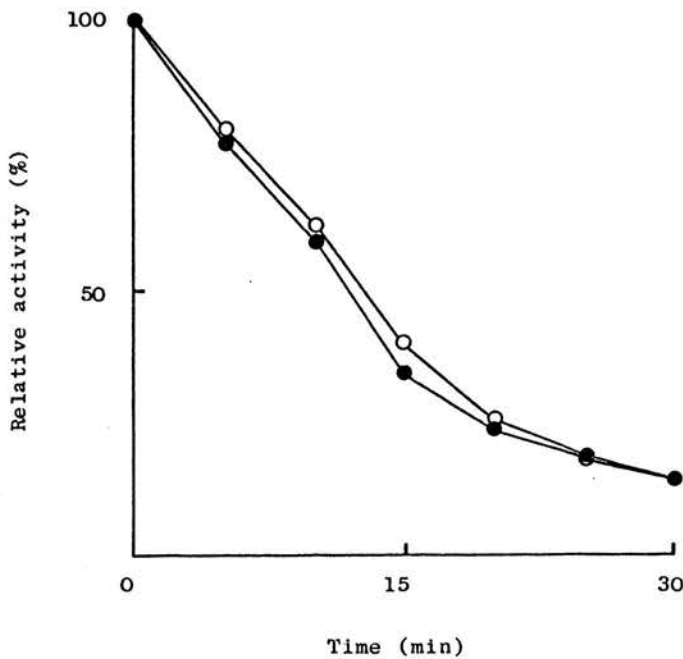


Fig. 19 Heat treatment of human kidney dipeptidase, assayed against Pro-Ala (●) and Gly-Leu (○).

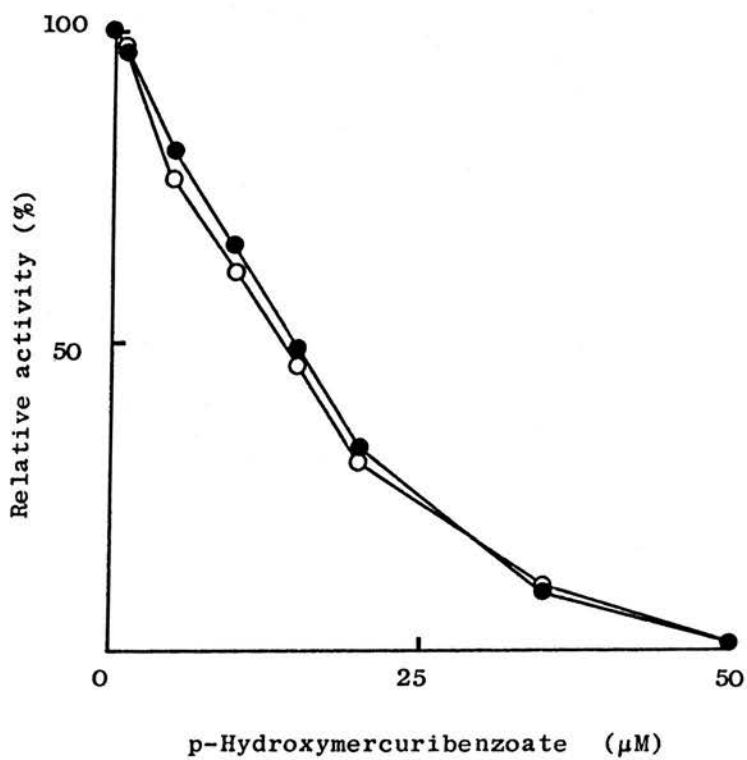


Fig. 20 Inhibition of human kidney dipeptidase by p-hydroxymercuribenzoate. (●) Pro-Ala, (○) Gly-Leu.

4.3.3. Enzyme activities with different substrates

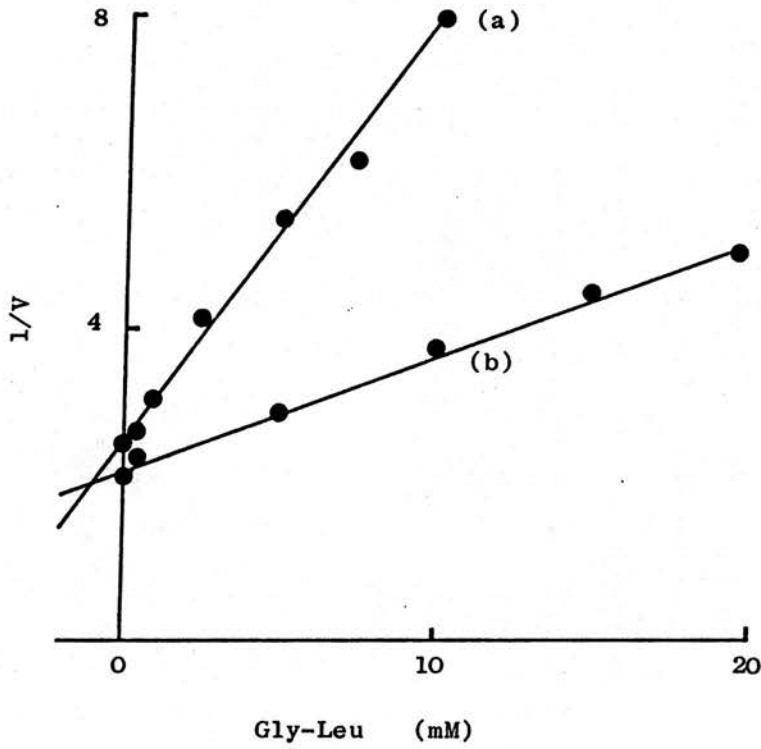
The activity of the final enzyme preparation against a range of potential substrates (10 mM) is given in Table 5. Of the non-specific dipeptidase substrates the highest activity was against Gly-Leu, whilst Phe-Gly was not hydrolysed. Three of the prolinase substrates (Pro-Leu, Pro-Phe and Pro-Val) gave activities that were higher than the activity towards Pro-Ala, the substrate used to follow the enzyme purification. The enzyme had no activity towards Pro-Gly-Gly, the substrate for proline iminopeptidase, and Gly-Pro and Phe-Pro, substrates for prolidase.

Substrate	Enzyme Activity (units/ml)	Relative Activity (%)
Gly-Leu	3.23	100
Ser-Leu	1.29	40
Val-Leu	0.96	30
Ala-Leu	0.89	28
Phe-Ala	0.32	10
Phe-Gly	0	0
Leu-Gly-Gly	0.12	4
Pro-Leu	1.55	48
Pro-Phe	1.42	44
Pro-Val	1.30	40
Pro-Ala	0.43	13
Pro-Gly	0.29	9
Hypro-Gly	0.12	4
Pro-Gly-Gly	0	0
Gly-Pro	0	0
Phe-Pro	0	0

Table 7. Hydrolysis of dipeptides and tripeptides by the purified human kidney enzyme.

4.3.4. Substrate kinetics and inhibition studies

Analysis of substrate interactions by Dixon plots (Dixon, 1953) showed that Gly-Leu was a competitive inhibitor of enzyme activity towards Pro-Ala with an apparent K_i of 1.1 mM at pH 9.2 (Fig.21) and Pro-Ala was a competitive inhibitor of enzyme activity towards Gly-Leu with an apparent K_i of 0.22 mM at pH 8.0 (Fig.22) and 2.2mM at pH 9.2. The K_m for the enzyme against Pro-Ala was 0.47 mM at pH 9.2 and the K_m against Gly-Leu was 0.79 mM at pH 8.0 and pH 9.2. The expected hydrolysis of equimolar concentrations of two substrates by a single enzyme in an extract can be calculated using the experimentally determined values of V_{max} and the K_m s for the two substrates (Dixon and Webb, 1964, pp 84-87; see appendix, section 11.5.) The present data gave a calculated total activity of 8.9 units/ml. As the observed total activity was 9.2 units/ml, it seems highly probable that the preparation contained a single enzyme hydrolysing both substrates.



apparent
 Fig. 21 Determination of K_i for Gly-Leu inhibition of
 prolinase activity against Pro-Ala by the
 method of Dixon (1953). a) 2 and b) 5 mM
 Pro-Ala, V = units/ml.

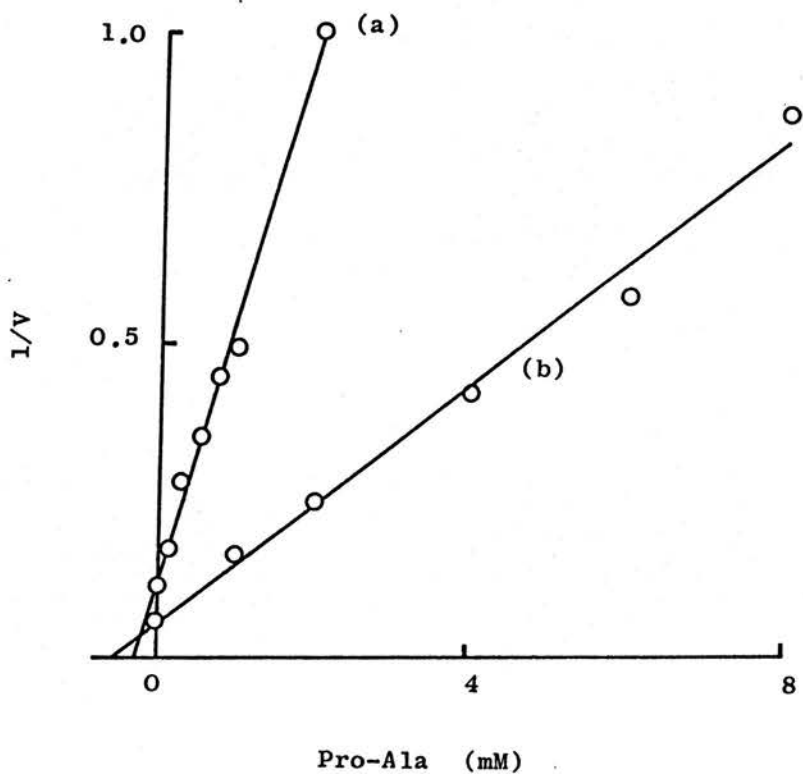


Fig. 22 Determination of K_i^{apparent} for Pro-Ala inhibition of non-specific dipeptidase activity against Gly-Leu by the method of Dixon (1953).
 a) 0.5 and b) 2 mM Gly-Leu, $V = \text{units/ml}$.

4.4. DISCUSSION

4.4.1. Purification and properties

Non-specific dipeptidase purified from pig and Guinea pig intestinal mucosa (Norén et al., 1973; Piggott and Fottrell, 1975) and mouse ascites tumour (Hayman and Patterson, 1971) hydrolysed prolinase substrates. Conversely, prolinase purified from pig kidney (Sarid et al., 1962; Mayer and Nordwig, 1973), bovine kidney (Akrawi and Bailey, 1976) and mouse brain (Reith and Neidle, 1979) hydrolysed non-specific dipeptidase substrates. Also, the major peak of human skin fibroblast prolinase coincided with a peak of non-specific dipeptidase on DEAE-Cellulose chromatography (Butterworth and Priestman, 1984). Generally, the two activities had been considered to be due to at least two enzymes and whether there is an enzyme capable of hydrolysing both prolinase and non-specific dipeptidase substrates is undecided, particularly for human tissues.

Prolinase and non-specific dipeptidase from human kidney were soluble in agreement with results for human skin fibroblasts (section 3.2.1.) and pig kidney (Mayer and Nordwig, 1973). The accuracy of the molecular weight calibration curve was demonstrated by obtaining the expected molecular weights for α -D-mannosidase and β -hexosaminidase (Marinkovic and Marinkovic, 1976a,b).

The determined molecular weight of 100,000 for the human kidney dipeptidase was similar to the value reported for bovine kidney prolinase (Akrawi and Bailey, 1976), but was lower than the value of 300,000 estimated for pig kidney prolinase (Mayer and Nordwig, 1973). The inhibition of human kidney dipeptidase by p-hydroxymercuribenzoate indicates the presence of a thiol group at the active site, as previously suggested for monkey intestinal (Das and Radhakrishnan, 1972) and pig kidney enzyme (Sarid et al., 1962).

4.4.2. Chromatography

The elution position of the human kidney enzyme on DEAE cellulose chromatography corresponded with that of the major peak of human skin fibroblast prolinase (see Fig. 12; Butterworth and Priestman, 1984).

Metal chelate chromatography separates proteins on the basis of their affinity for heavy metal ions (Porath et al., 1975; Porath and Olin, 1983). Histidine and cysteine form complexes with Zn^{2+} , Co^{2+} , Hg^+ and Ni^{2+} and proteins containing these amino acids on their surface can potentially bind to metal chelated Sepharose (Porath et al., 1975). Although Mn^{2+} activates prolinase (Fig.6) and a number of other enzymes (Dixon and Webb, 1964, pp. 672-785) the weak capacity of gel-immobilised Mn^{2+} for protein binding

(Porath et al., 1975) could explain why the Mn^{2+} gel did not adsorb prolinase. Affinity elution of human kidney dipeptidase from a Ni^{2+} gel by imidazole strongly suggests the presence of histidine residues on the surface of the enzyme (Porath and Olin, 1983). The human kidney dipeptidase results demonstrate the usefulness of the inclusion of a metal chelate chromatography step in enzyme purification.

The binding of human skin fibroblast prolinase to butyl-agarose (Fig.16) and of pig intestinal non-specific dipeptidase to hydroxylapatite (Norén et al., 1973) was the same as that of the human kidney. Although bovine (Akrawi and Bailey, 1976) and pig (Mayer and Nordwig, 1973) kidney gave a pI of 4.3 on isoelectrofocusing, the pI value of 5.3 for the human kidney preparation was within the range 5.05-5.3 obtained by chromatofocusing of cultured human skin fibroblasts, liver and kidney (Butterworth and Priestman, 1984). The marked loss of human kidney enzyme activity on chromatofocusing was probably due to exposure to acidic pH, which is known to inactivate the enzyme (see Fig.10). The presence of multiple bands of activity, probably owing to subunits and reaggregation, on polyacrylamide gel electrophoresis of purified pig kidney prolinase (Mayer and Nordwig, 1973) was not a feature of the human kidney enzyme.

4.4.3. Substrate specificity

Although Gly-Leu was the best of the substrates tested for human kidney dipeptidase, the hydrolysis of non-specific dipeptidase substrates varies widely depending upon conditions of assay and source of enzyme (Hayman and Patterson, 1971; Mayer and Nordwig, 1973; Norén et al., 1973). The ability of the human kidney enzyme to hydrolyse prolinase substrates was similar to that of human skin fibroblast prolinase (Table 5). In contrast with the human enzyme, pig kidney (Mayer and Nordwig, 1973) and Guinea pig intestinal mucosal (Piggott and Fottrell, 1975) prolinase had very little activity toward Pro-Leu and Pro-Phe. The failure of the human kidney enzyme to hydrolyse Pro-Gly-Gly, the substrate for proline iminopeptidase, and Gly-Pro and Phe-Pro, substrates for prolidase, agrees with the previous findings for pig and bovine kidney prolinase (Sarid et al., 1962; Mayer and Nordwig, 1973; Akrawi and Bailey, 1976).

4.4.4. Conclusion

Throughout the purification procedures the human kidney prolinase and non-specific dipeptidase activities could not be separated. The enzyme peaks were congruent and the ratio of their activities was constant following each of the separative procedures.

In addition, the responses of the two activities to PHMB and to heat treatment were the same. These data, together with the competitive inhibition of prolinase and non-specific dipeptidase activity by Gly-Leu and Pro-Ala respectively and the analysis of substrate interaction (Dixon and Webb, 1964, pp 84-87), provide firm evidence for the existence of an enzyme hydrolysing both prolinase and non-specific dipeptidase substrates in human kidney.

5. ASSAYS FOR PROLIDASE

5.1. INTRODUCTION

Three methods were developed for the assay of prolidase. Firstly, a modification of the ninhydrin method of Nordwig and Mayer (1973) for proline-detection was developed. The other two methods, modified from the assays of Shoaf et al. (1974) and Matsuomoto et al. (1982), detect the C-terminal amino acid liberated from the substrate. These latter two methods are novel for the assay of prolidase and are more sensitive than the first. Conditions of assay were determined in cultured skin fibroblasts from controls and from two unrelated cases of prolidase deficiency using Glycyl-Proline (Gly-Pro) and Phenylalanyl-Proline (Phe-Pro) as substrates. Like prolinase, prolidase is also activated by manganese ions (Walter et al., 1980), so a detailed study was made of its manganese requirements.

5.2. METHODS

5.2.1. Enzyme source and preparation of extracts

Human skin fibroblasts were cultured as described in the appendix (Section 11.1.) and extracts prepared as described in section 2.2.1. Skin fibroblast cultures were obtained from a male Turkish patient (Case 1, Pedersen et al., 1983) and a male Italian patient (Case 2, Sheffield et al., 1972). The patients had imidodipeptiduria and a marked deficiency of prolidase activity against Gly-Pro in erythrocytes and cultured skin fibroblasts. Protein was estimated by the method of Lowry et al. (1951) using bovine serum albumin as standard (see appendix, section 11.3.)

5.2.2. Assays for prolidase

5.2.2.1. Ninhydrin method

25 μ l extract was incubated with 50 μ l 50 mM barbital / HCl pH 8.0 containing 2 mM $MnCl_2$ and the assay started by the addition of 25 μ l 80 mM Gly-Pro or Phe-Pro_N (Sigma Ltd). After incubation at 37°C for 30 min 200 μ l 15% TCA was added and the liberated proline estimated using a modification of the method of Mayer and Nordwig (1973). After stopping the assay with TCA, 250 μ l 3.6 mM glycine was added followed by 1.0 ml ninhydrin

reagent (1% ninhydrin (w/v) in acetic acid / phosphoric acid / distilled water, 12:3:5 by volume) and mixed thoroughly on a vortex mixer before heating in a boiling water bath for 30 mins. The tubes were then left to cool down to room temperature, mixed again, and the absorbance read at 515 nm on a Perkin-Elmer dual-beam spectrophotometer. The rate of development of colour was assessed by heating a proline standard at 100°C for up to 90 min. Gly-Pro was also heated for this length of time to determine the extent of hydrolysis due to heat.

5.2.2.2. L-amino acid oxidase method

Phenylalanine liberated from the hydrolysis of phenylalanyl-proline (Phe-Pro) was estimated spectrophotometrically using a micro-method based upon the non-specific dipeptidase assay of Shoaf et al. (1974). The assay mixture was the same as for the ninhydrin method above (5.2.2.1.). After 30 min incubation, the reaction was stopped by heating at 100°C for one minute. 250 µl reagent (25 mM barbital / HCl pH 8.0 containing 0.27 g/l L-amino acid oxidase type VI (Sigma Ltd); 20 mg/l horseradish peroxidase type II (Sigma Ltd); and 2 g/l o-dianisidine.2HCl (Sigma Ltd)) was added and after 10 min incubation at 37°C, 250 µl 50% (v/v) sulphuric acid added. The

colour was read at 530 nm and compared with a phenylalanine standard curve.

5.2.2.3. Homovanillic acid method

The method of Matsuomoto et al. (1982) for monoamine oxidase was applied to the fluorimetric estimation of phenylalanine. The assay mixture was the same as for the ninhydrin method above (5.2.2.1.). After heating the assay tubes at 100°C for one minute to stop the reaction, 400 µl reagent (25 mM barbital / HCl pH 8.0 containing 0.1 g/l L-amino acid oxidase type VI; 10mg/l horseradish peroxidase type II; 50 mM EDTA; and 0.5 µM homovanillic acid (Sigma Ltd)) was added. After incubation at 37°C for 10 min, 500 ul 0.5 M NaOH was added and the fluorescence read on a Perkin-Elmer 1000 fluorimeter (excitation 323 nm; emission 426 nm) and related to a phenylalanine standard curve. Addition of EDTA was needed to prevent precipitation of manganese on addition of NaOH.

5.2.3. pH optimum, time and protein linearities

The optimum pH for prolidase activity was determined for a control fibroblast culture and for prolidase-deficient fibroblasts by assay with a range of buffers (25 mM barbital / HCl) pH 7.0-9.0. Time and protein linearities for the hydrolysis of Phe-Pro were determined by incubating at 37°C for up to 60 min and with protein levels up to 1.6 mg/ml.

5.2.4. Effect of manganese

Extracts from control fibroblasts and from the two cases were preincubated with 1.33 mM $MnCl_2$ (1 mM final concentration in the assay) using Phe-Pro as substrate. A control was also preincubated with this level of manganese and assayed with a range of other prolidase substrates (Gly-Pro, Ala-Pro, Met-Pro, Leu-Pro, Val-Pro and Ser-Pro). To determine the optimal level of manganese for the assay, control fibroblasts were assayed with 0-5 mM $MnCl_2$ with or without preincubation for 5 min using Gly-Pro and Phe-Pro as substrates. The effect of preincubation for 5 min with manganese levels up to 0.5 mM was measured for fibroblasts from a control and for the two cases with Phe-Pro.

5.3. RESULTS

5.3.1. Prolidase assays

The ninhydrin reaction for proline detection was linear for at least 100 nmoles in the assay giving an optical density of about 1.2 after heating in a boiling water bath for 30 min (fig. 23a). The development of colour over a period of 90 min is shown in Fig. 23b, which also shows the rapid hydrolysis of Gly-Pro on heating in acid conditions ^{of the assay} (about 200 nmoles after 90 min). All the other substrates were more stable and gave lower substrate blanks than Gly-Pro.

The L-amino acid oxidase colorimetric method gave an optical density (OD) of 0.285 for 10 nmoles phenylalanine (Fig. 24a) and was linear up to at least an OD of 1.0 equivalent to 35 nmoles phenylalanine. The L-amino acid oxidase fluorimetric method gave a reading of 900 fluorescent units for 10 nmoles phenylalanine (Fig. 24b) with the fluorimeter standardised to 200 units with 1 mg/l quinine.

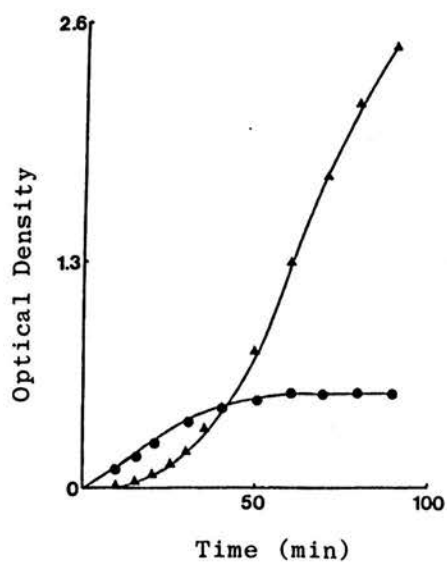
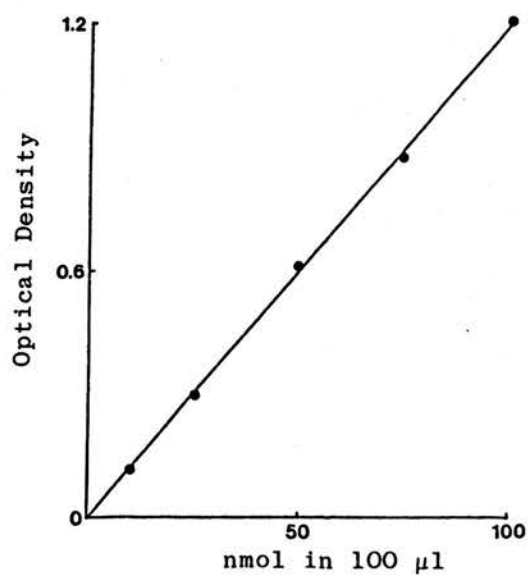


Fig. 23 a) Standard curve for proline reacting with ninhydrin reagent.
 b) Development of colour with time for a proline standard (50 nmol) (●) and for Gly-Pro hydrolysis (▲).

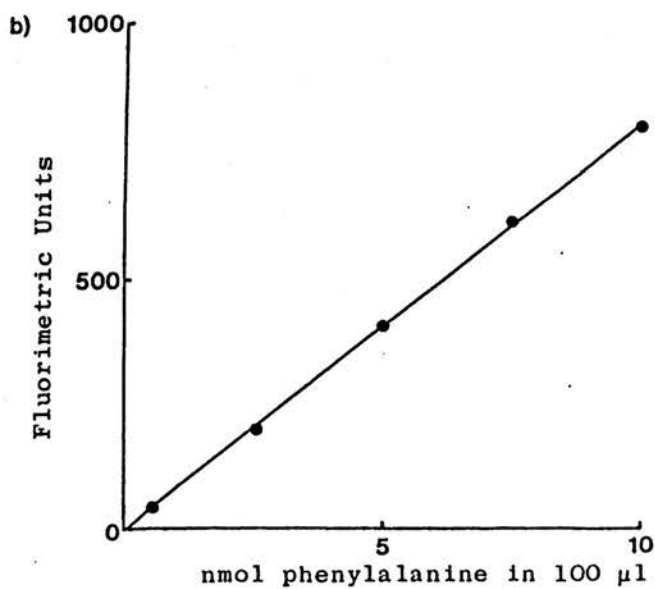
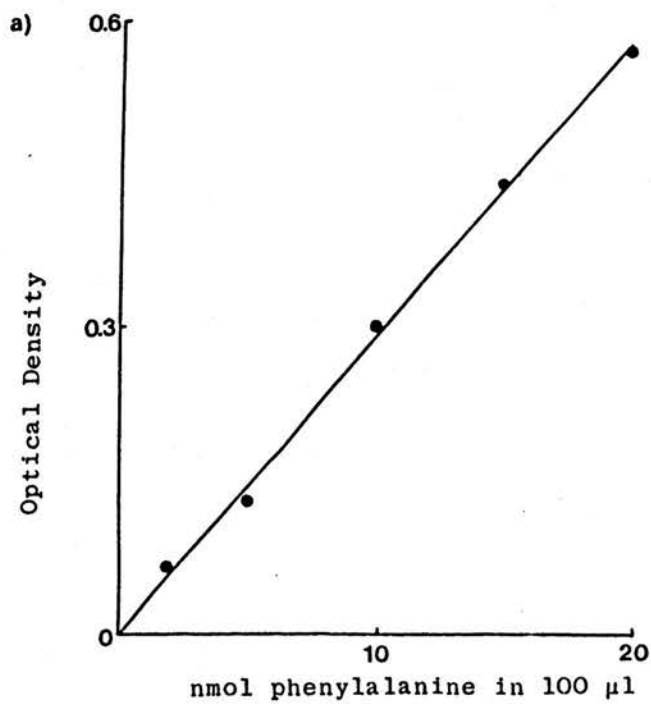


Fig. 24 a) Standard curve for phenylalanine in L-amino acid oxidase reagent.

b) Standard curve for phenylalanine in homovanillic acid reagent.

5.3.2. pH optimum, time and protein linearities

An optimum of pH 8.0 was obtained for both normal and abnormal fibroblast prolidase (Fig. 25). Prolidase activity was linear with time for at least 60 min and with protein levels up to 40 μg in the assay (Fig. 26). A range of 5-15 μg protein per assay was used for the L-amino acid oxidase methods and 15-40 μg for the ninhydrin method.

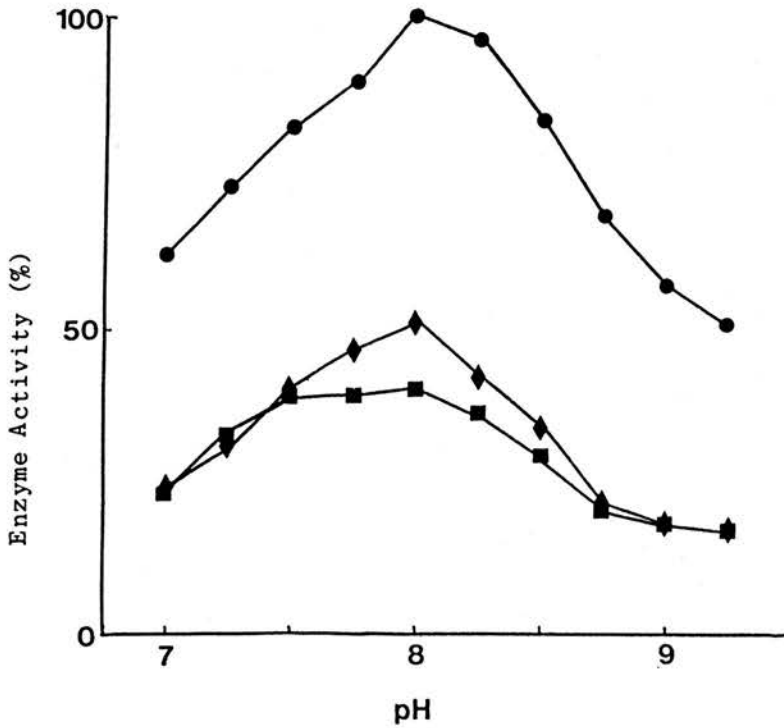


Fig. 25 pH profiles for prolidase from control (●), case 1 (◆) and case 2 (■) fibroblasts using Phe-Pro as substrate (section 5.2.2.2.). (100 % = 97 nmol/min/mg protein)

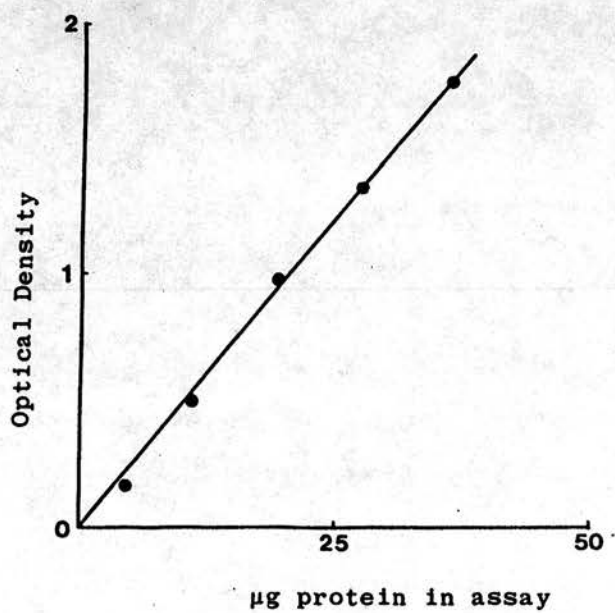
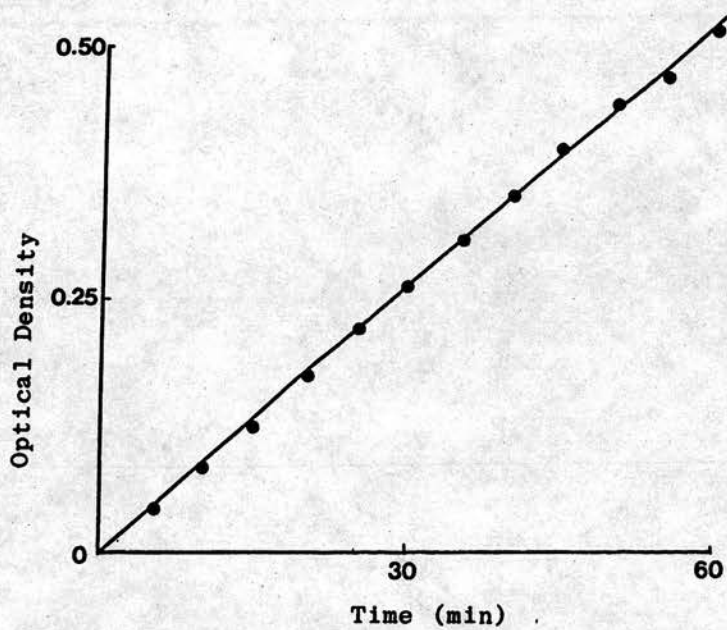


Fig. 26 a) Time and b) protein linearities for prolidase using Phe-Pro as substrate (section 5.2.2.2.).

5.3.3. Manganese

Whereas control prolidase activity was unaltered by preincubation in buffer before addition of manganese, preincubation with 1.33 mM MnCl_2 for up to 60 min showed that optimal activation for Phe-Pro was achieved after about 5 min. This was followed by a gradual return to the original activity (Fig. 27). A similar pattern was observed for all the other substrates studied (Fig. 28) except Gly-Pro. The profile for Gly-Pro was markedly different, increasing rapidly in the first 15 min and then rising at a slower rate up to 60 min when its activity was ten-fold higher than without preincubation (Fig. 28). The abnormal prolidase was stable in buffer but was progressively inhibited in the presence of manganese (Fig. 27, case 1 shown). Optimal activity for control prolidase against Phe-Pro was observed with 1 mM MnCl_2 final concentration in the assay (Fig. 29a) with a small fall off in activity with higher concentrations. This was true whether the enzyme was preincubated or not. The hydrolysis of Gly-Pro required a higher level of 2.5 mM MnCl_2 for optimal activity (Fig. 29b).

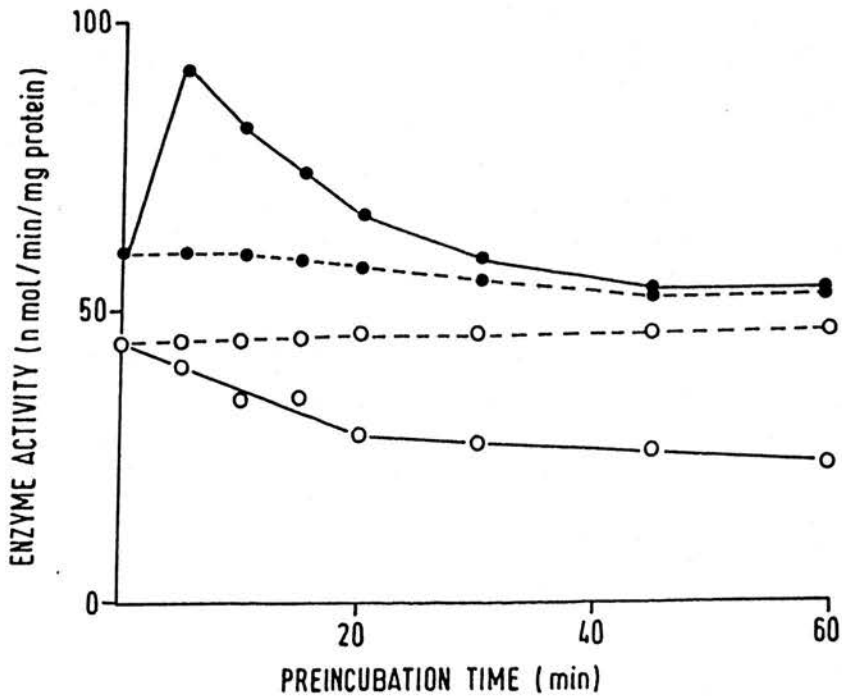


Fig. 27 Effect of preincubation of control (●) and case 1 (○) fibroblast extracts for up to 60 min with 1.33 mM MnCl₂ (—) or without MnCl₂ (---) on prolidase activity assayed with Phe-Pro.

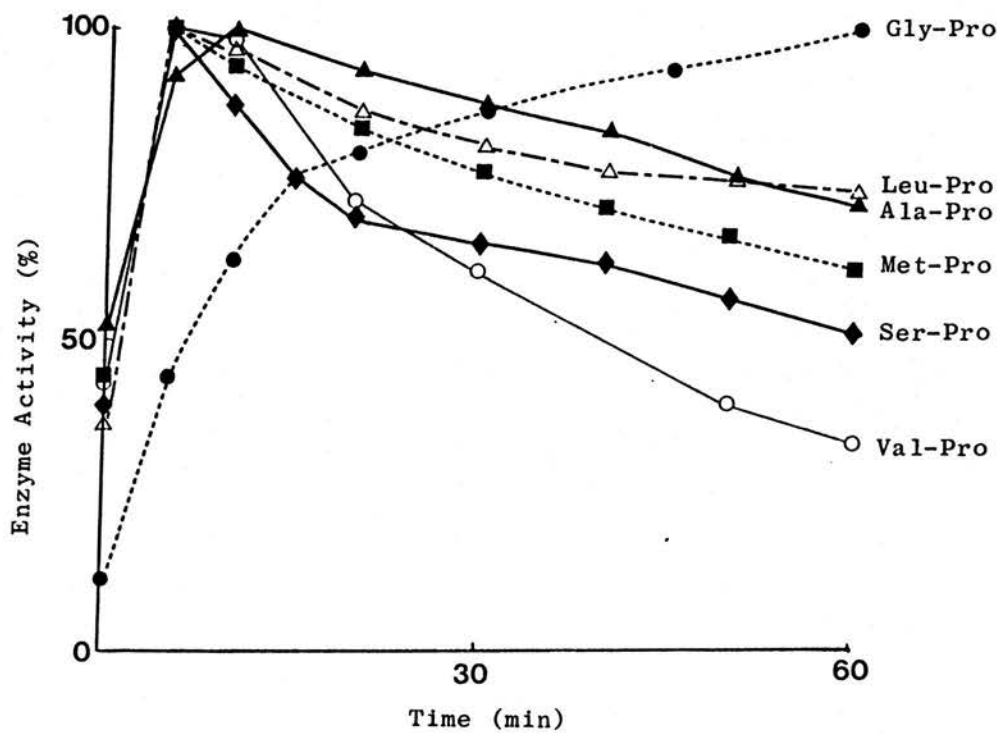


Fig. 28 Effect of preincubation of prolidase with 1.33 mM $MnCl_2$ for up to 60 min for a range of substrates.

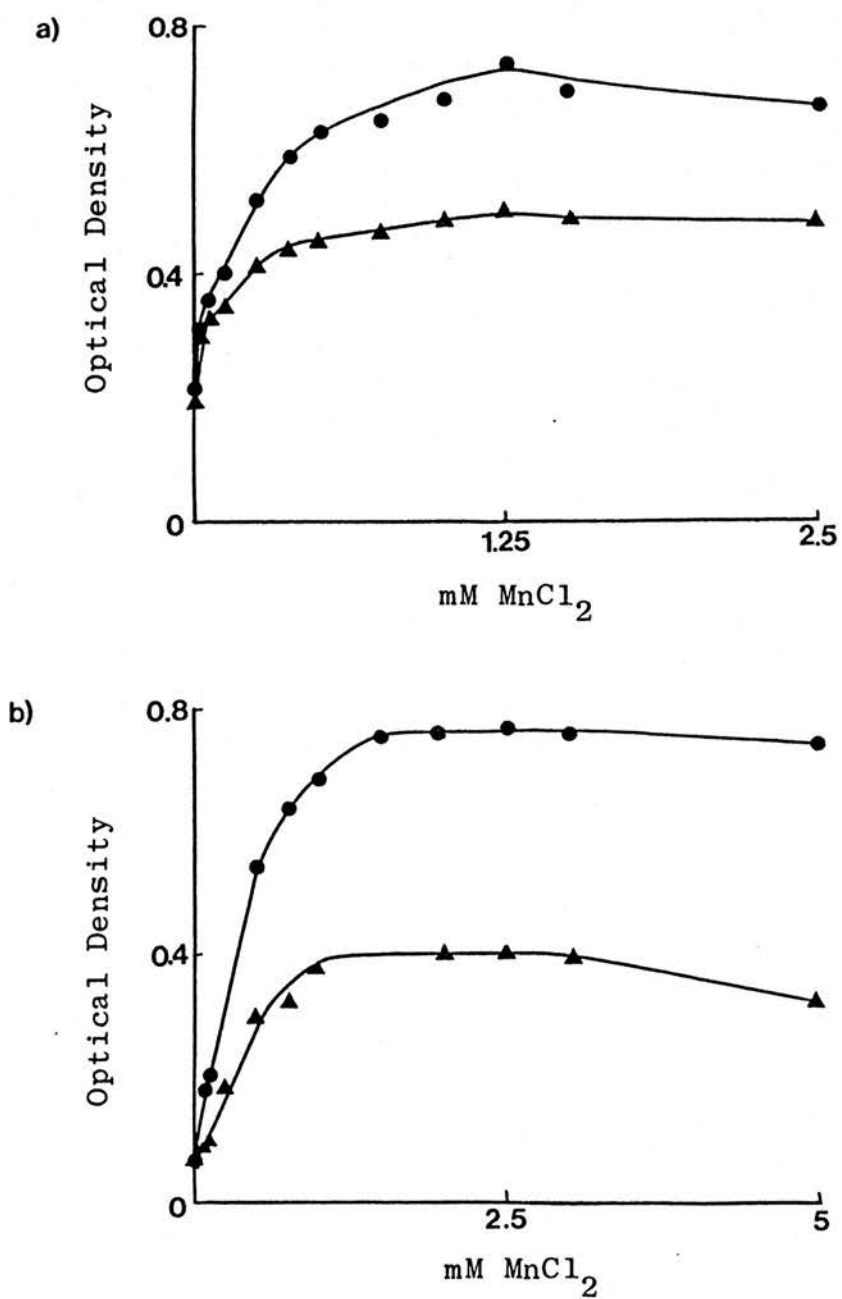


Fig. 29 Effect of manganese on prolidase activity with (●) and without (▲) 5 min preincubation for a) Phe-Pro and b) Gly-Pro.

The abnormal prolidase was activated by the addition of manganese but the optimal level for case 1 activity against Phe-Pro was 0.05 mM whereas case 2 had about 90% maximal activity at this level (Fig. 30). Abnormal prolidase activity was always reduced by preincubation irrespective of the level of manganese. However, both cases still required 60 min preincubation with 0.05 mM $MnCl_2$ for the optimal hydrolysis of Gly-Pro (Fig. 31).

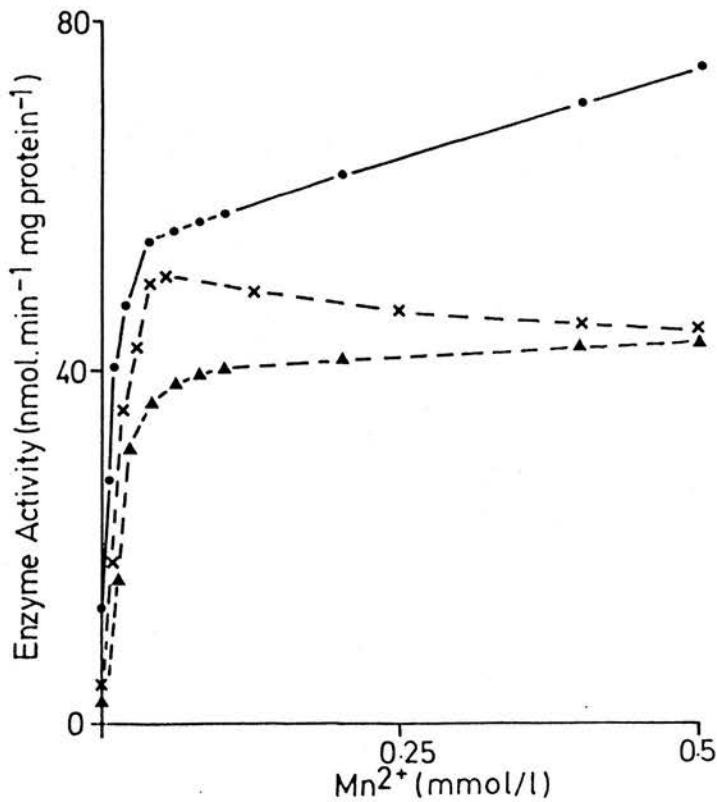


Fig. 30 Effect of $MnCl_2$ on control (●), case 1 (x) and case 2 (▲) prolidase activity. Extracts were preincubated with $MnCl_2$ for 5 min at 37°C at the levels shown before the addition of Phe-Pro resulting in a 25% reduction of the manganese concentration shown in the assay.

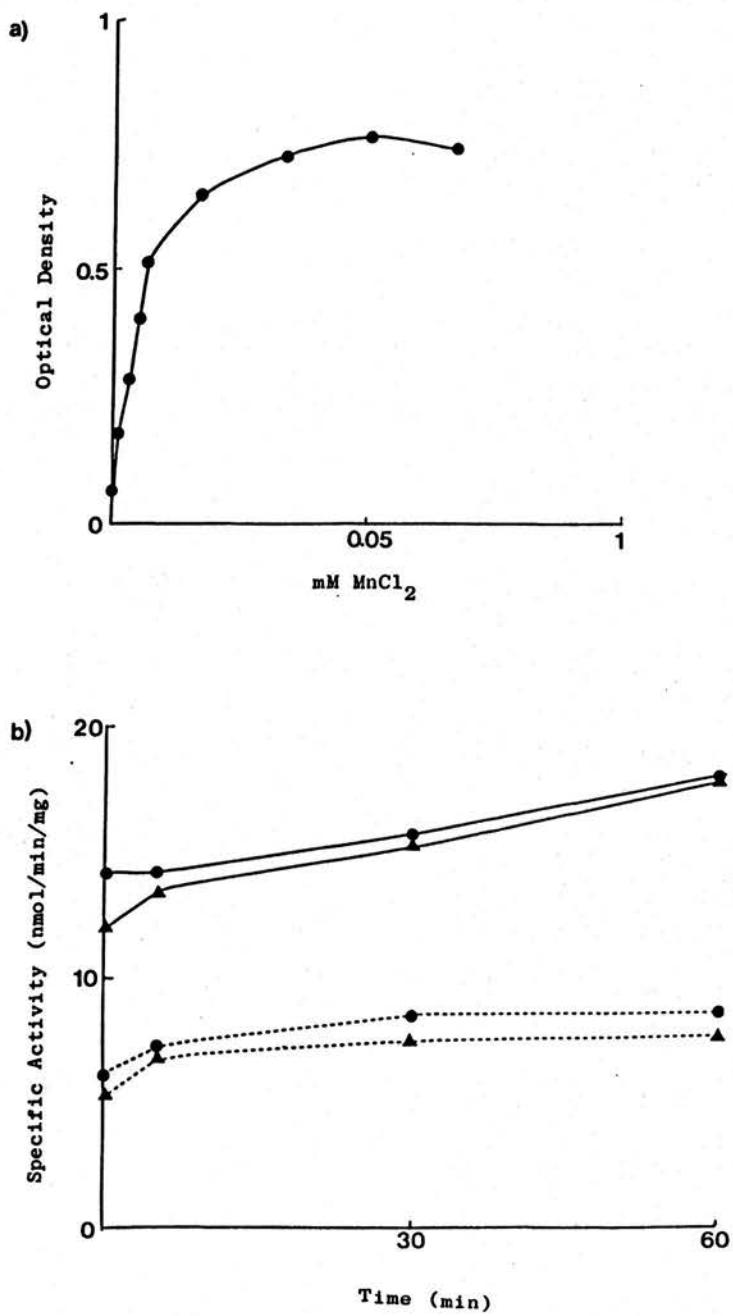


Fig. 31 a) Effect of preincubation for 5 min with different levels of MnCl₂ for case 1 assayed with Gly-Pro.
 b) Preincubation of case 1 (●) and case 2 (▲) fibroblasts with 0.05 mM (—) and 1.33 mM (---) MnCl₂.

5.4. DISCUSSION

5.4.1. Prolidase assays

As both L-amino acid oxidase methods gave larger readings for a given enzyme level than the proline method, they were more suitable for the rapid analysis of large numbers of samples such as may be generated by kinetic and chromatographic studies. However, the ninhydrin assay is the only method which can be used with Gly-Pro as substrate. Glycine in the ninhydrin reaction increased the proline standard reading and was used in the method as recommended by Mayer and Nordwig (1973). This eliminated any tendency for glycine liberated from Gly-Pro to increase the reading. Because of the rapid hydrolysis of Gly-Pro with heating under acid conditions assays were heated in the boiling water bath for 20 instead of 30 min. This resulted in lower substrate blanks as noted by Myara et al. (1982). Gly-Pro was used in the studies to enable comparison with previous studies. Phe-Pro, which is more easily assayed with the L-amino acid oxidase methods, is a novel substrate for studying prolidase.

The pH optimum of 8.0 for hydrolysing Phe-Pro was similar to that of 7.8 reported for human erythrocyte prolidase using Gly-Pro as substrate (Endo et al., 1982). In contrast, bovine prolidase had optimal activity at pH 7.0 (Yoshimoto et al., 1983).

5.4.2. Manganese

In previous studies Gly-Pro has been used with a wide range of different levels of manganese in the assay and have shown an almost complete absence of the enzyme in the disease. The conditions of assay have varied from no manganese (Arata et al., 1979), 10 mM (Sheffield et al., 1977), or 16 mM (Endo and Matsuda, 1981) with no preincubation to 1 mM preincubated for 2 hr (Powell et al., 1974; Ogata et al., 1981), 0.9 mM preincubated for 24 hr (Myara et al., 1982) or 10 mM for 1 hr at 37°C (Endo and Matsuda, 1981). Based on the present findings, these variable manganese conditions would partly explain the very variable prolidase levels reported in the literature (Powell et al., 1974; Sheffield et al., 1977; Arata et al., 1979; Endo and Matsuda, 1981; Ogata et al., 1981; Myara et al., 1982; Pedersen et al., 1983; Lombeck et al., 1986). In addition, the manganese levels which are optimal for control prolidase are not necessarily those required by the abnormal enzyme.

6. STUDIES ON PROLIDASE

6.1. INTRODUCTION

Prolidase deficiency can be diagnosed by assaying prolidase in cultured skin fibroblasts (Sheffield et al., 1977; Arata et al., 1979; Myara et al., 1983) as well as in red and white blood cells (Powell et al., 1974; Endo and Matsuda, 1981; Ogata et al., 1981). As noted in section 5.4.2. all these studies utilised Gly-Pro as substrate and indicated an almost complete deficiency of prolidase activity. However, there has been no research into the the properties of the residual prolidase activity in the disease or any measurements made using other imidodipeptide substrates. Having determined the optimal conditions of assay for control prolidase, a number of characteristics of the normal and abnormal enzyme were therefore studied, enabling diagnosis of the disease to be made using substrates in addition to Gly-Pro.

6.2. METHODS

6.2.1. Substrate specificity

The substrate specificity of prolidase was determined as previously described (5.2.2.1.) in control (n=10) and prolidase deficient fibroblasts using Gly-Pro, Phe-Pro, Ala-Pro, Val-Pro, Leu-Pro, Ser-Pro, Met-Pro (Sigma Ltd) and Glu-Pro (BaChem Ltd). After preincubation of an extract with 1.33 mM $MnCl_2$ at 37°C for 5 min, substrate was added (final concentration 20 mM) and incubated for a further 30 min. Liberated proline was then estimated by the ninhydrin method (5.2.2.1.). Gly-Pro, Phe-Pro, Leu-Pro and Ala-Pro were also assayed after 5 min preincubation without manganese, adding manganese with the substrate, and without any additional manganese in the assay.

6.2.2. pH stability

The pH stability of human skin fibroblast prolidase was determined using Phe-Pro as substrate. The pH of an extract (0.5 ml) was adjusted with 0.01 M HCl or NaOH to pHs from 4.0-10.0, left at room temperature for 30 min and then neutralised prior to assay.

6.2.3. Heat stability

The effect of heat on prolidase activity in control and prolidase deficient fibroblasts was investigated by heating extracts in 50 mM barbital / HCl pH 8.0 in a water bath at 30-60°C for 5 min or at 48°C for up to 60 min. This was carried out in the presence (1.33 mM) or absence of manganese. For samples treated without manganese an equivalent amount of $MnCl_2$ was added with the substrate.

6.2.4. Substrate kinetics

Substrate kinetics of control and abnormal prolidase were studied after preincubation with 1.33 mM $MnCl_2$ for 5 min for all the substrates over the concentration range 1-20 mM. K_m and V_{max} were calculated by constructing Eadie-Hofstee plots using a Hewlett-Packard HP97 (Zivin and Waud, 1982).

Product formation studies were also performed for the hydrolysis of Phe-Pro using the method of Chrastil and Wilson (1982)(see appendix, section 11.6.). Product (phenylalanine) formation was followed for up to 24 hr with a limiting substrate level (0.5 mM) for control and prolidase deficient fibroblasts with the same extract protein level.

6.2.5. Effect of PHMB

The effect of p-hydroxymercuribenzoate (PHMB) on prolidase activity against Phe-Pro was assessed by adding 0.01-0.5 mM PHMB before or after treatment with 1.33 mM $MnCl_2$. Based upon these results, 0.05 mM PHMB was added to normal and abnormal prolidase following preincubation with manganese and assayed with all the substrates except Glu-Pro.

6.2.6. Chromatography

6.2.6.1. Ion-exchange chromatography

Ion-exchange chromatography was performed using DEAE-Cellulose prepared as described in section 3.2.7. in a 1 x 25 cm column. The extract (2 ml) was dialysed for 60 min against the elution buffer, centrifuged at 100,000 x g for 30 min, then applied to the column. Elution was carried out with ^{300 ml of} a 0-250 mM NaCl linear gradient in 10 mM barbital / HCl buffer pH 7.4 and 1.25 ml fractions collected.

6.2.6.2. Gel filtration

Gel filtration was performed with Sephadex G 150-120 (Sigma Ltd) in a 2.5 x 55 cm column. 2ml extract was dialysed against elution buffer for 60 min,

centrifuged at 100,000 x g for 30 min and applied to the column. Elution was carried out with 10 mM barbital / HCl buffer pH 7.4 containing 100 mM NaCl with an ascending flow-rate of 4 ml/hr and 1.25 ml fractions collected.

6.2.6.3. Isoelectrofocusing

Sucrose density isoelectrofocusing was performed in 'J' tubes (see appendix, section 11.4.) by applying 0.75 ml samples of control and case 1 fibroblast extracts with 'narrow-range' ampholine pH 4-6. Fractions (0.22 ml) were collected and assayed for prolidase using Phe-Pro with 5 min preincubation or Gly-Pro with 60 min preincubation with 1.33 mM $MnCl_2$. Control was assayed for 30 min and case 1 for 60 min. Prolidase was also assayed with Leu-Pro and Ala-Pro under the same conditions as for Phe-Pro.

6.3 RESULTS

6.3.1. Prolidase activities

Skin fibroblast prolidase activated by preincubation with manganese showed a tight range of values for each of the eight substrates studied (Fig. 32). The highest specific activity was against Ser-Pro whilst the lowest was against Glu-Pro, the only substrate which wasn't activated by manganese. Activity against Gly-Pro, when fully activated by 60 min preincubation with 2.5 mM $MnCl_2$, was increased about 2.5 fold to a mean specific activity of 220 nmol/min/mg protein. Abnormal prolidase activity against Gly-Pro was reduced to 3-6% of control activity depending upon the manganese conditions used. The reduction in activity against the other substrates was not nearly as marked, ranging from 27 to 80% of control (see Fig. 32).

Prolidase activities against four different substrates in fibroblasts from controls and the two cases with and without manganese are shown in Table 8. Clearly there is a marked deficiency in the cases against each of the four substrates when assayed without manganese. After preincubation with manganese, the deficiency was still marked for Gly-Pro (94%), but less so for Phe-Pro (55%), Leu-Pro (40%) and Ala-Pro (70%).

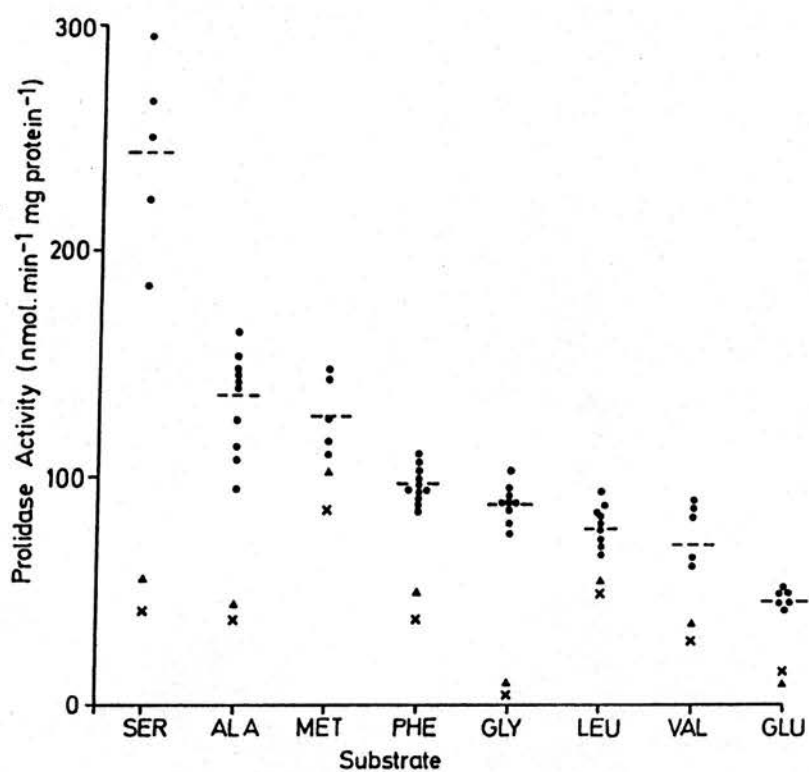


Fig. 32 Prolidase activity of control (●), case 1 (x) and case 2 (▲) skin fibroblasts (---; mean activity) following preincubation with 1.33 mM MnCl₂ for 5 min at 37°C against 20 mM substrate (X-Pro; X-amino acid shown).

6.3.2. pH and Heat stability

Prolidase was stable over the range pH 4.5-9.5 with a fall off to less than 50% of the neutral pH activity at pH 4.0 (Fig. 33).

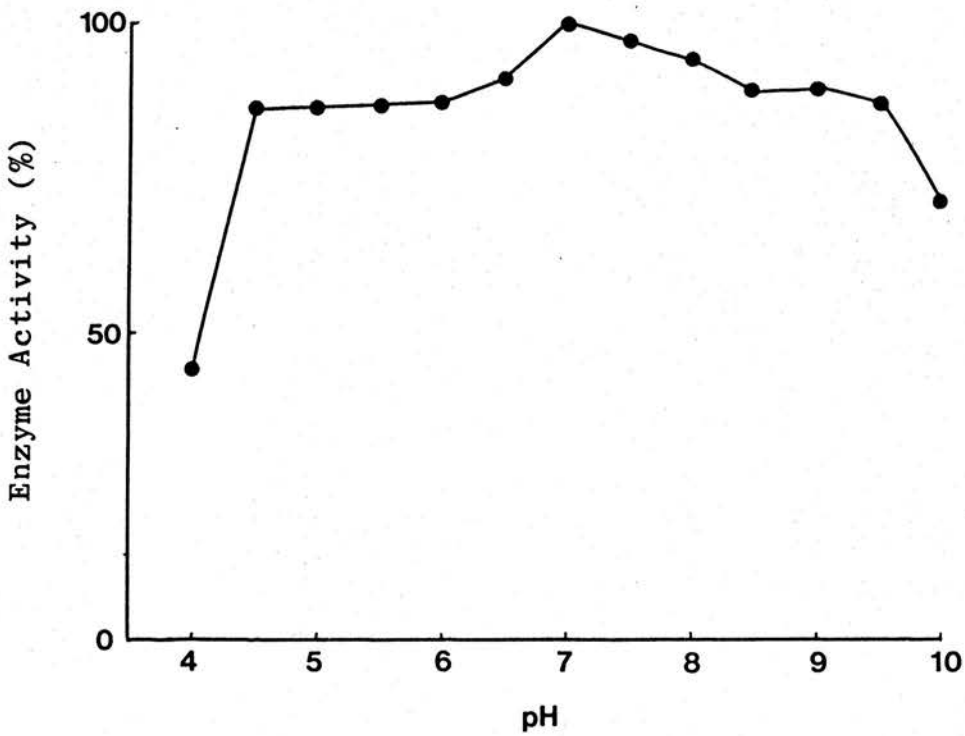


Fig. 33 pH stability of skin fibroblast prolidase.

Both normal and abnormal prolidase activities against Phe-Pro were thermolabile above 45°C with only about 10% residual activity at 55°C after 5 min (Fig. 34a). Following the loss of activity at 48°C (Fig. 34b) showed that 70-90% of control enzyme activity (example shown was typical of 6 controls studied) was lost after 60 min without manganese. However, if manganese was present the enzyme was protected and only lost about 25% of activity. The two cases (case 2 shown) showed a similar pattern of loss of enzyme activity as in the controls in the absence of manganese, but in the presence of manganese the abnormal enzyme was initially rather more labile. Hence the addition of manganese increased the heat stability of the normal, but decreased that of the abnormal enzyme.

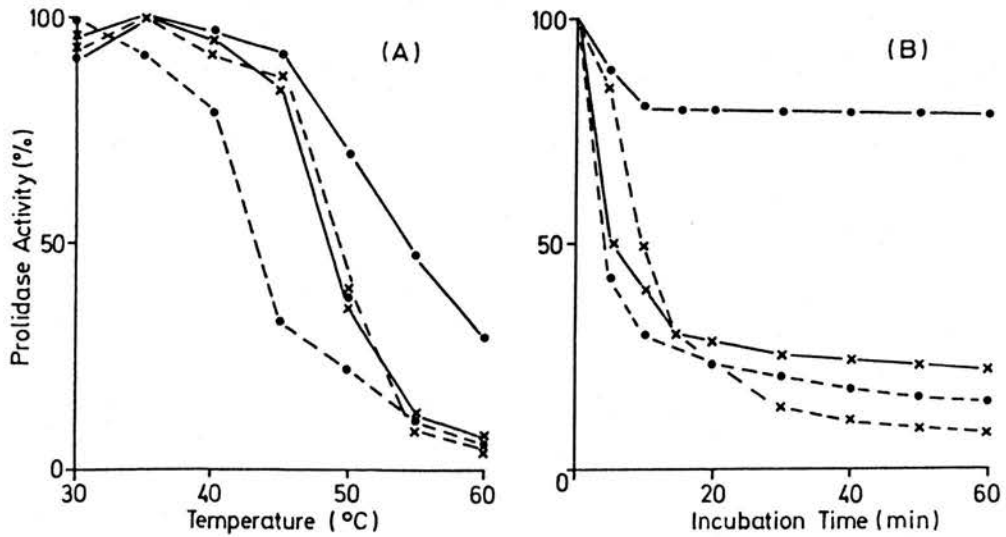


Fig. 34 Heat stability of control (—) and case 2 (---) prolidase activity against Phe-Pro without (×) or with (●) 1.33 mM MnCl_2 . (a) Effect of 5 min at 30-60°C. (b) Effect of preincubation at 48°C for up to 60 min.

6.3.3. Substrate kinetics

The response of normal and abnormal prolidase (case 2), following preincubation with 1.33 mM $MnCl_2$ for 5 min, to a number of substrates was analysed by Eadie-Hofstee plots. The results for the corrected K_m and V_{max} are given in Table 9. The estimates for data variation ($SD(E)$ rad; Zivin and Waud, 1982) was less than 0.07 for all the substrates, indicating the results to be acceptable. The normal enzyme exhibited biphasic kinetics for Phe-Pro (Fig. 35a), whilst the abnormal enzyme showed only a single line (Fig. 35b). Plots for all the other substrates were monophasic for controls and cases. The abnormal enzyme had a lowered affinity for all the substrates, the smallest change being for Glu-Pro and the largest for Ser-Pro, Phe-Pro and Leu-Pro. The maximum activity of the abnormal enzyme, calculated from the Eadie-Hofstee plots was increased for Met-Pro, unchanged for Phe-Pro and Leu-Pro, somewhat reduced for Ser-Pro and Val-Pro and most reduced for Ala-Pro, Glu-Pro and Gly-Pro (Table 9).

Substrate	Km (mM^{-1})		Vmax (nmol/min/mg protein)	
	Control	Case2	Control	Case2
Ser-Pro	3.8	36.2	279	142
Ala-Pro	2.3	8.1	221	62
Met-Pro	4.5	14.4	147	210
Phe-Pro	2.8, 6.8	24.9	136	147
Gly-Pro	2.8	13.7	150	20
Leu-Pro	1.3	10.4	80	72
Val-Pro	0.9	3.3	68	40
Glu-Pro	4.4	6.1	33	6

Table 9. Substrate kinetics of control and case 2 prolidase. 1-20 mM substrate analysed by Eadie-Hofstee plots (Zivin and Waud, 1982).

25 μl extract was preincubated with 50 μl 50 mM barbital / HCl pH 8.0 containing 2 mM MnCl_2 for 5 min and the assay started by the addition of substrate and incubation at 37°C for 30 min.

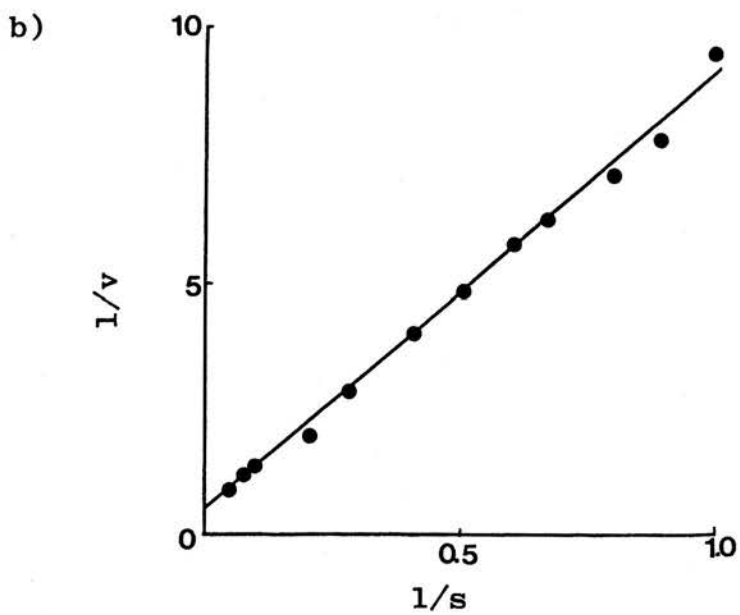
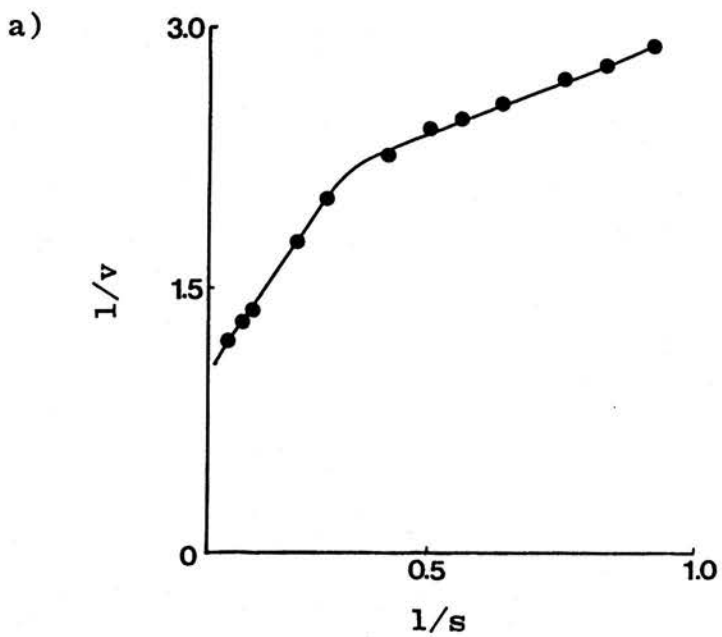


Fig. 35 Lineweaver and Burk plots for a) control and b) case 1 skin fibroblast activity against Phe-Pro.

Analysis of product formation (Fig. 36), using Phe-Pro as substrate by the method of Chrastil and Wilson (1982)(see appendix, section 11.6.), indicated the abnormal enzyme had an unchanged sterical constant value (n) of over 0.9 and a maximum product formation value (p_{∞}) of 0.35 mM. The rate constant related to the diffusion coefficient (k) was reduced to 0.0023 as compared with 0.0086 l/g/sec for control enzyme.

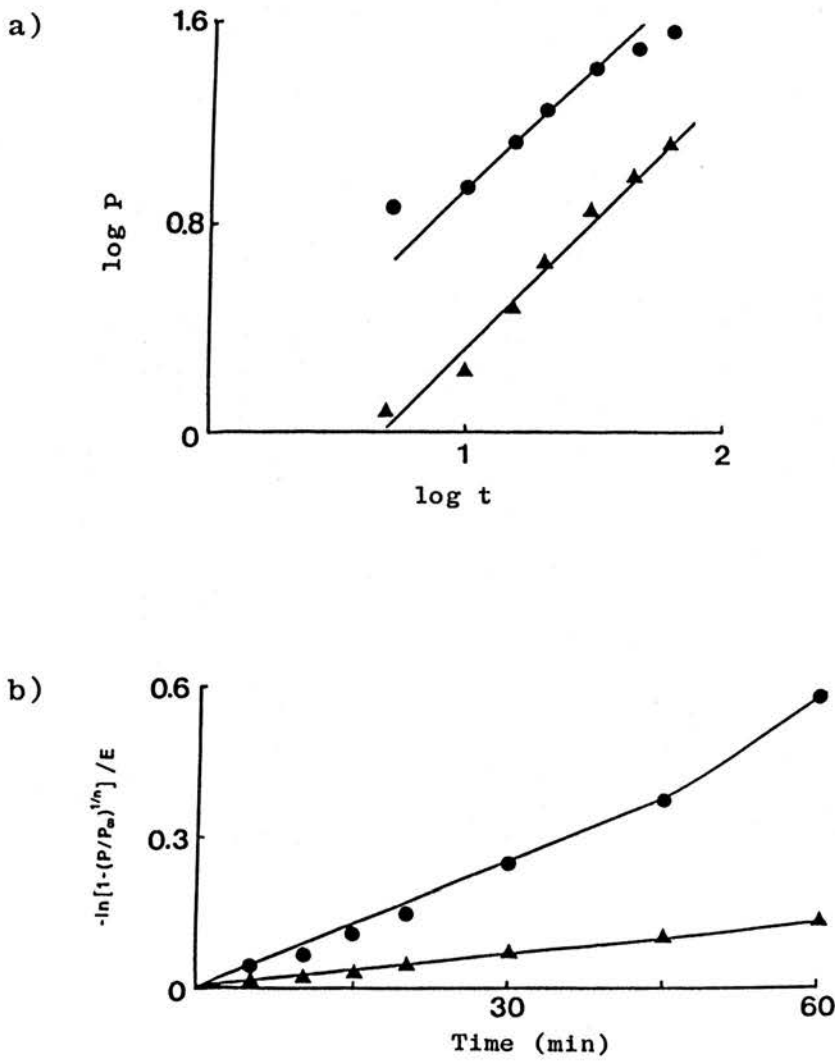


Fig. 36 Determination of a) sterical structure constant n , and b) rate constant k , for prolidase in control (●) and case 1 (▲) fibroblast extracts assayed with Phe-Pro. The constants are obtained as the slopes of the linear part of each plot (see appendix, section 11.6.).

6.3.4. Inhibition with PHMB

Both control and case prolidase activity against Phe-Pro was strongly inhibited by treatment with PHMB prior to incubation with manganese. However, preincubation with 1.33 mM $MnCl_2$ prior to addition of PHMB, protected the normal enzyme but not the abnormal enzyme from inhibition (Fig. 37). Addition of PHMB and manganese simultaneously gave an intermediate result for control prolidase with 0.05 mM PHMB plus 1.33 mM $MnCl_2$ inhibiting by 30% compared to less than 5% when PHMB was added after the manganese. The abnormal prolidase (cases 1 and 2) was inhibited by greater than 95% under both these conditions. Table 10 shows the effect of 0.05 mM PHMB on prolidase activity against a number of substrates after preincubation with manganese for three controls and for both cases. The addition of manganese prior to PHMB protected normal prolidase activity for all the substrates except for a small reduction with Gly-Pro and a moderate reduction for Met-Pro. However, the abnormal enzyme was markedly inhibited for all the substrates, except for a moderate reduction of the small residual activity against Gly-Pro.

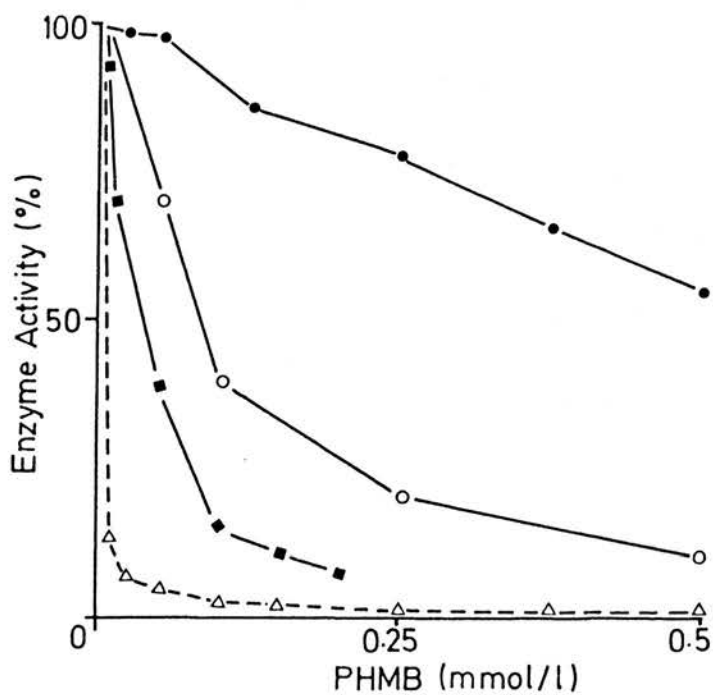


Fig. 37 Effect of PHMB on control (—) and abnormal (---) prolidase activity against Phe-Pro.
 ■, PHMB added before manganese;
 ○, PHMB added with manganese;
 ●, PHMB added after manganese;
 △, PHMB added before or after manganese.

Substrate	Controls			Cases	
	1	2	3	1	2
Ser-Pro	100	97	100	14	14
Ala-Pro	92	98	100	0	4
Met-Pro	45	53	56	4	3
Phe-Pro	113	108	100	6	3
Gly-Pro	76	81	80	58	58
Leu-Pro	117	93	75	2	4
Val-Pro	131	126	120	4	3

Table 10. Relative activity (%) of prolidase against different substrates following PHMB treatment. Prolidase activated by preincubation with 1.33 mM $MnCl_2$ for 5 min at 37°C and PHMB added with substrate to give a concentration of 0.05 mM.

6.3.5. Chromatography

DEAE-Cellulose ion-exchange chromatography gave a single peak of prolidase eluting at 135 mM NaCl, just after the second peak of prolinase at 130 mM NaCl (Fig. 38). Sephadex gel filtration also gave a single peak of prolidase eluting just before the first prolinase peak (Fig. 39). As all the prolidase activity was found to be below pH 6.0 on a preliminary run, isoelectrofocusing was carried out with a narrow-range pH 4-6 ampholite gradient. Control fibroblast prolidase showed a peak for Gly-Pro and Phe-Pro at pI 4.5 (Fig. 40). Prolidase-deficient fibroblasts (case 1) showed a peak at pI 4.6 for both these substrates. The prolidase activity against Leu-Pro and Ala-Pro coincided with these peaks. The minor peak at about pI 5.0 was associated with a large precipitated protein band and was probably artifactual.

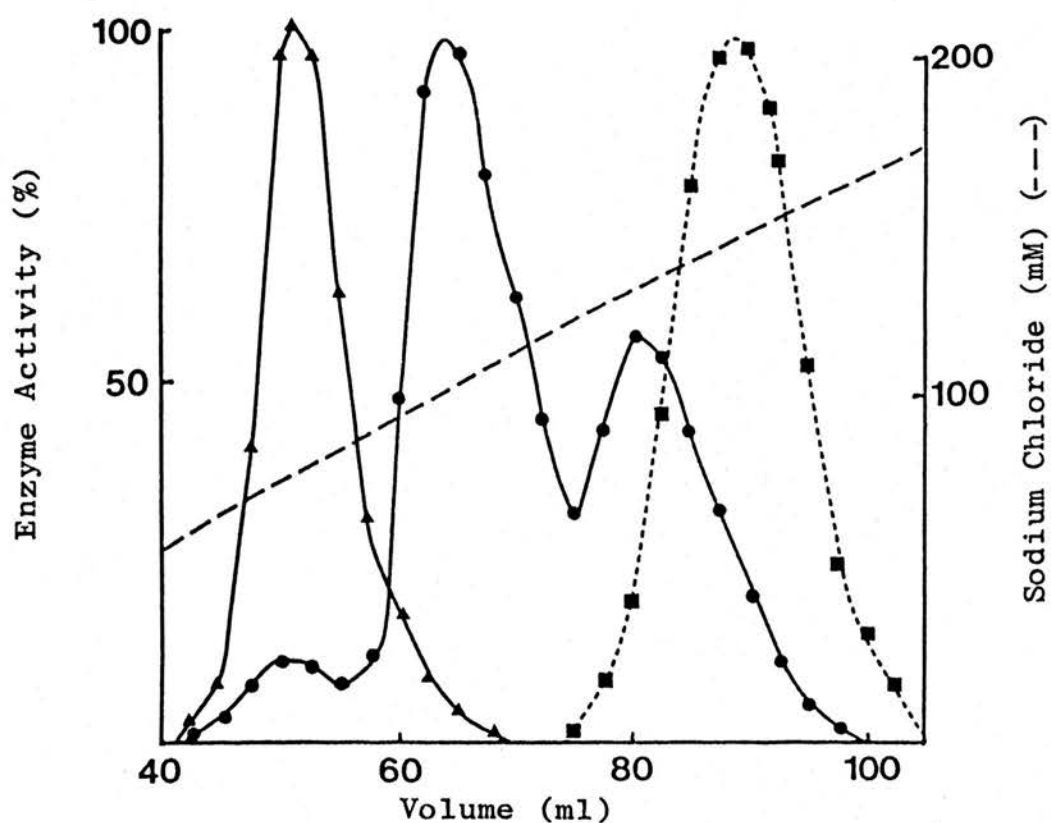


Fig. 38 DEAE-Cellulose ion-exchange chromatography of human skin fibroblast prolidase (Phe-Pro, ■); prolinase (Pro-Gly, ●) and proline imino-peptidase (Pro-Gly-Gly, ▲). 1 x 25 cm column eluted with 0-250 mM NaCl in 10 mM barbital / HCl buffer pH 7.4.

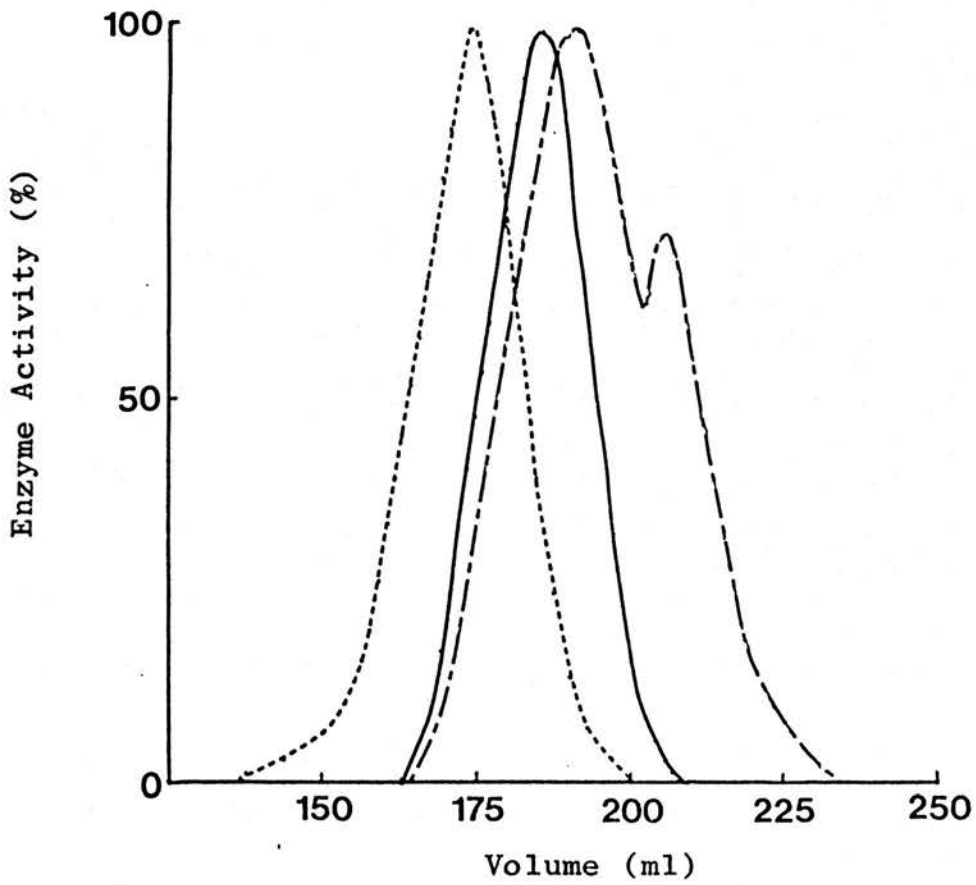


Fig. 39 Sephadex G150-120 gel filtration of human skin fibroblast prolidase (Phe-Pro, —); prolinase (Pro-Gly, ---); and β -hexosaminidase (-----). 2.5 x 55 cm column eluted with 10 mM barbital / HCl buffer pH 7.4 containing 100 mM NaCl.

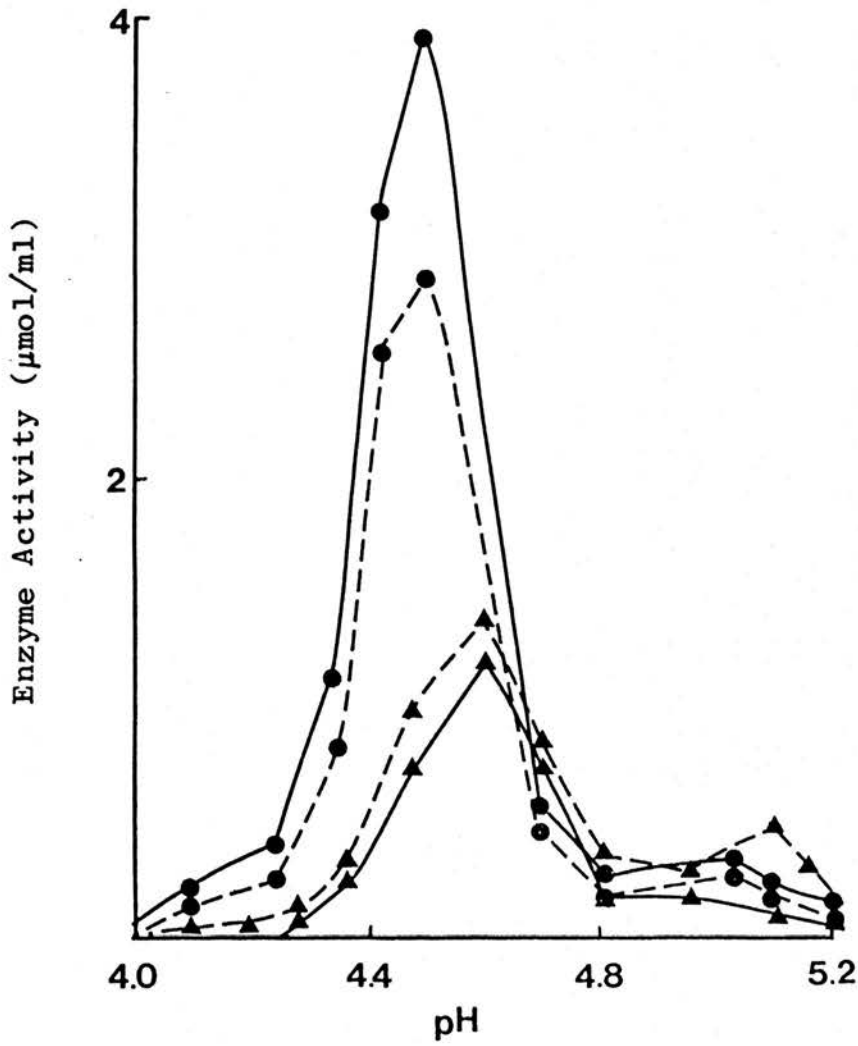


Fig. 40 Sucrose density isoelectrofocusing of control (●) and case 1 (▲) fibroblast prolidase assayed with Gly-Pro (—) or Phe-Pro (---). Isoelectrofocusing was performed as described in the appendix, section 11.4.

6.4. DISCUSSION

6.4.1. Prolidase activities

The reported mean specific activity (nmol/min/mg protein) of control skin fibroblast prolidase against Gly-Pro ranged from 7.2 (Arata et al., 1979), 20 (Sheffield et al., 1977), 50 (Myara et al., 1982) to 154 (Pedersen et al., 1983), all less than for the present level of 220 under optimal conditions. This wide range of activities is a reflection of the variable manganese conditions used. Abnormal prolidase activity against Gly-Pro has consistently been reported to be absent or very low (Powell et al., 1974; Sheffield et al., 1977; Arata et al., 1979; Endo and Matsuda, 1981; Ogata et al., 1981; Myara et al., 1982; Pedersen et al., 1983) and in the present study both cases also had reduced activities, 3-6% of control depending upon assay conditions. The marked deficiency of activity against Gly-Pro in the two cases was not a feature of the other substrates tested and indicates a change in substrate specificity rather than a complete absence of the enzyme. The deficiency against substrates with a polar amino acid (glycine, serine and glutamic acid) was greater than against those with a non-polar amino acid (alanine, valine, phenylalanine, leucine and methionine)(Fig. 32). Thus it seems that

the disease is a result of a change in the structure of the enzyme at or close to the active site.

6.4.2. Heat stability

The results for the heat stability of prolidase may indicate that the normal enzyme consists of two forms, one labile and one stable in the presence of manganese (Fig. 34b). In prolidase deficiency it appears that this stable form is largely absent.

6.4.3. Substrate kinetics

Owing to the altered K_m values, the deficiency in abnormal prolidase activity would be more marked the lower the substrate level used. The substrate (Gly-Pro) levels used in previous work has varied from 1 (Sheffield et al., 1977), 3.7 (Powell et al., 1974), 7 (Arata et al., 1979), 10 (Endo et al., 1981) to 47 mM (Myara et al., 1982) which would differentially affect the level of control and abnormal prolidase activity. As with manganese conditions (see section 5.4.2.), the level of substrate optimised for the assay of control prolidase would not be optimal for the abnormal enzyme.

The biphasic kinetics of control prolidase against Phe-Pro (Fig. 35a) provide further evidence for the existence of two enzymes, though it is interesting

that, of the substrates tested, this is only observed for Phe-Pro.

The data for product formation studies indicated no major alteration in enzyme structure or absorption affinity for the substrate, but faulty activation or presence of inactive enzyme (Chrastil and Wilson, 1982).

6.4.4. Inhibition with PHMB

The effect of PHMB on prolidase was tested as PHMB has been shown to bind to pig intestinal prolidase (Sjöström and Norén, 1974) and bovine intestinal prolidase (Yoshimoto et al., 1983). Also, sulphhydryl reagents such as iodoacetamide have been reported to inhibit pig kidney prolidase (Davis and Smith, 1953) and this inhibition was noted to be prevented by manganese. In keeping with these findings, human prolidase was inhibited by PHMB and protected by manganese ions. Since PHMB and iodoacetamide react with SH groups, the prevention of PHMB inhibition of control prolidase activity by manganese ions indicates that manganese activates prolidase by binding to SH groups. Prolidase activity in the two cases, however, was much more susceptible to inhibition by PHMB after preincubation with manganese (see Table 10). The failure of manganese to protect the activity of the two cases indicates the absence of this form of prolidase

or that the genetic alteration in the enzyme may involve one or more SH groups.

6.4.5. Chromatography

The chromatographic profiles for DEAE-Cellulose, Sephadex and isoelectrofocusing all indicate that cultured skin fibroblasts have only a single enzyme, prolidase, for the hydrolysis of imidodipeptides. The pI of 4.5 observed in the present study is almost the same as that of 4.4 seen in guinea pig brain (Browne and O'Cuinn, 1983) and pig intestine (Sjöström and Norén, 1974). The pI of 4.6 for abnormal prolidase was not significantly different from the pI of the control enzyme.

6.4.6. Conclusions

From these results, a number of properties can be observed to distinguish control and abnormal prolidase. In addition to the differing responses to preincubation with manganese (section 5.3.3.), three other properties may be considered to be diagnostic: 1) substrates: abnormal enzyme has reduced specific activities and increased K_m values compared to control; 2) heat stability: control is more stable, but abnormal more labile to heat treatment in the presence of manganese and 3) PHMB: inhibition of control prevented by

manganese, abnormal more susceptible to inhibition in presence of manganese. These results may be explained by either a change in the properties of a single form of prolidase or the presence of two isoenzymes, at least one of which is affected in the disease. Although only a single form was observed with the separative techniques used here and in human erythrocytes (Endo et al., 1982), an unstable second form may be losing its activity at some stage in the preparative procedure. Also the results here indicate that a second form would not be able to hydrolyse Gly-Pro efficiently and would require the use of other substrates for detection. Which of these hypotheses is correct will be resolved by the use of further chromatographic procedures and assay of prolidase with a number of substrates.

7. A SECOND COMPONENT OF PROLIDASE ACTIVITY

7.1. INTRODUCTION

Although only one form of prolidase was found in the previous studies (section 6.3.5.), the changed properties of prolidase in the disease state (section 6.3.) could be most easily explained by the presence of more than one form of prolidase in controls. Hence a further chromatographic study was undertaken to see if a second form had not been found for methodological reasons. Indeed a second form of prolidase was found and a DEAE-Cellulose batch method was developed to enable the rapid separation of the two forms of prolidase. The distribution of the two forms was studied in a range of human cells and tissues. Properties of the two prolidasases in controls and the two cases of prolidase-deficiency were studied in order to determine how the two prolidasases were altered in the disease.

7.2. METHODS

7.2.1. DEAE-Cellulose

7.2.1.1. Column chromatography

Cell and tissue extracts were prepared by the addition of ice-cold 10 mM barbital / HCl buffer pH 7.4 containing 50 mM NaCl, sonication at 4-6 μ m for 30 sec for cells or homogenisation in a Potter-Elvehjem homogeniser followed by sonication for 60 sec for tissues. Extracts were then centrifuged at 100,000 x g for 60 min.

The separation of cell and tissue prolidase into two components was achieved by applying 1 ml fibroblast, amniotic fluid or white cell extract (\sim 5 mg/ml), 1 ml red blood cell extract or serum (50 mg/ml) or 1 ml (10% w/v) tissue extract in 10 mM barbital / HCl buffer pH 7.4 containing 50 mM NaCl to a 0.8 x 5 cm DEAE-Cellulose column, eluting with 70 ml of a 50-300 mM NaCl linear gradient and collecting 1.5 ml fractions.

7.2.1.2. DEAE batch method

A DEAE-Cellulose batch method was then developed for the rapid separation and estimation of the two prolidase activities. A mixture of 0.25 ml 50% (v/v)

DEAE-Cellulose slurry in 10 mM barbital / HCl pH 7.4, 0.125 ml NaCl and 0.125 ml 2% (w/v) extract in the same buffer was mixed for 15 min at 10°C, centrifuged at 1,000 x g for 10 min and the amount of unbound prolidase estimated against Phe-Pro. The concentration of NaCl added was varied from 400 to 1200 mM to find the optimal levels for recovery of peak I without peak II and for total recovery. The addition of 480 mM NaCl released peak I prolidase and 1000 mM NaCl released both peaks and their sequential use allowed the separation of both peaks. The reproducibility of the method was checked by estimating the percentage of each of the peaks in a cerebral frontal cortex extract ten times. The percentage of the two peaks was also estimated for a number of cells and tissues using the batch method. The volumes used in the method can be proportionately increased to allow the preparation and characterisation of the separated peaks.

7.2.2. Substrate specificity

Prolidase activity of the peaks, separated by column chromatography or the batch method, was measured with a variety of substrates (Gly-Pro, Phe-Pro, Ala-Pro, Val-Pro, Met-Pro and Leu-Pro; Sigma Ltd) by estimating liberated proline (section 5.2.1.1.) or phenylalanine (section 5.2.1.2.). Activities were related to protein using bovine serum albumin as

standard (Lowry et al., 1951; see appendix, section 11.3.).

7.2.3. Properties

The manganese requirement, heat stability at 48°C, inhibition by p-hydroxymercuribenzoate and substrate kinetics (Km) of the two peaks of prolidase from controls and the two cases were studied as previously described in sections 5.2.3. and 6.2.

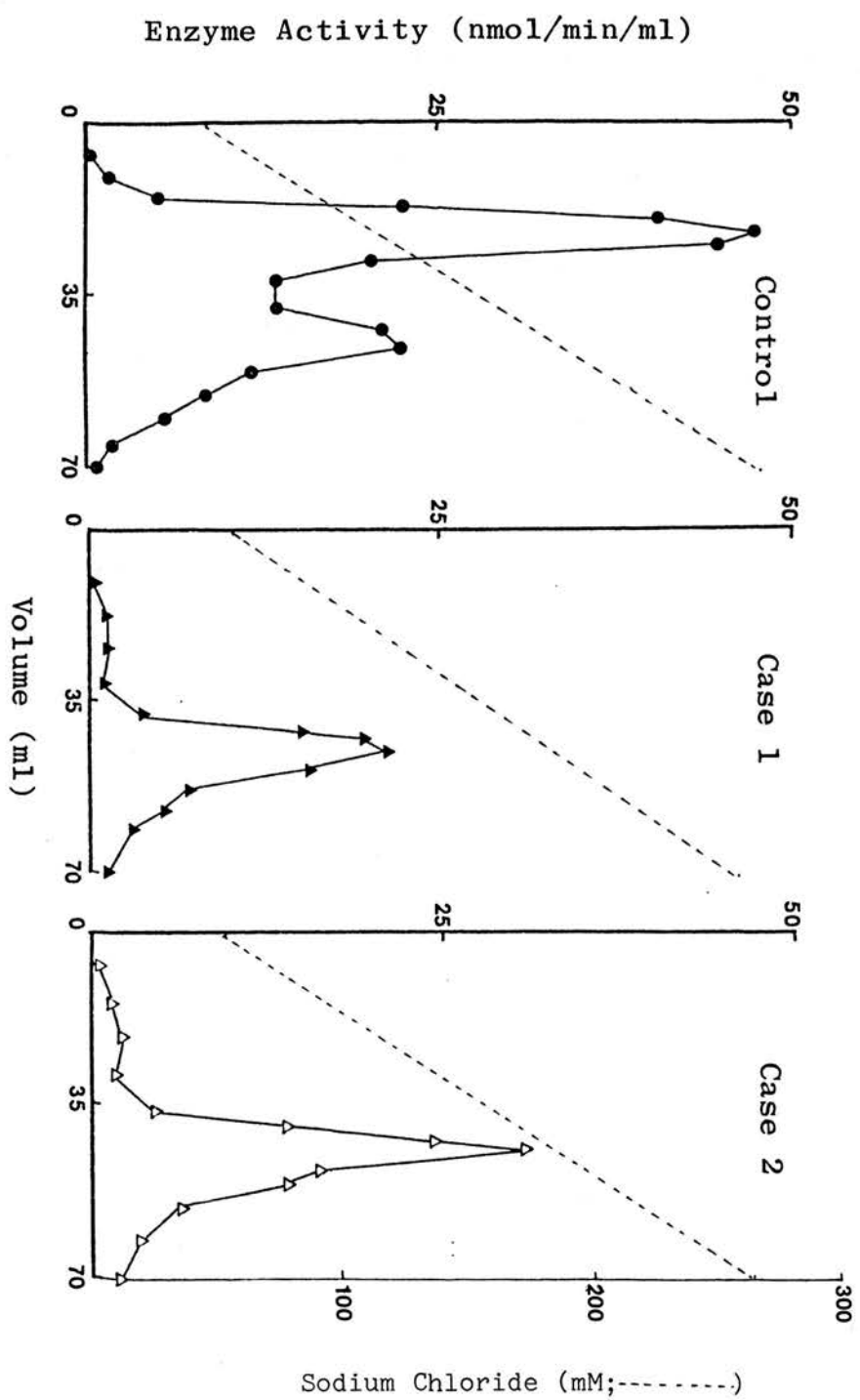
7.3. RESULTS

7.3.1. Column chromatography

Initial work using a 1 x 25 cm DEAE-Cellulose column showed two peaks of prolidase activity in cultured skin fibroblasts, eluting at 135 and 210 mM NaCl respectively. Dialysis of an extract against (10 mM Barbitol / HCl pH 7.4 containing 50 mM NaCl) buffer, inactivated peak II, but the inclusion of 0.1 mM dithiothreitol during dialysis prevented this loss. Chromatography of fibroblast prolidase activity of the prolidase-deficient cases against Phe-Pro showed a marked deficiency of peak I as compared to control activity, although a very small residual activity could be detected (Fig. 41). Peak II was not reduced in the two cases. In control fibroblasts peak I was very active against Gly-Pro compared with Phe-Pro, whereas peak II was relatively less active against Gly-Pro (Fig. 42). The activity against Phe-Pro was split about 60% peak I and 40% peak II estimated by calculation of areas under the elution curve. Activity against Gly-Pro was split about 85% peak I, 15% peak II.

The activity against six other substrates was also investigated, though not for peak I in the cases owing to insufficient activity for reliable measurements using the proline detection method. The main features were an increased activity of case peak

Fig. 41 DEAE-Cellulose chromatography of control, case 1 and case 2 fibroblast prolidase activity against Phe-Pro.



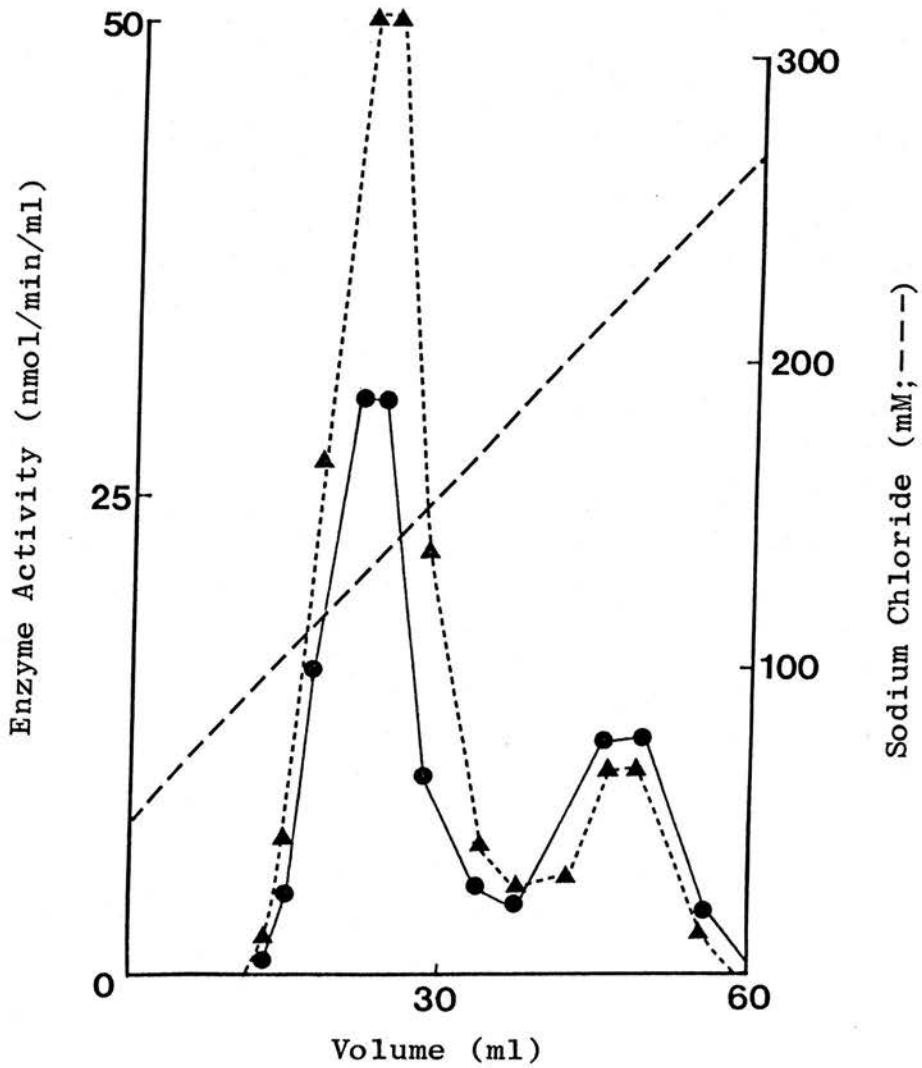


Fig. 42 Skin fibroblast prolidase separated by DEAE-Cellulose chromatography. Prolidase was assayed with Gly-Pro (\blacktriangle --- \blacktriangle) and Phe-Pro (\bullet — \bullet).

II against Met-Pro and a markedly reduced activity against Gly-Pro as compared to control peak II (table 11).

In order to compare the proportions of the two peaks in a number of different cells and tissues, small 0.8 x 5 cm DEAE-Cellulose columns were used to reduce the sample size required. Recovery of prolidase activity was above 70% and all the control cells and tissues examined had both peaks of prolidase. Red blood cells, kidney, liver and spleen had about two-thirds peak I, cerebral frontal cortex slightly more peak II and pancreas about two-thirds peak II (Fig. 43). Serum had a very low prolidase specific activity (1.0 nmol/min/mg protein) and showed a single peak eluting prior to peak I at 85 mM NaCl.

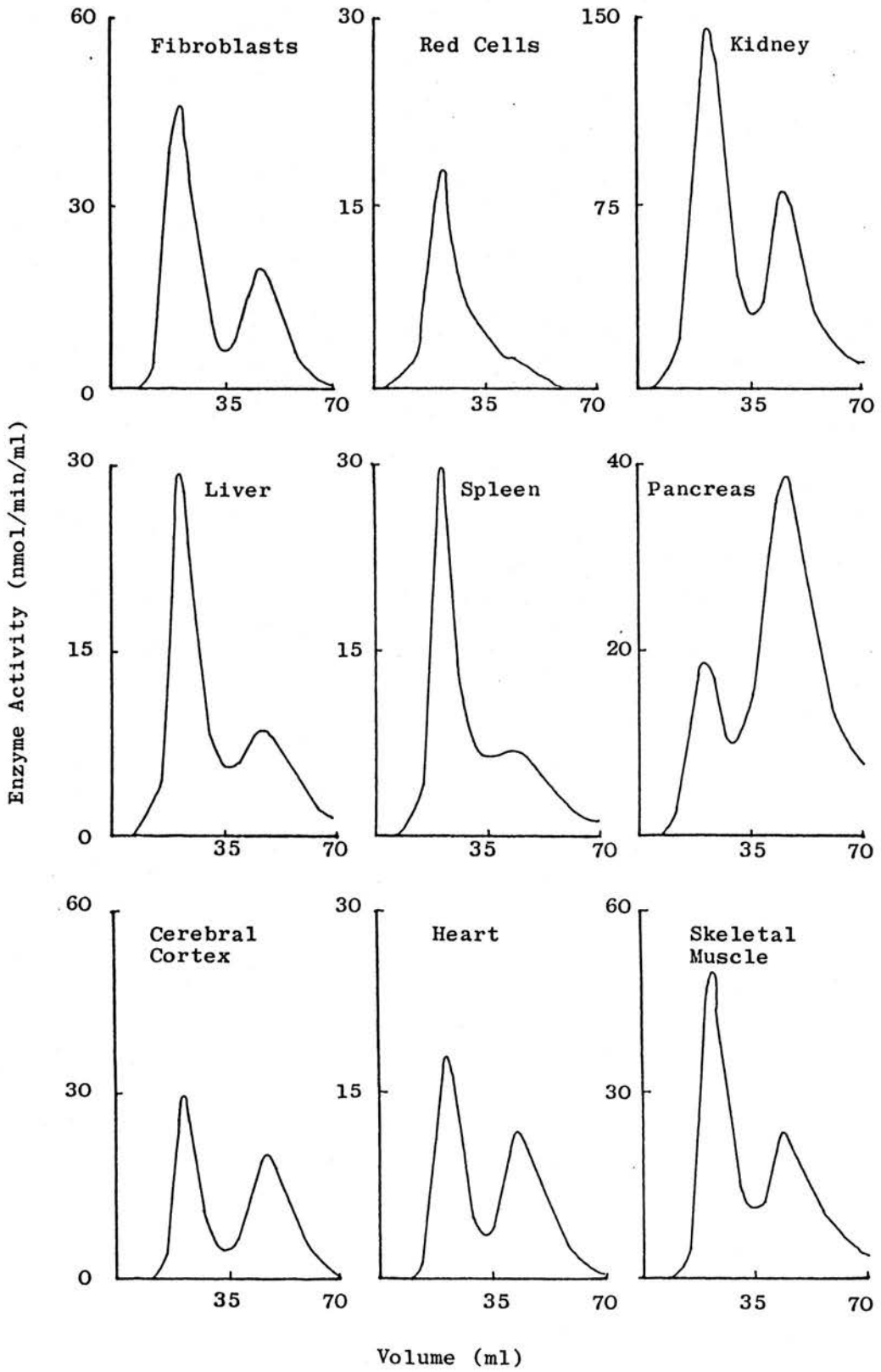
Substrate	CONTROL				CASE 1		CASE 2	
	Activity I	Relative %age I	Activity II	Relative %age II	Activity II	Relative %age II	Activity II	Relative %age II
Phe-Pro	64	100	32	100	38	100	46	100
Val-Pro	45	71	24	74	25	66	34	74
Leu-Pro	54	84	30	95	44	116	56	122
Met-Pro	90	141	48	150	85	224	99	215
Ala-Pro	125	196	42	130	39	103	56	122
Ser-Pro	154	240	41	128	44	116	56	122
Gly-Pro	105	164	26	80	5	13	5	11

Table 11. Activity* and relative percentage† of prolidase peaks in control and prolidase-deficient fibroblasts separated by DEAE-Cellulose column chromatography.

* Activity (nmol/min) after 5 min preincubation with 1 mM MnCl₂ corrected for 70% recovery from columns.

† Percentage activity against different substrates relative to Phe-Pro.

1 ml of a 5 mg/ml fibroblast extract was applied to a 0.8 x 5 cm DEAE-Cellulose column, eluting with 70 ml of a 50-300 mM NaCl linear gradient, collecting 1.5 ml fractions.



7.3.2. DEAE-Cellulose batch chromatography

Whilst column chromatography is ideal for fine analytical work, the technique does not readily permit analysis of small quantities of many samples in a short time. The DEAE-Cellulose batch method was therefore developed to allow rapid analysis of large numbers of samples. To follow the binding of the two peaks, advantage was taken of their differing responses to manganese in that peak I needs preincubation for full activity, whilst peak II does not (see section 7.3.3.2. below). Without preincubation there was a plateau of prolidase activity at 120-140 mM NaCl representing peak I rising to a new plateau at about 250 mM NaCl, the difference representing peak II (Fig. 44). On preincubation with manganese, prolidase activity increased to a new level at 120-130 mM NaCl representing peak I activation which was also reflected in the increased level at 250 mM NaCl. A final concentration of about 120-130 mM NaCl would, therefore, separate peak I from peak II and 250 mM NaCl liberate both peaks. By the sequential use of 120 and 250 mM NaCl the two peaks can be obtained separately. As the recovery of prolidase activity was greater than 95%, the estimation of the two peaks would be more reliable with the batch than the column method. Following separation by the DEAE-Cellulose batch method the two peaks could be mixed together to give the

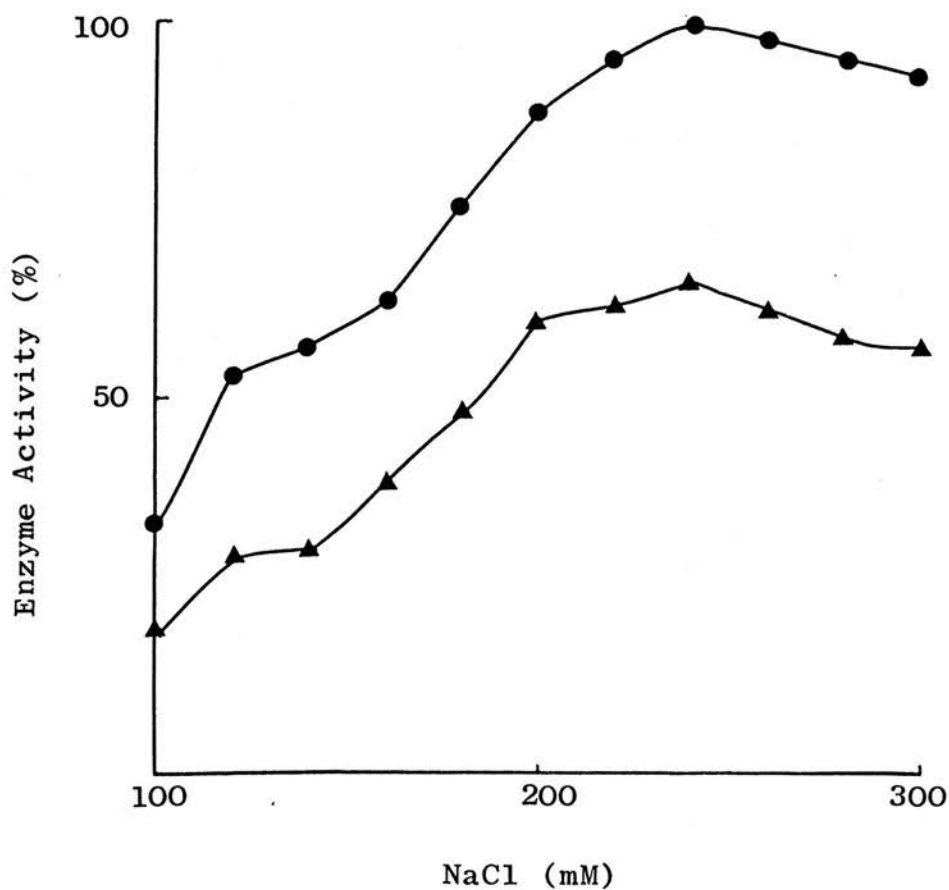


Fig. 44 Activity of fibroblast prolidase assayed against Phe-Pro unbound to DEAE-Cellulose in the batch method at different final concentrations of NaCl. (▲) no preincubation, $MnCl_2$ added with substrate. (●) preincubation with 1 mM $MnCl_2$ for 5 min.

expected prolidase activity (Fig. 45), indicating no interference with each other in the assay.

The reproducibility of the batch method, as checked by estimating the two peaks ten times for one cerebral frontal cortex extract, was excellent with the coefficient of variation for peak I being 2% and for peaks I and II 3%, the same as the 2-3% for the prolidase assay. The specific activities against Phe-Pro and the percentage of peak I determined by the batch method for a number of cells and tissues are given in Table 12. A 2% (w/v) extract was needed for tissues except kidney and pancreas (1% w/v), and about 1 mg protein for red and white blood cells, fibroblasts and amniotic fluid cells. An assay time of 20 min was required for all the enzyme sources except for red blood cells (30 min). The reduced requirement for material enabled white blood cells to be analysed and showed moderate enzyme activity with about equal amounts of the two peaks, making them a useful source of prolidase for analysis of the two peaks in the disease state. The specific activity and percentage of the two peaks for fibroblasts and amniotic fluid cells were similar, indicating that a prolidase abnormality detected in skin fibroblasts should also be present in amniotic fluid cells. The two cases had 16% and 11% peak I activity which was higher than indicated by the DEAE-Cellulose column method. The greater loss of

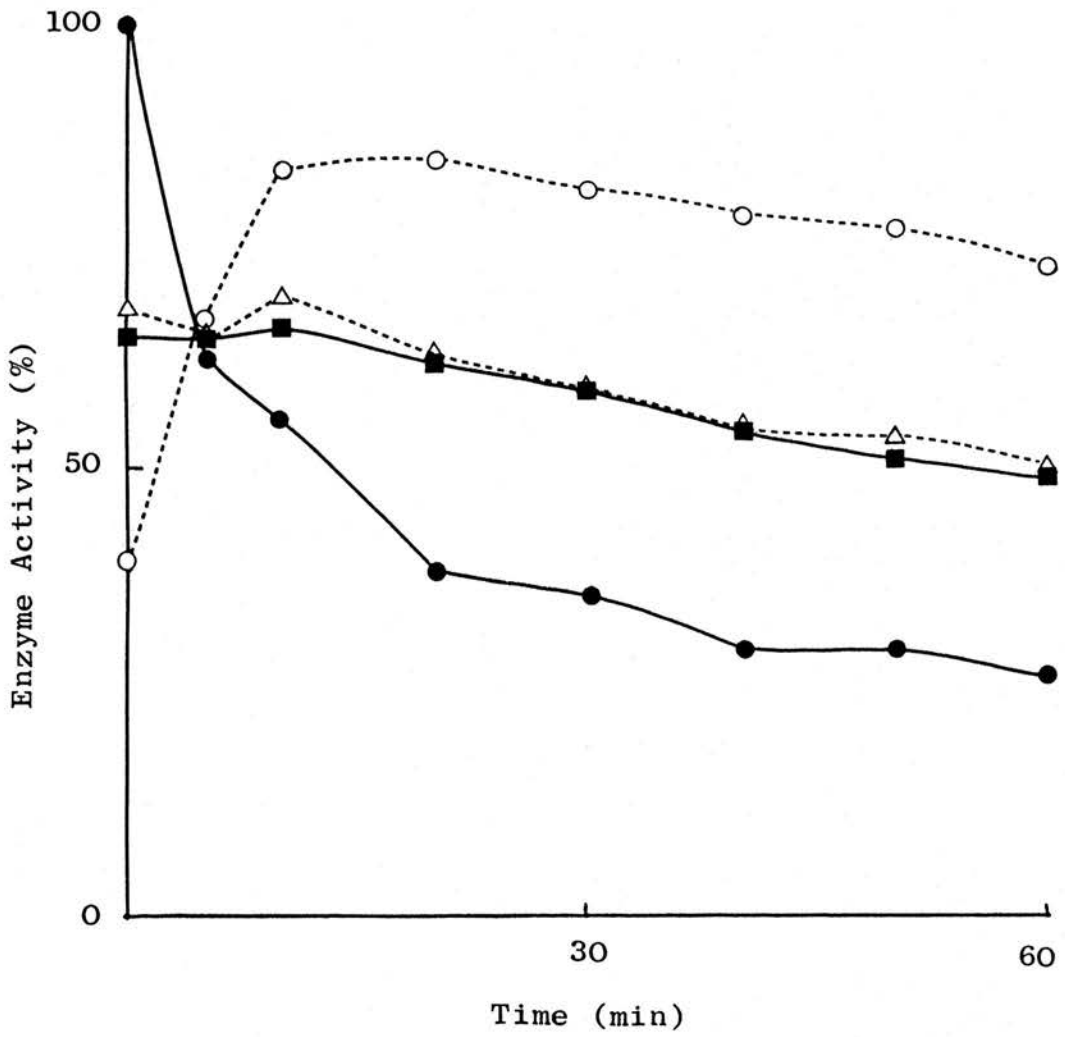


Fig. 45 Fibroblast prolidase activity against Phe-Pro after preincubation for up to 60 min with 1 mM $MnCl_2$ for peak I (O), peak II (●) and peak I +II estimated experimentally (■) and theoretically (Δ) on an equal activity basis after 5 min preincubation.

Sample	Specific Activity	%age Peak I
Fibroblasts		
Controls (n=10)	100 \pm 8 [†]	53 \pm 4
Case 1	42	16
Case 2	57	11
Amniotic fluid cells		
(n=10)	108 \pm 15	55 \pm 3
Red blood cells	5.7, 6.1, 6.7	68, 73
White blood cells	54, 58, 64, 66	42, 42, 44, 50
Kidney	190, 213, 236, 291	60, 68, 73
Liver	56, 63	77, 77
Spleen	57, 71	68, 74
Pancreas	235, 287	24, 34
Cerebral frontal cortex		
(n=10)	108 \pm 16	40 \pm 4
Heart	64, 77	53, 61
Skeletal muscle	55	50

Table 12. Cell and tissue prolidase activity against Phe-Pro* and percentage peak I estimated by the DEAE-Cellulose batch method.

* nmol/min/mg protein

† Mean \pm SD

enzyme in the column method may have resulted in an underestimate of abnormal peak I.

The DEAE-Cellulose batch method allowed the rapid preparation of the two peaks with excellent recovery permitting analysis of both peaks in the disease, as shown for case 1 in Table 13. Control peak I hydrolysed Ala-Pro and particularly Gly-Pro at a faster rate than peak II. In case 1, peak I residual activity against all five substrates was low compared to that of the control. Peak II residual and relative activities in case 1 were similar to those of the control, except that both these parameters were reduced to about 10% for Gly-Pro.

Substrate	CONTROL		CASE 1	
	Peak I Activity* Relative (%)	Peak II Relative (%)	Peak I Activity Relative (%)	Peak II Relative (%)
Phe-Pro	4.5 100	3.2 100	0.40 100 (9)	3.6 100 (113)
Val-Pro	3.1 69	2.6 81	0.11 28 (4)	2.5 69 (96)
Leu-Pro	3.3 73	3.0 94	0.26 65 (8)	3.4 95 (113)
Ala-Pro	9.4 211	4.3 135	0.16 40 (2)	4.3 119 (100)
Gly-Pro	16.4 365	3.4 106	0.46 115 (3)	0.34 9 (10)

Table 13. Activity, relative and residual percentages of prolidase peaks from control and case 1 prolidase-deficient fibroblasts separated by the DEAE-Cellulose batch method (residual activity shown in parentheses).

* nmol/min; peak I preincubated for 30 min with 1 mM MnCl₂; peak II no preincubation; Assays with Gly-Pro were preincubated for 60 min.

† Activity against substrate relative to Phe-Pro.

A protein concentration of 4 mg/ml was used for the estimation of the prolidase peaks in the batch method (section 7.2.1.2.).

7.3.3. Properties

A number of properties of peaks I and II prolidase activity of control and prolidase-deficient fibroblasts were studied. When compared, the results were the same for the peaks separated by both the DEAE-Cellulose column and batch methods.

7.3.3.1. pH profile

The pH optimum of control and abnormal prolidase activity against Phe-Pro was pH 8.0 following preincubation with 1 mM $MnCl_2$ (Fig. 46). Analysis of control peak I showed that without preincubation with manganese, the activity decreased with increasing pH, but that activation after preincubation with manganese increased with pH and an optimum of pH 8.0 was obtained. Although peak II also had a pH optimum of 8.0, higher activity was seen without preincubation. The pH profile of an extract would therefore depend upon the proportions of the two peaks and the assay conditions.

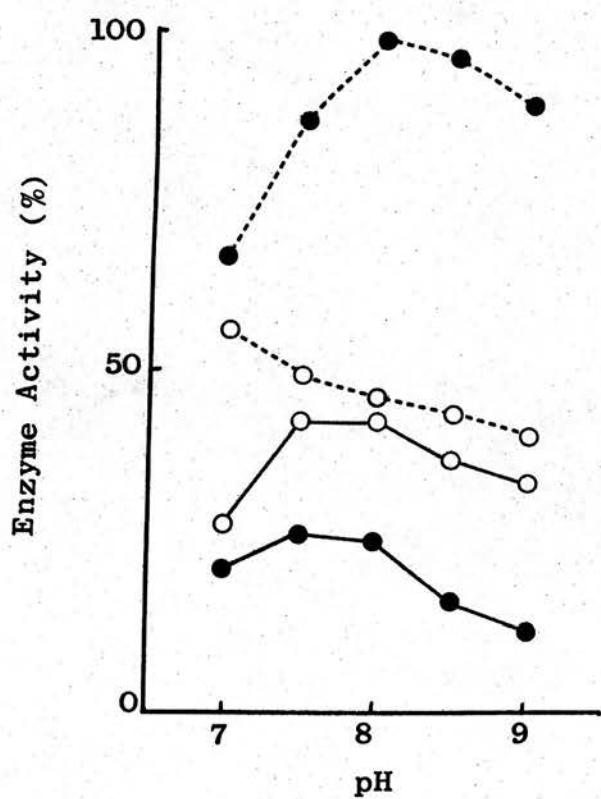


Fig. 46 pH profile of control fibroblast peak I (-----) and peak II (—) without (○) or with (●) preincubation for 30 min with 1 mM MnCl₂. Phe-Pro was used as substrate.

7.3.3.2. Manganese

Control peak I activity towards Phe-Pro was optimal following 10-20 min preincubation at 37°C with 1 mM manganese but was reduced by preincubation without manganese (Fig. 47A, B). In contrast, control peak II was inactivated by preincubation with manganese (Fig. 47A) and optimal activity was obtained by the addition of 0.5-1.0 mM $MnCl_2$ with the substrate (Fig. 47B). The two abnormal peaks had the same responses, except that peak II was even more unstable in the presence of manganese.

Control peaks I and II both needed preincubation with 5 mM $MnCl_2$ for 60 min for full activity against Gly-Pro (Fig. 47C). The very small amount of peak I in the cases also needed this level for optimal activity, whilst Peak II needed only 0.1 mM $MnCl_2$ (Fig. 47C)

As a consequence of these different effects of manganese on the two peaks of prolidase, the specific activity and proportions of the peaks of a particular cell-type or tissue will depend upon the conditions. In practise, estimates obtained after 5 min preincubation were not very different to values for optimised conditions.

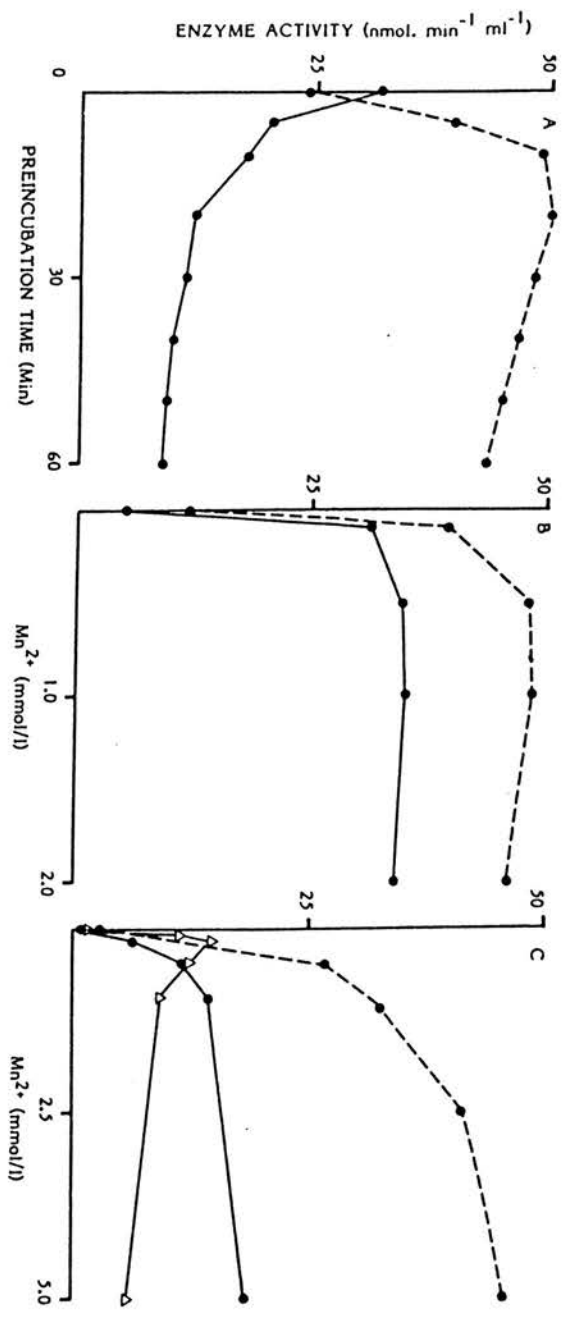


Fig. 47 Effect of Mn²⁺ on control (●) and abnormal (Δ) peak I (-----) and peak II (—) prolidase activity. A Phe-Pro preincubated with 1mM MnCl₂ for up to 60 min. B Phe-Pro: peak I preincubated 20 min with Mn²⁺; peak II Mn²⁺ added with substrate. C Gly-Pro: preincubated 60 min with MnCl₂.

7.3.3.3. Heat stability

At 48°C, loss of control peak I activity against Phe-Pro was completely prevented by the presence of 1 mM MnCl_2 , whereas control peak II activity was only partially protected. However, abnormal peak II was very labile in case 1 whether or not manganese was present, whilst in case 2 the activity was more stable in the absence of manganese and more labile in its presence than control peak II (Fig. 48). As indicated above, similar but smaller changes were seen at 37°C.

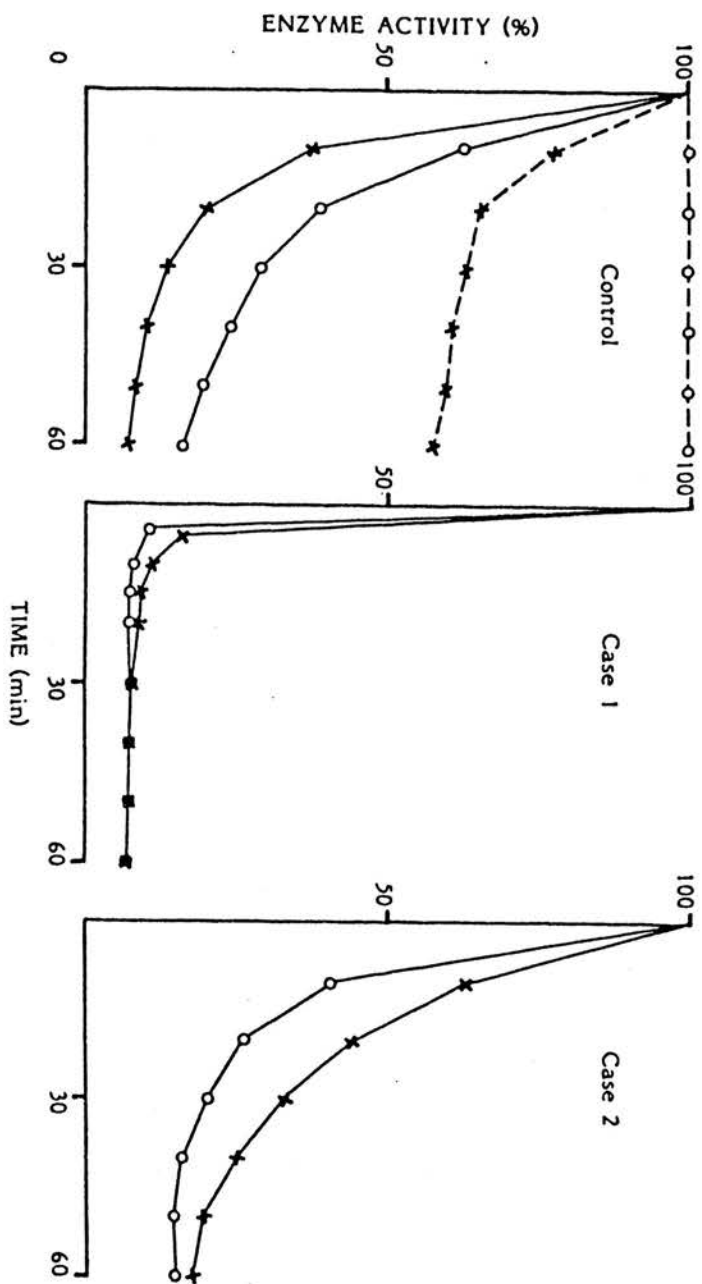


Fig. 48 Effect of heat on control, case 1 and case 2 fibroblast prolidase peak I (---) and peak II (—) in the presence (o) or absence (x) of 1 mM $MnCl_2$ and assayed with Phe-Pro.

7.3.3.4. Effect of PHMB

Control peak I preincubated for 30 min with 1 mM MnCl_2 for Phe-Pro and 60 min for Gly-Pro lost about 20% of activity against both substrates when treated with 0.05 mM PHMB (Fig. 49). Control peak II against Phe-Pro, which doesn't require preincubation with manganese, was inhibited 90% with 0.05 mM PHMB, whilst the activity against Gly-Pro, which does require preincubation, was only inhibited 50% following preincubation with 1 mM MnCl_2 for 60 min. However, peak II activity in both cases against Phe-Pro and Gly-Pro was completely abolished by 0.05 mM PHMB irrespective of the conditions.

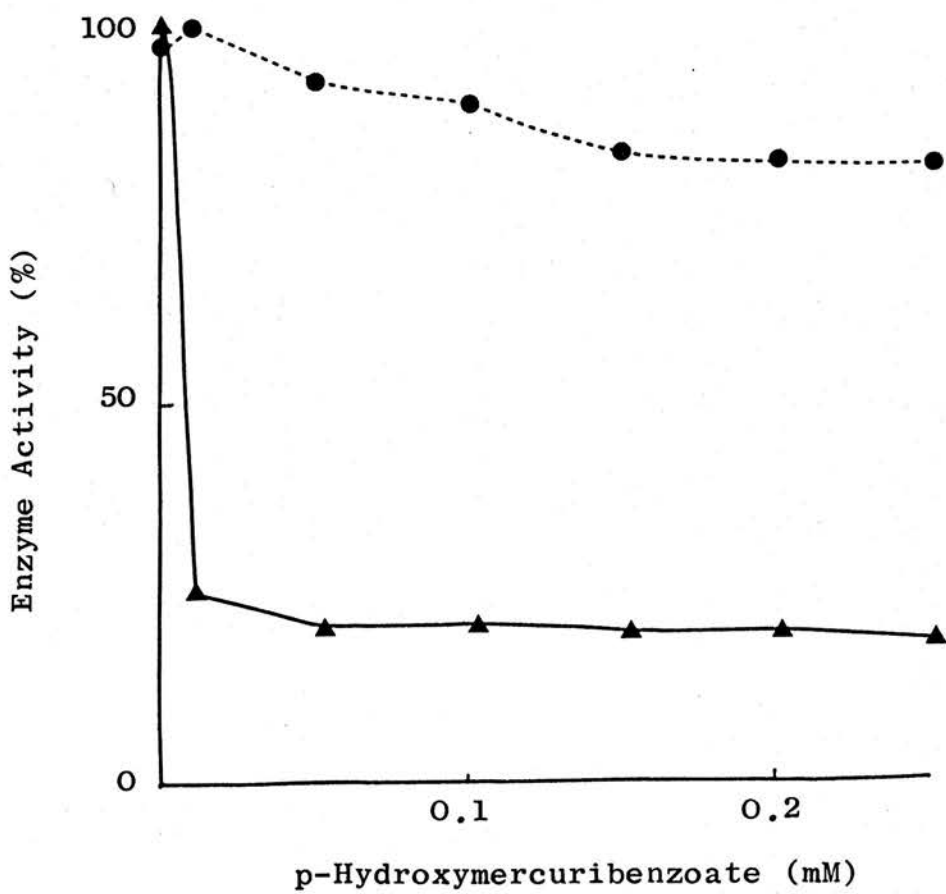


Fig. 49 Effect of different levels of PHMB on skin fibroblast prolidase peak I (●--●) and peak II (▲—▲) assayed with Phe-Pro after 5 min preincubation with 1 mM $MnCl_2$.

7.3.3.5. Substrate kinetics

The substrate kinetics of normal and abnormal prolidase for Gly-Pro and Phe-Pro were analysed by Eadie-Hofstee plots and the K_m values given in Table 14. The estimates of data variation (SD(E)Rad; Zivin and Waud, 1982) for both substrates was less than 0.07, indicating acceptable results. There was insufficient abnormal peak I activity for the reliable estimation of K_m values. The results indicate that peak II prolidase in the cases had decreased affinities for both Gly-Pro and Phe-Pro.

Substrate	Source	Preincubation (min)	MnCl ₂ (mM)	K_m
Gly-Pro	Control extract	5	5	2.5
	Control extract	60	5	9.0
	Control peak I	60	5	17.0
	Control peak II	60	5	7.0
	Case 2 peak II	60	5	33.0
Phe-Pro	Control peak I	30	1	0.5
	Control peak II	0	1	8.0
	Case 1 peak II	0	1	16.0
	Case 2 peak II	0	1	36.0

Table 14. K_m values of control and abnormal skin fibroblast prolidase.

7.4. DISCUSSION

7.4.1. DEAE-Cellulose

The finding of a second form of prolidase explains the presence of moderate prolidase activity in prolidase-deficient fibroblasts (6.3.1.). The pH optimum of both peaks against Phe-Pro was about 8.0 in agreement with the value for a fibroblast extract (5.3.1.) and similar to pH 7.8 for erythrocytes against Gly-Pro (Myara et al., 1984a). The isolated erythrocyte enzyme (Endo et al., 1982) is equivalent to the present peak I and showed comparable relative activities for Gly-Pro, Ala-Pro and Leu-Pro. Previous investigations of prolidase (Endo et al., 1982; Myara et al., 1984a; section 6.3.5.) would not have picked up peak II for a number of reasons: erythrocytes have little peak II and dialysis inactivates peak II, as does the use of high manganese levels and prolonged preincubation. That dithiothreitol prevents the loss of peak II activity on dialysis, indicates a need to preserve SH groups on the enzyme during dialysis.

The results with the batch method were in reasonable agreement with the column chromatography data, with erythrocytes, kidney, liver and spleen having more peak I and pancreas more peak II. The high level of peak II in the pancreas indicates a

particularly important role for this enzyme in the pancreas.

7.4.2. Substrate specificity

The previous finding that the enzyme in prolidase-deficient fibroblast extracts showed changes in the rate of hydrolysis of a number of substrates (section 6.3.1.) can be explained by the changed rate at which both peaks hydrolyse the various substrates. Indeed, the much reduced ability of abnormal peak II to hydrolyse Gly-Pro as compared with the other substrates explains why only activity against Gly-Pro showed a marked deficiency in the disease fibroblasts (6.3.1.). Reports showing residual activity against Gly-Pro in the disease (Gray et al., 1983; Pedersen et al., 1983; Naughten et al., 1984) can be attributed to the use of assay conditions capable of detecting peak II activity. That control and abnormal fibroblast prolidase have different K_m values for a number of substrates (Table 9) can be partially explained by the loss of peak I activity, as the two peaks have different K_m values, and by changes in the abnormal peak II values.

7.4.3. Manganese

A wide variety of manganese conditions have been used for the assay of prolidase against Gly-Pro (section 5.4.2.). Although optimal activity against Gly-Pro for both control peaks can be obtained under the same conditions, abnormal prolidase needed a much lower level of manganese (section 5.3.3.). This can be explained by the loss of peak I together with a reduction in the manganese required by peak II. As no one combination of manganese and preincubation time can give optimal activity against Phe-Pro for both peaks simultaneously, the conditions and response for an extract represent a compromise between the requirements for the two peaks. Hence the failure of prolidase-deficient fibroblast activity against Phe-Pro to be increased by preincubation with manganese can be explained by the loss of manganese-activated peak I in the disease.

7.4.4. Heat stability

Stabilisation of control fibroblast prolidase activity to heat by manganese (section 6.3.2.) is due to complete protection of peak I and partial protection of peak II. Although in abnormal fibroblasts an absence of peak I would result in a lower level of protection by manganese, abnormal peak II is more

labile in the presence of manganese giving an even greater reduction in prolidase activity on heat treatment.

7.4.5. Inhibition by PHMB

Protection of human fibroblast prolidase peak I activity by manganese against PHMB inhibition suggests that manganese ions bind to prolidase via sulphhydryl groups, as found for swine kidney prolidase (Davis and Smith, 1953). The inability of manganese to protect peak II activity and the need to add manganese with the substrate indicates that at least part of the manganese effect on peak II activity involves interaction with the substrate. The loss of peak I explains the failure of manganese to protect prolidase-deficient fibroblast activity against PHMB (section 6.3.4.).

7.4.6. Implications

The finding of a second form of prolidase explains the presence of a moderate prolidase activity for a number of substrates in prolidase-deficient cells (section 6.3.1.). The differences in the properties of the two prolidases and their changes in the disease mean that particular attention has to be paid to their estimation. This can be achieved most easily by the use of the DEAE-Cellulose batch method followed by

appropriate assay conditions. Alteration of both prolidases in prolidase deficiency indicates a structural relationship. As human prolidase (peak I) is known to consist of two subunits (Endo et al., 1982), the most likely explanation is a common subunit which is changed in the disease such as to alter substrate hydrolysis.

8. PURIFICATION OF HUMAN KIDNEY PROLIDASE AND RAISING OF SPECIFIC ANTISERA IN RABBITS

8.1. INTRODUCTION

Although prolidase has been purified from several animal tissues (Davis and Smith, 1957; Baksi and Radhakrishnan, 1974; Hui and Lajtha, 1980; Browne and O'Cuinn, 1983; Yoshimoto et al., 1983) and bacteria (Ryden, 1971; Kaminogawa et al., 1984), the human enzyme has only been purified from erythrocytes (Adams and Smith, 1952; Endo et al., 1981). As erythrocytes have very low prolidase activity (Table 12, section 7.3.2.), prolidase was purified from human kidney which had the highest activity of the range of tissues in Table 12. Purified prolidase was injected into rabbits to raise specific antisera, which were used to study the enzyme in control and prolidase-deficient fibroblasts.

8.2. METHODS

8.2.1. Extract preparation

Human kidneys were obtained at autopsy 24 hr after death and stored at -40°C . A 20% (w/v) extract was prepared by homogenising 1 Kg kidney in 4.5l 10 mM barbital / HCl buffer pH 7.4 containing 1 mM MnCl_2 in a Waring blender. The homogenate was then centrifuged at 23,000 x g for 2 hr and the supernatant stored at -40°C . The pellet was resuspended in 1.0 l buffer, disrupted further in a Silversen homogeniser and stored at -40°C overnight. This preparation and the original supernatant were then thawed and centrifuged at 23,000 x g for 2 hr.

8.2.2. Chromatographic procedures

All chromatography was performed at 4°C unless otherwise stated. Column fractions were diluted appropriately with bovine serum albumin (0.25 mg/ml) in 10 mM barbital / HCl buffer pH 7.4 containing 50 mM NaCl prior to assay of prolidase activity against Phe-Pro.

8.2.2.1. DEAE-Cellulose

4.5l kidney extract was applied to a 10 x 25 cm

DEAE-Cellulose column equilibrated with 10 mM barbital / HCl buffer pH 7.4. After washing with 5l of the same buffer containing 50 mM NaCl (buffer A), the enzyme was eluted with 4.5l buffer containing 0.25M NaCl and 250 ml fractions collected.

1.62 Kg $(\text{NH}_4)_2\text{SO}_4$ was added to the pooled enzyme peak (3.75l) to bring it to 60% saturation and left for 24 hr at 4°C to precipitate. The precipitate was removed by centrifugation at 18,000 x g for 2 hr and then dialysed against 4 x 5l buffer A. The dialysate (580 ml) was then applied to a 4.5 x 75 cm DEAE-Cellulose column equilibrated with buffer A. After washing with 2.5l of the same buffer, the column was eluted with 3l of a 50-500 mM NaCl linear gradient and 10 ml fractions collected.

8.2.2.2. Concanavalin-A Sepharose

3 ml buffer A containing 100 mM MnCl_2 , MgCl_2 and CaCl_2 was added to 300 ml of the pooled DEAE-Cellulose enzyme peak and applied to a 2 x 35 cm Concanavalin-A Sepharose column equilibrated with buffer A containing the above salts at a concentration of 1 mM. The enzyme passed straight through the column and 10 ml fractions collected.

8.2.2.3. Metal-ion-chelate chromatography

For metal-chelate chromatography a Ni^{2+} gel was prepared as previously described (section 4.2.3.3.). 300 ml of the eluate from the Concanavilin-A column was applied to a 1.4 x 50 cm column equilibrated with buffer A. After washing with 200ml of the same buffer, the column was eluted with a 0-150 mM Imidazole linear gradient in buffer A and 4.75 ml fractions collected.

8.2.2.4. Hydroxylapatite chromatography

The prolidase peak (60 ml) from the nickel-chelate gel was applied to a 2.4 x 35 cm hydroxylapatite column equilibrated with buffer A. The column was washed with 100 ml buffer A, eluted with 200 ml of a 0-50 mM sodium phosphate linear gradient followed by 50 ml 50 mM sodium phosphate and 5 ml fractions collected.

8.2.2.5. Gel filtration

The hydroxylapatite peak (75 ml) was concentrated five-fold using a PM 10 ultrafiltration membrane system, applied to a 2.5 x 90 cm Ultrogel AcA 44 column and eluted with buffer A at an ascending flow-rate of 26 ml/hr and 5 ml fractions collected.

8.2.2.6. Hydrophobic chromatography

The prolidase peak (47 ml) after gel filtration was applied to a 1 x 25 cm ω -aminohexyl-agarose (Miles Ltd) column equilibrated with 10 mM barbital / HCl pH 7.4 containing 10 mM NaCl. After washing with 100 ml buffer the column was eluted with 70 ml of a 10-300 mM NaCl linear gradient and 1.6 ml fractions collected.

8.2.2.7. Preparative polyacrylamide gel electrophoresis

The prolidase peak (19.5 ml) after hydrophobic chromatography was concentrated ten-fold in the PM10 ultrafiltration membrane system. 0.5 ml electrophoresis sample buffer (2% bromophenol blue in 10% (w/v) sucrose) was added to 1.5 ml of the concentrated enzyme peak and applied to the surface in a preparative polyacrylamide gel electrophoresis apparatus (Shandon Ltd) set up as described by Smith (1976). The acrylamide to bis-acrylamide ratio was 30:0.8% (w/w) and the gel concentration was 7.5%. Electrophoresis was carried out at 30 mA for 60 min followed by 80 mA for 6 hr, collecting 1.9 ml fractions. The fractions containing the enzyme were pooled (9.5 ml) and used for studying its properties and for raising antibodies in rabbits.

8.2.3. Properties of purified prolidase

8.2.3.1. Electrophoresis

The purified enzyme was studied by analytical disc gel electrophoresis in gel rods (Davis, 1964) to assess the purity of the final preparation. Rods were stained with Coomassie blue and also cut into slices for estimating enzyme activity. The molecular weight of prolidase subunits was determined under denaturing conditions by SDS PAGE, performed by the method of Weber and Osborn (1969) in a Bio-Rad Protean slab electrophoresis cell. The stacking and separating gels were 5.5% and 12% acrylamide respectively and the buffer system as described by Laemmli (1970). Purified prolidase, Dalton Mark VII-L molecular weight standards (Sigma Ltd) and samples from various stages of the purification were mixed with sample buffer, heated at 95°C for 5 min, and electrophoresed at 10 mA for 60 min followed by 15 mA for 3 hr. The gel was then transferred to fixing solution (40% (v/v) methanol, 5% (v/v) acetic acid) before staining with 0.25% (w/v) Coomassie blue in this solution. After destaining, the gel was laid flat on a sheet of filter paper (Whatman 3 mm) and dried in a Bio-Rad heated slab gel drier under reduced pressure.

8.2.3.2. Specific activity

The specific activity of the purified preparation against Gly-Pro, Phe-Pro, Ala-Pro, Val-Pro and Met-Pro (20 mM final in the assay) was determined with 60 min preincubation (5 mM MnCl_2) for Gly-Pro and 20 min preincubation (1 mM MnCl_2) for the other substrates. Liberated proline was estimated by the ninhydrin method described in section 5.2.1.1.

8.2.3.3. Manganese

The manganese requirements for purified prolidase were studied for the hydrolysis of Gly-Pro and Phe-Pro. The enzyme was preincubated for 0, 10, 30 and 60 min with 0, 0.1, 1.0, 2.5 and 5.0 mM MnCl_2 . The manganese concentration during the preincubation was maintained in the assay by adding an appropriate level of MnCl_2 with the substrate. A 1/500 dilution of the purified enzyme was used for Gly-Pro and a 1/100 dilution for Phe-Pro.

8.2.3.4. Substrate Kinetics

Substrate kinetics of purified prolidase with Gly-Pro after 60 min preincubation with 5 mM MnCl_2 and for Phe-Pro after 30 min preincubation with 1 mM MnCl_2 were studied over the range 1-20 mM substrate. Kms

were calculated from Lineweaver and Burk plots (1934).

8.2.4. Immunological studies

8.2.4.1. Immunisation of rabbits

Antisera to the purified prolidase were raised in two male New Zealand white rabbits, 1 year old, fed on a normal diet. The animals were bled prior to immunisation to obtain samples of pre-immune serum. Purified prolidase was mixed with an equal amount of Freund's complete adjuvant (Behring Ltd), sonicated at 4-6 μ m for 30 sec and injected intradermally into the rabbits at multiple sites on the back (approximately 10 μ g pure enzyme per site). Injections were repeated at 2 and 4 weeks, using Freund's incomplete adjuvant for the booster at 4 weeks. A week later blood was taken from the ear vein of the animals and the serum tested for immunoreactivity with pure and crude prolidase preparations. A further booster was injected after 7 weeks and 10 days later blood was taken by cardiac puncture under anaesthesia. Antisera from the rabbits was stored at -40°C. Immunoreactivity was checked by immunotitration of successive dilutions of the antisera with the purified enzyme.

8.2.4.2. SDS-Polyacrylamide gel electrophoresis

Extracts of control fibroblasts, brain, kidney and the purified enzyme were immunoprecipitated prior to SDS-PAGE and Western blotting. 175 μ l immunoprecipitation buffer (0.4M Tris / HCl pH 7.4 containing 4% Triton-X-100 and 1.6 M KCl) and 25 μ l antiserum or pre-immune serum was added to 0.5 ml of sample and left at 4°C overnight to precipitate. The mixture was then centrifuged at 8,800 x g for 15 min (Eppendorf 5413 centrifuge), washed twice with 10 mM phosphate buffer pH 7.0, given a final wash with acetone and dried under nitrogen. The precipitate was then dissolved in 50 μ l sample buffer (62.5 mM Tris / HCl buffer pH 6.8 containing 2% (w/v) SDS, 10% glycerol, 5% 2-mercaptoethanol and 0.002% bromophenol blue) and heated at 95°C for 5 min. Molecular weight standards were similarly prepared. SDS-PAGE was then performed as described in section 8.2.3.1. with the acrylamide to bis-acrylamide ratio changed to 30:0.312%. After electrophoresis of the immunoprecipitates the gel was removed and prepared for Western blotting.

8.2.4.3. Western Blotting

Western blotting was carried out in an Electroblot Transfer Apparatus (Bio-Rad Ltd) according to the manufacturers instructions. Blotting was performed

overnight at 0.1 A then for 60 min at 0.25 A as described by Towbin et al. (1979). Following blotting, the nitrocellulose was removed and prepared for immunoblotting.

8.2.4.4. Immunoblotting

The nitrocellulose paper was washed in 20 mM Tris / HCl pH 7.5 containing 0.5 M NaCl (TBS) for 10 min. A thin strip containing the molecular weight standards at the edge of the nitrocellulose was cut off and stained with amido black as described by Gershoni and Palade (1983) as follows: The strip was immersed in 0.1% (w/v) amido black in 45% (v/v) methanol / 10% (v/v) acetic acid for 2-3 min, followed by rinsing in distilled water for 30 sec. The strip was destained in 90% (v/v) methanol / 2% (v/v) acetic acid with 3 x 1 min washes, followed by a final rinse in distilled water for 1-2 min and left to dry. The remainder of the nitrocellulose sheet was immersed in 3% (w/v) gelatin in TBS containing 0.05% (v/v) Tween-20 (TTBS) for 60 min to block unfilled sites with protein. After blocking, the nitrocellulose was immersed in 0.5% (v/v) antiserum in antibody buffer (TTBS containing 2% (w/v) gelatin) and agitated for 3 hr at room temperature. The nitrocellulose was then rinsed in distilled water and washed in TTBS for 20 min before transferring to 0.033% (v/v) goat anti-rabbit IgG-peroxidase (Bio-Rad

Ltd) for 60 min with agitation. After rinsing with distilled water, washing with TTBS for 10 min twice and a rinse in TBS, the nitrocellulose was transferred to freshly prepared colour reagent (60 mg chloronaphthol dissolved in 20 ml methanol mixed with 100ml TBS containing 60 μ l ice-cold 30% hydrogen peroxide) and developed for 5-15 min.

8.3. RESULTS

8.3.1. Purification of prolidase

The chromatographic profiles obtained during the purification of prolidase peak I are shown in Fig. 50, the hatched areas represent the fractions pooled for the next chromatographic step. The purification achieved and yield of prolidase at each step are shown in Table 15. As with prolinase, to assay the fractions after each stage of the purification it was necessary to dilute to very low protein concentrations at which the enzyme was unstable. Addition of bovine serum albumin (0.25 mg/ml) stabilised prolidase to dilution.

More than 90% of protein was removed by DEAE-Cellulose including peak II prolidase which may comprise 30% of the enzyme activity in human kidney (Table 12, section 7.3). The Concanavalin-A step was included to specifically remove glycoproteins which accounted for a quarter of the protein in the pooled DEAE-Cellulose fractions. Gel filtration with a calibrated Ultrogel AcA 44 column indicated a molecular weight of 110,000 for the native enzyme. After preparative PAGE, less than 0.005% of the original protein remained and the enzyme had an activity of 225 $\mu\text{mol}/\text{min}/\text{mg}$ protein with Phe-Pro as substrate. The purified prolidase after the final step showed a single band of activity in gel slices after PAGE in rods and a

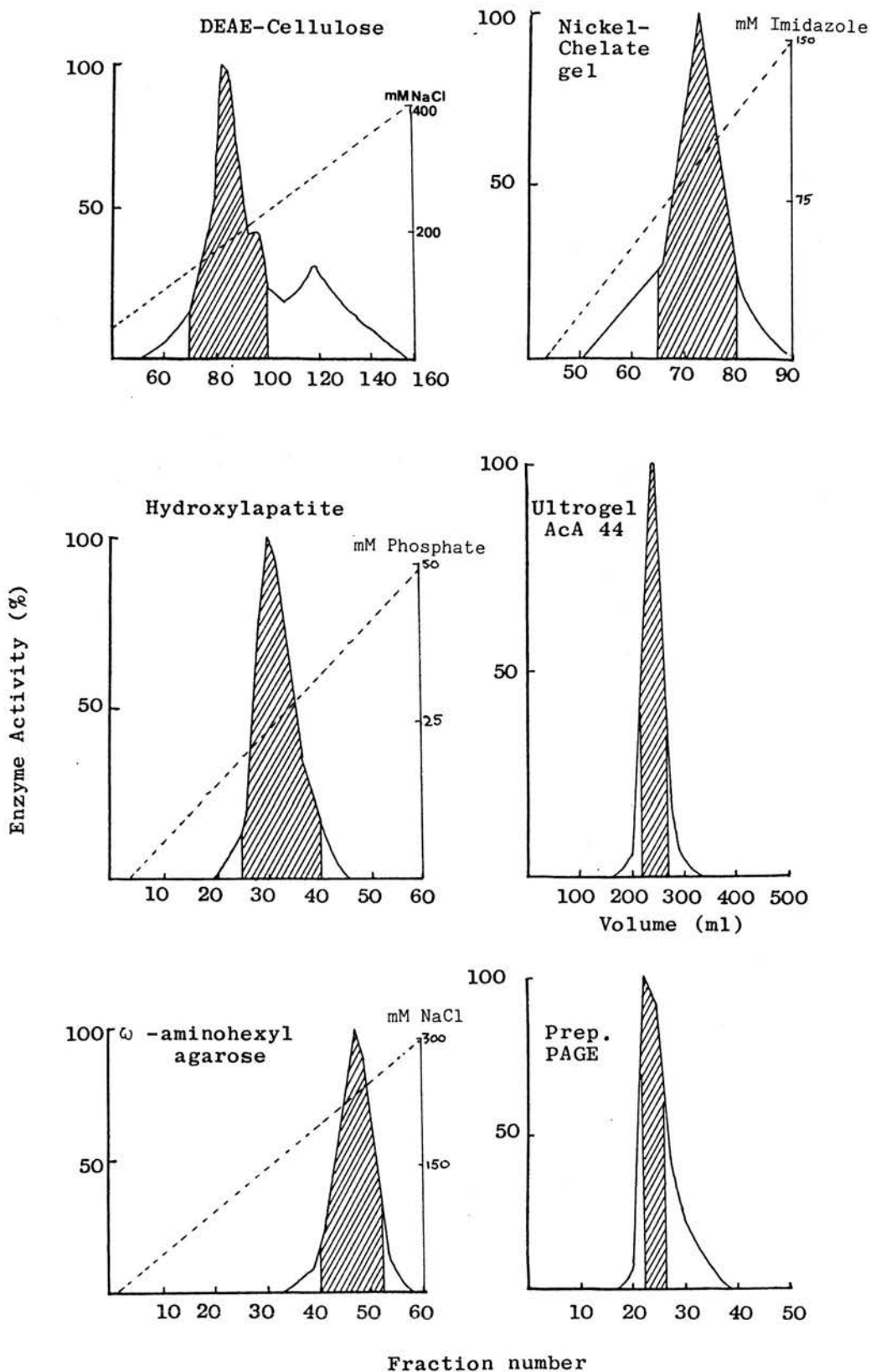


Fig. 50 Chromatographic profiles obtained during the purification of prolidase from human kidney. The hatched areas represent fractions pooled.

single band on staining with Coomassie blue.

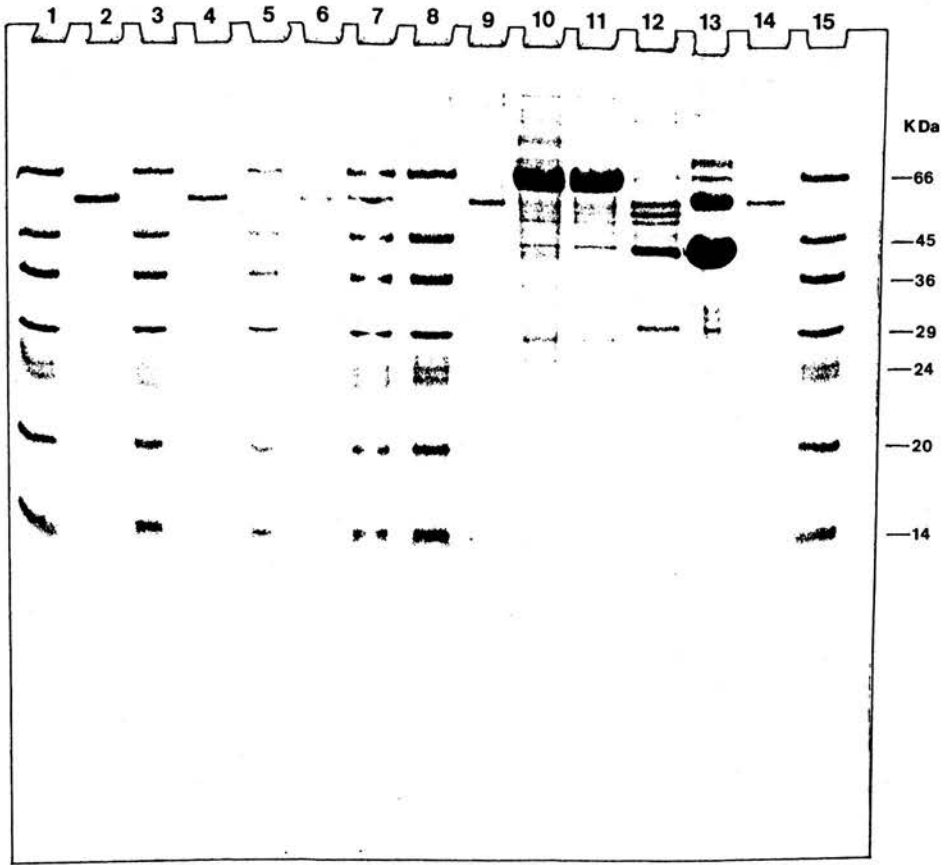
8.3.2. Properties of purified prolidase

The purified enzyme also showed a single band after SDS-PAGE (Fig. 51). The molecular weight of the subunit was calculated to be 55,000, indicating that the native enzyme comprises two identical subunits. Figure 51 also shows electrophoresis of samples from the pooled enzyme peaks after DEAE-Cellulose, Concanavalin-A Sepharose, nickel-chelate chromatography and hydrophobic chromatography. As with crude enzyme preparations, the purified enzyme was optimally active at about pH 8 (Fig. 52). Of five substrates studied, Gly-Pro had the highest specific activity of 921 $\mu\text{mol}/\text{min}/\text{mg}$ protein and Val-Pro was the weakest with about 15% of this activity (Table 16).

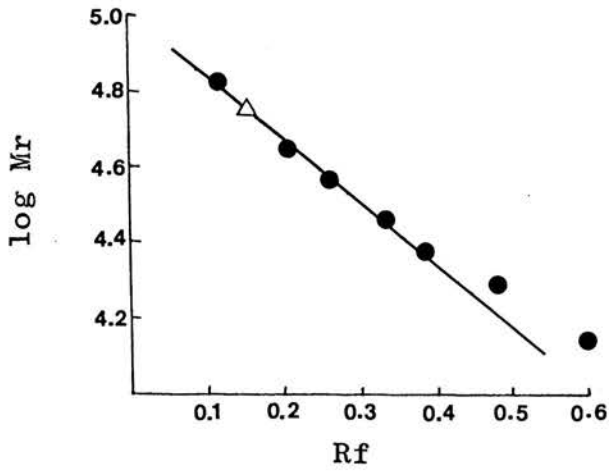
Substrate	Specific Activity $\mu\text{mol}/\text{min}/\text{mg}$	Relative Activity (%)
Gly-Pro	921	100
Ala-Pro	471	51
Phe-Pro	225	24
Leu-Pro	195	21
Val-Pro	142	15

Table 16. Specific activities of purified prolidase against various substrates.

a)



b)



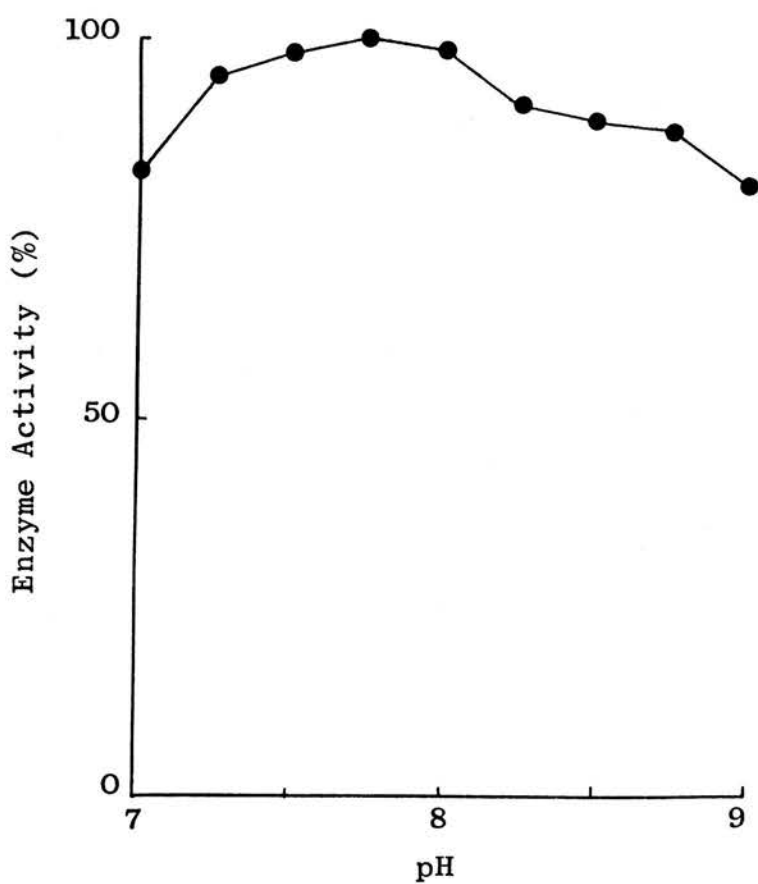


Fig. 52 pH profile for purified prolidase assayed with Phe-Pro in 25 mM Barbital / HCl.

The effect of preincubation with different levels of manganese for Gly-Pro and Phe-Pro is shown in Fig. 53. Activity against Gly-Pro was highest when preincubated with 2.5 mM MnCl_2 for 60 min, though 1 mM and 5 mM MnCl_2 gave similar activities. Phe-Pro activity was highest after 30 min preincubation with 2.5-5 mM MnCl_2 .

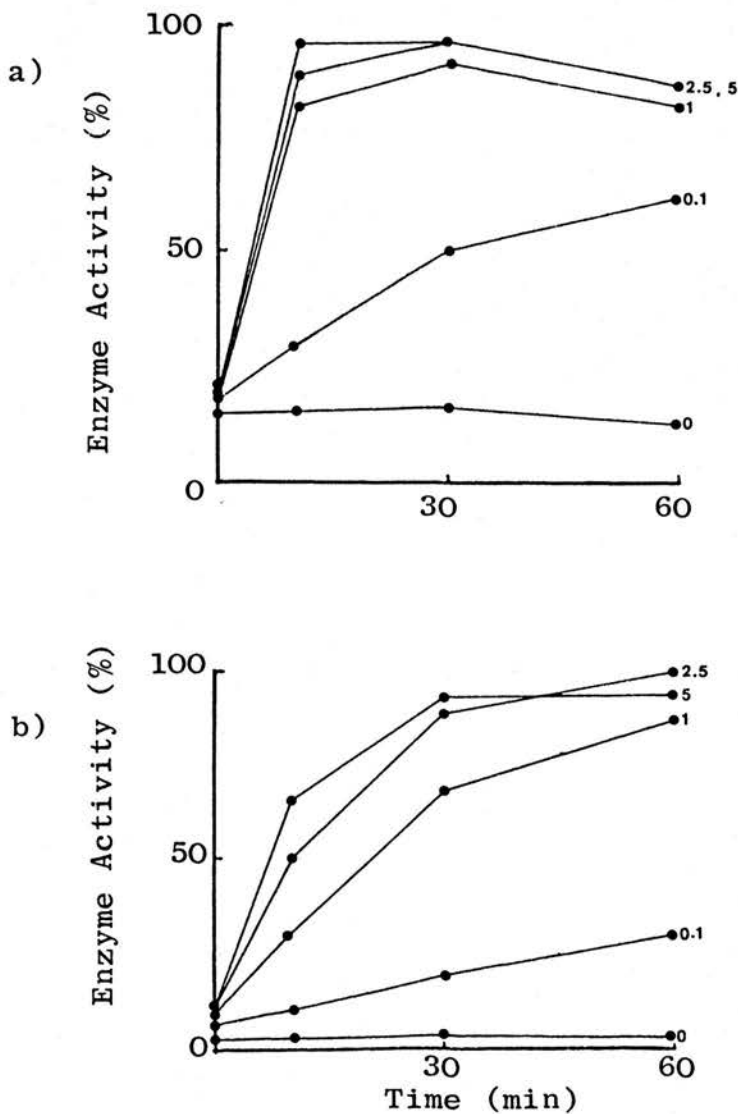


Fig. 53 Preincubation of purified prolidase with different levels of MnCl_2 (mM as shown) for a) Phe-Pro and b) Gly-Pro.

Substrate K_m s for Phe-Pro and Gly-Pro were determined from Lineweaver and Burk plots (Fig. 54). After preincubation of the purified enzyme for 60 min with 5 mM $MnCl_2$, the K_m for Gly-Pro was 22 mM whereas 30 min preincubation with 1 mM $MnCl_2$ resulted in a K_m of 3.1 mM for the hydrolysis of Phe-Pro.

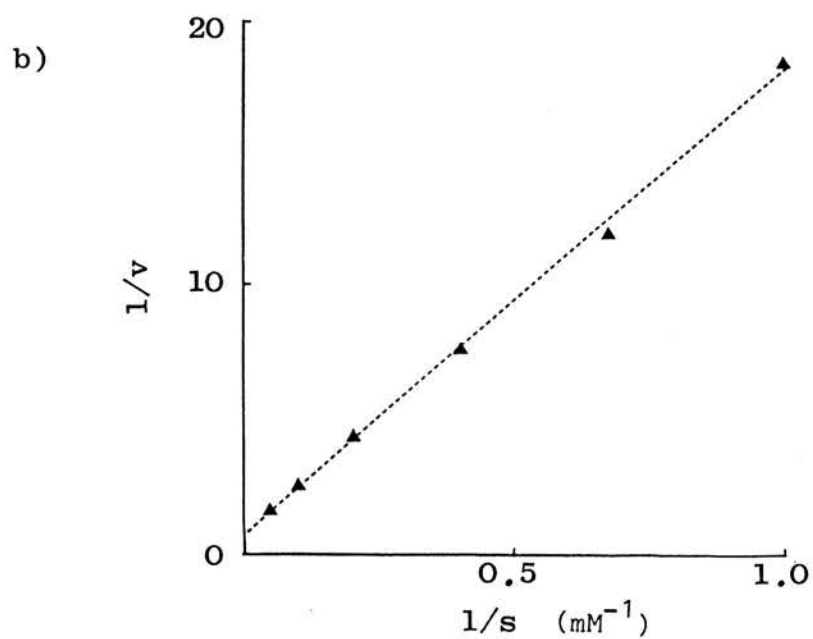
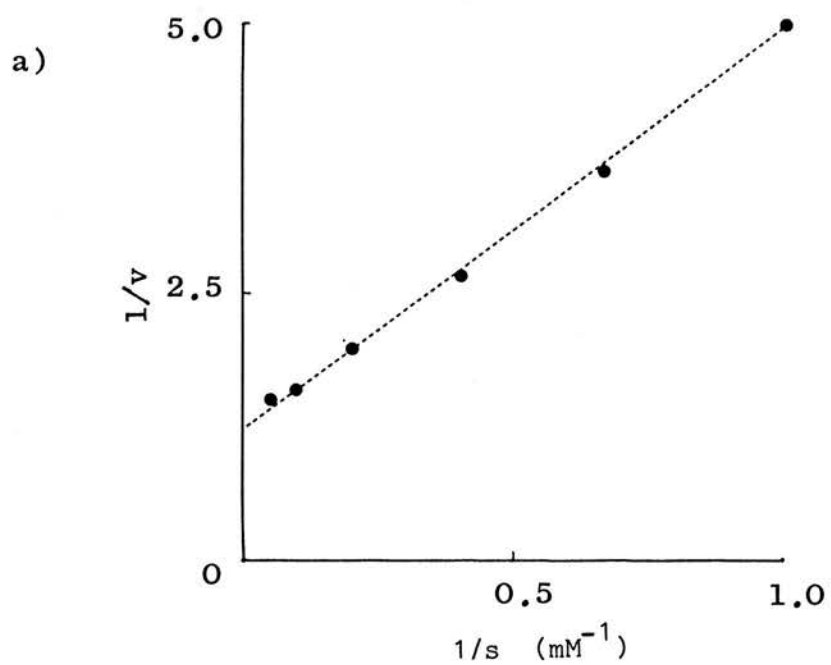


Fig. 53 Lineweaver and Burk plots for purified prolidase assayed with a) Phe-Pro and b) Gly-Pro.

$v = \text{units/ml}$

8.3.3. Immunological studies

8.3.3.1. Immunotitration

The immunoreactivity of the antisera raised in the two rabbits was measured by immunotitration with the purified enzyme and the results shown in Fig. 55. The antisera from the two rabbits showed similar immunotitration curves.

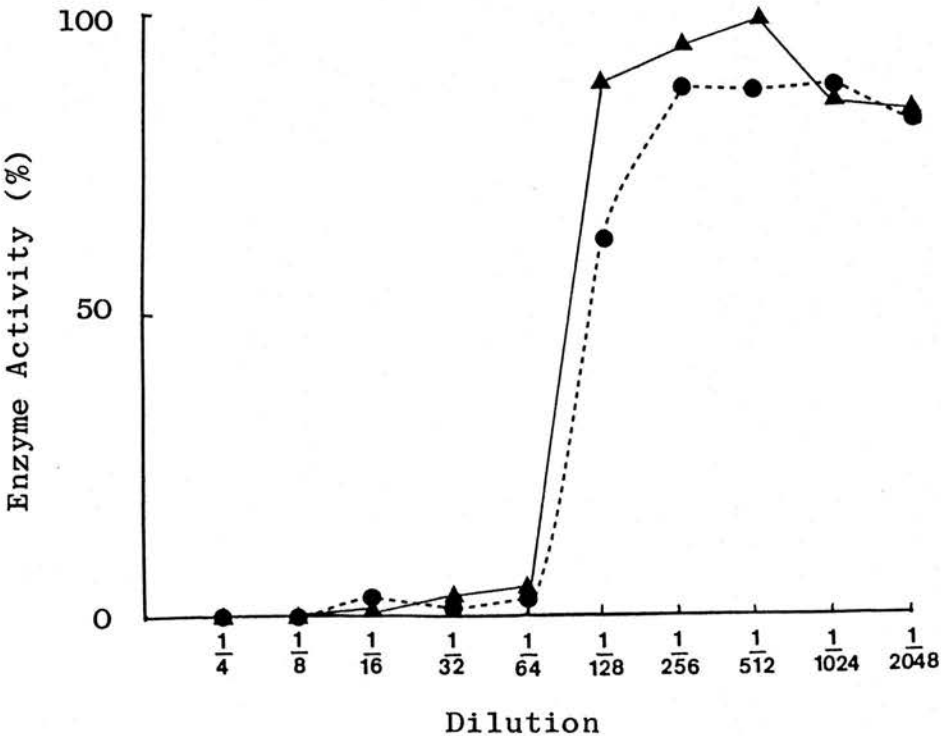
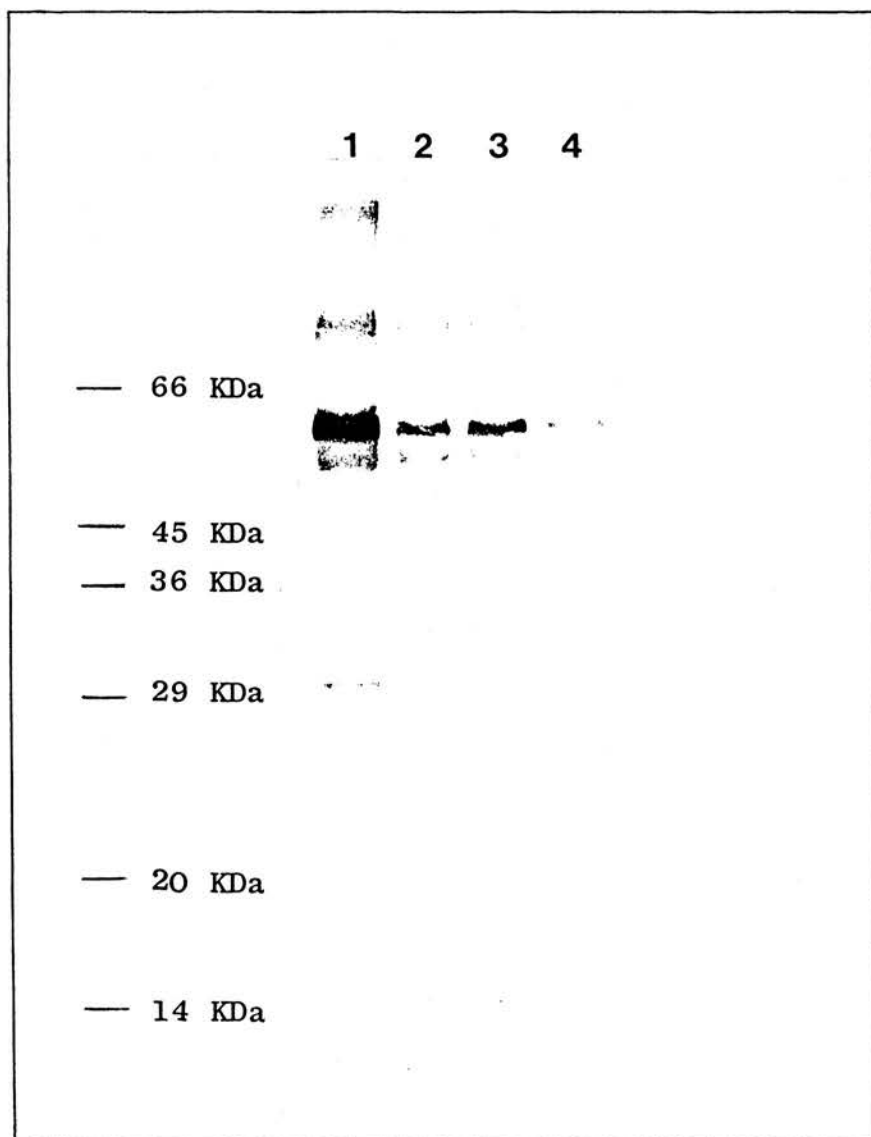


Fig. 55 Immunotitration of purified prolidase. 50 μ l purified prolidase (1/50 dilution in 0.2 M Tris / HCl pH 7.4 containing 2% Triton-X-100 and 0.8 M KCl) was mixed with 50 μ l antiserum at various titres as indicated. After incubation at 4°C overnight the mixtures were centrifuged at 8,800 x g for 15 min and 25 μ l supernatant assayed with Phe-Pro.

8.3.3.2. Immunoblotting

The results for immunoblotting following immunoprecipitation of pure prolidase, kidney, cerebral cortex and fibroblasts are shown in Fig. 56. The major band in each lane was prolidase with a calculated subunit molecular weight of 55,000. The band of approximately 30,000 molecular weight seems to be a contaminating protein which has copurified with prolidase and is highly immunoreactive. However it was not present in enough quantity to be seen in the tissue preparations. The other three bands which can be seen are due to rabbit immunoglobulin and were observed when the first antibody step was omitted from the procedure.

As yet it has not been possible to immunoprecipitate prolidase peak II obtained from skin fibroblasts and pancreas using the DEAE batch method. However, in preliminary studies with prolidase-deficient fibroblasts, it appears that prolidase peak I is present in normal quantities. To establish this will require further studies.



8.4. DISCUSSION

8.4.1. Purification and properties

Prolidase peak I was successfully purified 640-fold from human kidney. As with the purification of prolinase (section 4.3.1.), Ni^{2+} chelate gel chromatography was a highly effective preparative step removing more than 90% of protein with little loss of activity. Although the purification factor was much lower than that achieved for prolidase from human erythrocytes (Endo et al., 1982), the specific activity against Gly-Pro in the present study was four times the activity of the purified erythrocyte enzyme. Indeed this is the highest specific activity obtained for any of the purified preparations from animal tissues previously reported (Sjöström et al., 1973; Hui and Lajtha, 1980; Browne and O'Cuinn, 1983; Yoshimoto et al., 1983). The relative specific activities for the substrates Gly-Pro, Ala-Pro and Leu-Pro (Table 16.) were almost the same as for the purified erythrocyte enzyme (Endo et al., 1982). This contrasts with pig intestinal prolidase which hydrolysed Gly-Pro at less than a third of the rate of Ala-Pro (Sjöström et al., 1973).

SDS polyacrylamide gel electrophoresis showed a single band of molecular weight 55,000, indicating prolidase peak I to be a dimer consisting of two

identical subunits. This is the same as the result for erythrocyte prolidase (Endo et al., 1982) and compares with the subunit molecular weight of 56,000 reported for bovine intestinal prolidase (Yoshimoto et al., 1983). In contrast, the enzyme purified from guinea pig brain was reported to be a dimer comprising two dissimilar subunits of molecular weights 64,000 and 68,000 (Browne and O'Cuinn, 1983).

The optimal pH for purified prolidase activity was between 7.5 and 8.0 which is similar to crude enzyme preparations (sections 5.3.2. and 7.3.3.1.), erythrocyte enzyme (Endo et al., 1982) and the guinea pig brain enzyme (Browne and O'Cuinn, 1983).

Manganese levels and preincubation times required for optimal activity of the purified prolidase against Phe-Pro and Gly-Pro were similar to those found for peak I prolidase in cultured skin fibroblasts (section 7.3.3.2.). Endo et al. (1982) used 16 mM $MnCl_2$ and didn't specify any preincubation which may partly account for the lower specific activity against Gly-Pro with their purified preparation. Prolidase purified from bovine intestine didn't require manganese ions for activation and was inhibited 65% by preincubation with 1 mM $MnCl_2$ for 10 min (Yoshimoto et al., 1983).

The K_m of 22 mM for Gly-Pro was of a similar order to that of 14 mM obtained for the erythrocyte enzyme (King and Kuchel, 1984), but was ten-fold higher than the K_m of 1.9 mM reported for rat brain prolidase

(Middlehurst et al., 1984).

8.4.2. Immunological studies

The subunit molecular weight of 55,000 determined after immunoblotting confirmed the result obtained with SDS polyacrylamide gel electrophoresis.

That prolidase peak II doesn't appear to react with the antisera may imply that peaks I and II do not have a common subunit as proposed earlier (section 7.4.6.). However, if peak II is a dimer like peak I, it may be possible that the second subunit is occluding immunoreactive sites on the common subunit. If there proves to be no common subunit, changes in the properties of peak II prolidase in the prolidase-deficient fibroblasts (section 7.3.) may possibly be a result of a defect in the processing of precursor forms of the enzyme.

The presence of prolidase peak I in cultured skin fibroblasts from the two cases of prolidase deficiency, implies that the primary genetic defect may be causing a change in molecular structure resulting in a distortion of the active site. Using antisera to the erythrocyte enzyme, Endo et al. (unpublished observations) have found the prolidase subunit to be absent in their patient with prolidase deficiency. This seems to be due to a different mutation from the two cases studied here.

At the present time, only preliminary studies have been completed using the antisera raised to purified prolidase. It is hoped that a variety of further studies will elucidate the relationship between the two components of prolidase and the primary defect causing prolidase deficiency.

9. CONCLUSION

9.1. Prolinase and prolidase in human tissues

The initial work for the present study required the development of a reliable, sensitive method for the assay of prolinase. Most previous studies, carried out with animal tissue extracts (Davis and Smith, 1953; Sarid et al., 1962; Mayer and Nordwig, 1973), used Pro-Gly as substrate and were relatively insensitive. A number of methods, based on the reaction of a primary amine with o-phthalaldehyde in the presence of a sulphhydryl reagent, have been developed and used to study peptidases (Reeder et al., 1978; Porter et al., 1982). This reaction was considered to be ideally suited for the assay of prolinase because of the lack of a primary amine on proline dipeptide substrates. On hydrolysis of the substrate the C-terminal amino acid liberated reacts with the o-phthalaldehyde whereas proline is inert. A modification of the method of Roth (1971) was then successfully developed and subsequently used to study prolinase in human tissues. The method also proved to be suitable for the assay of proline iminopeptidase (PIP) and enabled the study and characterisation of this enzyme (Butterworth and Priestman, 1984a). With the use of the new method it was possible to determine optimal assay conditions for

studying human prolinase in both cultured cells and tissues.

An investigation of the physicochemical properties of the enzyme showed it was a soluble dipeptidase activated by manganese ions in common with prolinase in animal tissues (Mayer and Nordwig, 1973; Akrawi and Bailey, 1977). DEAE-Cellulose ion-exchange and gel filtration chromatography showed prolinase to consist of two components of activity, a property unobserved in previous studies.

The coincidence of one component of prolinase with a non-specific dipeptidase, used as a reference enzyme in the chromatographic studies, raised the possibility of proline dipeptides being hydrolysed by a broad specificity dipeptidase. However, the rate of hydrolysis was almost a tenth of that of other dipeptide substrates such as Gly-Leu (section 4.3.3.). Several authors have suggested that proline dipeptide substrates may be hydrolysed by a non-specific dipeptidase (Hayman and Patterson, 1971; Mayer and Nordwig, 1971; Das and Radhakrishnan, 1973) in animal tissue extracts, but no conclusion has yet been established.

Unlike cultured human skin fibroblasts, human kidney had only a single component of prolinase activity and this was the highest of a range of tissues studied (Table 3, section 2.3.4.), so it was decided to purify prolinase from human kidney and study its

properties to determine the specificity of the enzyme. Throughout the purification it was impossible to separate the activities against Pro-Ala and Gly-Leu and the ratio of the activities was constant for each successive separative procedure. The responses of the two activities to heat and inhibition by PHMB were the same and substrate kinetic studies indicated a single enzyme for the hydrolysis of both prolinase and non-specific dipeptidase substrates in human kidney.

Having studied prolinase three methods were developed for assaying prolidase and optimal assay conditions for enzyme activity were determined. The enzyme was then studied in cultured skin fibroblasts from controls and two unrelated cases of prolidase deficiency using a variety of imidodipeptide substrates. The two cases showed a marked loss of activity against Gly-Pro but only moderate reductions in activity against other substrates (section 6.3.1.). A study of the properties of prolidase including heat stability, substrate kinetics and inhibition with PHMB, showed considerable differences between controls and the cases (section 6.3.). Incubation with high levels of manganese inactivated the abnormal enzyme and made it more susceptible to heat inactivation. Optimal manganese levels for control prolidase activity were therefore not optimal for the abnormal enzyme, explaining the variable activities reported in the literature (Powell et al., 1974; Sheffield et al.,

1977; Arata et al., 1979; Myara et al., 1982). The relationship between cell density in culture and fibroblast activity as noted by Myara et al., (1984c, 1985a) was not observed in the present study and implies some difference in tissue culture technique.

Increased Km values in the prolidase-deficient fibroblasts (Table 9, section 6.3.3.) indicated that the disease results from production of enzyme with altered properties rather than a marked deficiency of normal enzyme.

The heat stability results and biphasic kinetics for Phe-Pro pointed towards the existence of a second component of prolidase activity previously unobserved. A second form of prolidase was then discovered after DEAE-Cellulose ion-exchange chromatography of cultured skin fibroblast extracts. The previous inability to detect it in this study and previous studies (Endo et al., 1982; Myara et al., 1984a) was due to a variety of possible reasons. In the present study, dialysis of the extract prior to chromatography inactivated peak II. In other studies Gly-Pro is the most common substrate used but is a relatively poor substrate for peak II, also, erythrocytes have little peak II and prolonged incubation with high levels of manganese inactivates peak II. Having found a second component of prolidase, the two cases of prolidase deficiency were shown to be deficient in peak I activity against all the substrates tested, whereas peak II had a much

reduced activity only against Gly-Pro. This, therefore, provided an explanation for the presence of moderate activities in the cases with substrates other than Gly-Pro. Also, Gly-Pro was the only imidodi-peptide detectable in the plasma and erythrocytes in the patients of Freij et al. (1984). As the hydrolysis of Gly-Pro in the disease is reduced much more than the other substrates tested, it seems likely that this shortfall in activity would be important in the clinical manifestations of the disease. This may be reflected in the most promising results for treatment of the leg ulcerations with a topical ointment containing both glycine and proline (Arata et al., 1986).

A DEAE-Cellulose batch method was also developed to enable rapid separation of the two peaks for further studies. Control peak II was then found to be more labile than peak I, being less stable to heat and unprotected by manganese in the presence of PHMB (section 7.3.). The properties of peak II in prolidase-deficient fibroblasts were found to differ from control peak II, implying a possible structural relationship between the two peaks.

Prolidase peak I was then purified from human kidney and injected into rabbits to raise specific antisera to the enzyme for further studies. It has not yet been possible to show a structural relationship between the two peaks of prolidase as peak II does not

appear to react with the antisera. However, preliminary studies indicate that prolidase peak I does seem to be present though inactive, in the two cases of prolidase deficiency. This is in contrast with the recent findings of Endo et al. (unpublished results) for a Japanese patient in whom the subunit protein is apparently absent, representing a different genetic defect causing prolidase deficiency.

In conclusion, the hydrolysis of the two types of proline dipeptides in human tissues now appears more complex than was originally thought. The results presented in this thesis show that there are two components of enzyme activity for both prolinase and prolidase and that at least one form of prolinase activity is due to a non-specific dipeptidase. The existence of more than one form of enzyme must therefore be borne in mind when studying possible deficiencies of either enzyme as activity may still be present due to the other component as found with some substrates in prolidase deficiency.

9.2. Suggestions for further research

Although the present study has elucidated many of the properties of prolinase and prolidase, there is still a wide scope for continuing research with both enzyme activities.

As the major peak of prolinase has been shown to be a non-specific enzyme, it is necessary to determine the full range of its substrate specificity with homogeneous pure enzyme. The specificity of the enzyme may also be dependent on metal ions other than manganese as found for pig kidney dipeptidase (Capobianco and Vescia, 1967). The second DEAE-Cellulose peak of prolinase present in some tissues, also deserves further study to establish its substrate specificity and the effect of metal ions. It is possible that this peak is a "true" proline-specific enzyme or it may also be non-specific with a different range of specificity.

Similar investigations on the specificity of the two components of prolidase activity may help clarify the relationship between them and their exact roles. The high level of peak II activity in pancreas certainly seems to indicate a particularly important role for the enzyme in this tissue. Pancreas would also be a useful source for the purification of peak II prolidase after which antibodies could be raised and used to study the possibility of a common subunit with

peak I. This still seems to be the most likely reason for the change in the properties of peak II in prolidase deficiency. The other possibility of a processing defect which may be present, will be studied using pulse-chase H^3 -Leucine labelling experiments with cultured skin fibroblasts.

It has been suggested that prolidase may be of some clinical importance in chronic liver disease and could be used as an index of collagen catabolism in hepatic fibrosis (Myara et al., 1984d). Although plasma prolidase in rats seems to reflect liver cell leakage rather than active stages of fibrosis (Zuyderhoudt et al., 1985), Myara et al. (1984d) found an increased plasma prolidase activity in the early stages of hepatic fibrosis. As there are no reliable markers of hepatic fibrosis in plasma, such a marker would be most useful in the clinical and experimental evaluation of liver disease and should therefore be investigated further.

The recent observation that the antihypertensive drug Captopril (D-3-mercapto-2-methyl propanoyl-L-proline, SQ 14,225) is an effective inhibitor of prolidase in human kidney, liver and intestine (Ganapathy et al., 1985) also deserves further study. Captopril therapy is often associated with adverse side effects including urticarial and maculopapular eruptions. These are similar to the skin lesions seen

in prolidase-deficient patients and may be a result of prolidase inhibition by the drug.

These are some of the possibilities for future research into prolinase and prolidase which should give us a greater understanding of their physiological roles in human cells and tissues.

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11. APPENDIX

11.1. Tissue culture

Tissue culture was carried out by tissue culture and cytogenetics staff of the Pathology Department of the Royal Hospital for Sick Children, Edinburgh.

The tissue culture medium consisted of Ham's F10 containing 1.6 mM glutamine (BDH Ltd), 20% foetal calf serum, Kanamycin sulphate BPC ('Kanasyn', Winthrop Ltd) or Penicillin-streptomycin ('Crystamycia', Glaxo Ltd) and 0.09% NaHCO₃. The medium was prepared non-sterile and filtered through a Millipore filter (pore size 0.22 μ), and then sterility tested.

Cells were cultured in babys' feeding bottles of 225 ml capacity and 40 cm² growth surface area. Amniotic fluid cell primary cultures were in Nunclon-delta petri dishes of 5 cm diameter.

Skin biopsies were cut up into small pieces in thrombin and placed on drops of chicken plasma on one face of a baby's feed bottle. Excess liquid was removed with a Pasteur pipette and the plasma drops containing the skin left to clot. When clotted, 10 ml medium was added and the bottle gassed with 5% CO₂ in air. Outgrowths of fibroblasts spread from the pieces of skin and these were subcultured.

Amniotic fluid cells were precipitated by centrifugation at 300 x g for 10 min, the supernatant

was poured off and the cells resuspended in medium supplemented with 30% foetal calf serum. The resuspended cells were put into petri dishes (5 ml in each), placed in anaerobic jars (modified McIntosh and Fildes design) and gassed with 5% CO₂ in air.

Cells were subcultured by trypsinisation using 0.25% (w/v) trypsin solution (Trypsin 1:250, Difco Ltd) in phosphate buffered saline (Dulbecco 'A', Oxoid Ltd). After centrifugation and resuspension, the cells were transferred to two culture vessels. Incubation of cells was at 37°C at all times.

11.2. Preparation of cultured cells

Cultured cells were harvested at confluency by trypsinisation in 0.25% trypsin and 0.04% (w/v) EDTA in PBS. The cells were then transferred to a 10 ml conical centrifuge tube and sedimented by centrifugation at 400 x g for 5 min. Finally, the cells were washed twice with isotonic saline and stored at -70°C prior to enzyme studies.

11.3. Lowry protein assay

The protein concentration of cell or tissue extracts was determined by a modification of the method of Lowry et al. (1951) as follows:-

Reagents

- A 10% (w/v) Na_2CO_3 in 0.5M NaOH
- B 0.5% (w/v) $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ in 1% sodium tartrate
- C 9 ml A + 1 ml B, prepared just before use
- D Folin-Ciocalteu reagent (Sigma Ltd)
diluted 1/5 with distilled water prior to use

Protein standard was lyophilised bovine serum albumin (Sigma Ltd), 0.25 mg/ml in distilled water.

Procedure

0.4 ml solution C was added to 0.4 ml sample (0.05 ml extract + 0.35 ml distilled water) and mixed on a vortex mixer and left at room temperature. After 10 min, 0.4 ml solution D was added with thorough mixing on a vortex mixer and left at room temperature for 30 min. The extinction at 650 nm was read against a blank solution without any protein.

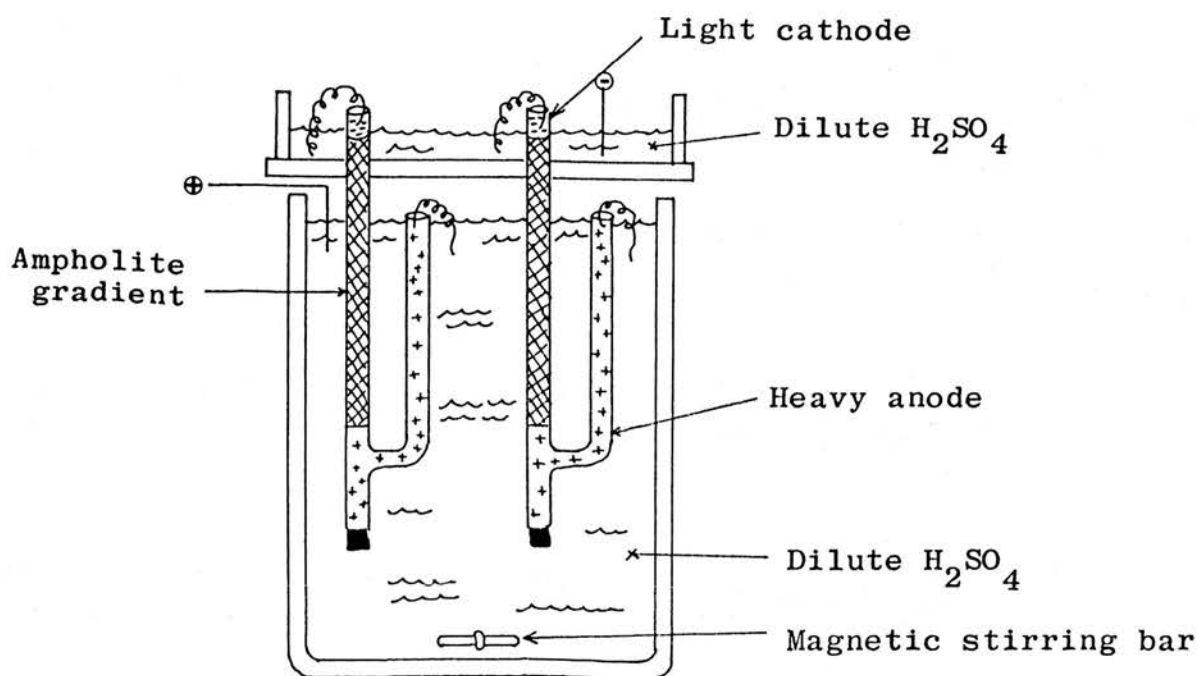
11.4. Isoelectrofocusing in a sucrose gradient

The method used for isoelectrofocusing was a modification of the method of Godson (1970) which enabled the use of small samples. The 'J-tubes' were as specified in the original method. Solutions were made up as follows:-

Anode solution	12g sucrose, 14 ml distilled water, 0.2 ml orthophosphoric acid (s.g. 1.7)
Cathode solution	2% (v/v) ethanolamine in distilled water
Heavy gradient	2.8g sucrose, 4.2 ml distilled water, 0.06 ml 10% (v/v) Triton-X-100, 0.75 ml 10% ampholine
Light gradient	sample made up to 6 ml with distilled water containing 0.06 ml 10% Triton-X-100 and 0.25 ml 10% ampholine
Electrolyte	dilute H_2SO_4 in tap water

The anode (14 ml) was run into the short arm of the J-tube. The sucrose gradient was made using a gradient mixer constructed from two 5 ml syringe barrels. Heavy gradient (4.5 ml) was added to the mixing chamber and allowed to fill the dead volume in

the exit tube and the tube connecting the two chambers before running the light gradient (5 ml) into the second chamber. The gradient was then run gently into the long arm of the J-tube, heavy to light. 1-2 ml cathode was then layered on top of the gradient. The final column consists of a heavy anode solution filling almost completely the short arm of the J-tube plus a few cm at the bottom part of the long arm. The ampholine/sucrose/sample gradient fills the remaining part of the long arm, supported on the heavy anode solution and with a light cathode solution layered on top (see figure below).



The anode and cathode were connected to a power pack with platinum wires dipped into the electrolyte in a small dish at the cathode and was used as a coolant for the J-tubes at the anode. The electrolyte was connected to the power pack by platinum wires. The current was raised step-wise until a voltage of 400 V was reached. This voltage was then maintained at 4°C for 18 hours.

On completion, an airtight connection was attached to the short arm and heavy sucrose (anode solution) was gently pumped in, displacing the sucrose gradient from the long arm. Fractions (0.25 ml) were collected and the pH of each read at 4°C.

11.5. Dixon and Webb substrate kinetics

The substrate kinetic studies as described by Dixon and Webb (1964, pp 84-87) were used to investigate the hydrolysis of Pro-Ala and Gly-Leu.

If an enzyme is not absolutely specific for one substrate only, it may act on two different substrates present at the same time. This cannot be considered as a simple case of two independent parallel reactions, as there will be competition between the substrates for the active site on the enzyme. For an enzyme acting on two substrates A and B, as far as A is concerned, B behaves simply as a competitive inhibitor and vice versa. The rate of breakdown of A in the presence of B as a competitive inhibitor is therefore given by analogy with the equation for competitive inhibition as given by Dixon (1953) :-

$$\text{I. } v_a = \frac{V_a}{1 + K_a (1 + [b] / K_b) / [a]}$$

and that of B by :-

$$\text{II. } v_b = \frac{V_b}{1 + K_b (1 + [a] / K_a) / [b]}$$

where v_a and v_b are the reaction velocities of A and B, V_a and V_b are the respective maximum velocities, K_a and K_b are the respective Michaelis constants, $[a]$ and $[b]$ are the substrate concentrations.

If Pro-Ala is substrate A and Gly-Leu substrate B, the experimentally determined values of K_m and V_{max} can be substituted into the equations to calculate the expected activities for each substrate.

$$V_a = 0.484 \text{ and } K_a = 0.47$$

$$V_b = 24.6 \text{ and } K_b = 0.79$$

$$[a] = [b] = 5 \text{ mM}$$

This gives a total estimated velocity ($v_a + v_b$) of 8.9 compared with the observed value of 9.2 units/ml.

11.6. Product formation kinetic studies

The kinetics for product formation as it relates to enzyme activity and concentration, reaction time and to substrate adsorption and affinity were studied according to Chrastil and Wilson (1982).

The movement of molecules in the pores of a protein is a diffusion process which can be expressed by the equation :

$$P = P_{\infty} [1 - \exp(-kEt)]^n$$

where P is a product which diffused in time t, P_{∞} is a product which diffused in $t = \infty$, k is a rate constant proportional to a diffusion coefficient D and n is a sterical constant dependent on the sterical structure of the system. This equation was found to accurately characterise many enzyme systems, except those with very short reaction times. The exponent n, and the rate constant k remained constant for a wide range of reaction times or enzyme concentrations.

A number of conclusions were reached:

a) If n and k were constant, and P_{∞} were found to be changed, then the number of free active centres (competitive inhibition or activation) would be changed.

b) If n and P_{∞} were constant, and k were found to be changed, then the surface reaction rate (non-competitive inhibition or activation) or the specific

activity of an enzyme (presence of inactive protein), and/or the diffusion coefficient would be changed.

c) If P_{∞} and k were constant, and n were found to be changed, then the overall sterical structure of the system would be changed.

Estimation of the constants

Constant P_{∞} (product formation in equilibrium) was estimated visually or by the higher order regression analysis of the corresponding time or enzyme concentration curves. To reach equilibrium in a reasonable time interval, it is recommended to use relatively high concentrations of an enzyme. Constant n was easily obtained as a slope of the linear part of the $\log t$ vs $\log P$ plot. Constant k was obtained as the slope of the linear part of t vs $-\ln[1-(P/P_{\infty})^{1/n}]/E$.

12. PUBLICATIONS SUBMITTED IN SUPPORT OF CANDIDATURE

1. Fluorimetric assay for prolinase.
J. Butterworth & D.A. Priestman;
J. Inher. Metab. Dis., 1982, 5, Suppl. 1, 9-10.

2. Fluorimetric assay for prolinase and partial characterisation in cultured skin fibroblasts.
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Fluorimetric assay for prolinase and partial characterisation in cultured skin fibroblasts

J. Butterworth* and D. Priestman

Department of Pathology, Royal Hospital for Sick Children and University of Edinburgh, Edinburgh EH9 1LF (UK)

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Summary

A sensitive assay for the estimation of prolinase has been developed, using *o*-phthaldialdehyde to detect the liberated amino acid. Substrates of the form proline-X (X = amino acid with one amino group) can be used. Prolinase can be estimated in cells used for diagnostic purposes with as little as 1 μ g cell protein. Partial characterisation of the enzyme indicates the presence of at least two forms of the enzyme in skin fibroblasts which needs consideration when testing for a prolinase deficiency.

Introduction

During collagen degradation dipeptides of the type proline-X (X = amino acid) are hydrolysed by prolinase (EC 3.4.13.8) and of the type X-proline by prolidase (EC 3.4.13.9). Existing methods [1-4] for prolinase, being relatively insensitive, are unsuitable for the characterisation of prolinase in the small quantities of human cultured cells available for diagnosis of collagen diseases. In addition, the described conditions of assay have been derived from animal tissues, as human prolinase has not been characterised [5-7]. Using a new simple and sensitive fluorimetric method, conditions of assay for prolinase in cultured skin fibroblasts were developed. Although a deficiency has been described for prolidase [1-3], no such deficiency has been reported for prolinase. The enzymic detection of Tay-Sachs disease only became possible with the realisation that there were two major forms of hexosaminidase, and the partial characterisation of prolinase was undertaken to see if the same held for prolinase.

* To whom correspondence should be addressed.

Materials and methods

Human amniotic fluid cells and skin fibroblasts were cultured as previously described [8], except that 20% (v/v) newborn calf serum was used. Tissue samples were obtained at autopsy 24 h after death. Red and white blood cells were prepared from fresh venous blood from children by dextran sedimentation [9]. All the samples were stored at -70°C . Extracts were prepared by the addition of ice-cold distilled water, sonication at 4–6 μm (M.S.E. Ultrasonic Disintegrator Mk. 2) for 30 s for cells and 60 s for tissues, and centrifugation at $1000 \times g$ for 10 min.

The following fluorimetric assay systems were developed to estimate prolinase in cultured cells, although the same procedures were used for other samples, (a) 50 μl extract (0.2 mg protein/ml), 50 μl 50 mmol/l veronal-HCl buffer pH 7.4 containing 4 mmol/l MnCl_2 and 100 μl 30 mmol/l dipeptide and (b) 50 μl extract, 50 μl 50 mmol/l veronal-HCl buffer pH 9.2 containing 0.1 mmol/l MnCl_2 and 100 μl 30 mmol/l dipeptide. Amino acids coupled to proline (Sigma Ltd.) included glycine, alanine, valine, leucine, phenylalanine and glutamic acid (Pro-Gly, Pro-Ala, Pro-Val, Pro-Leu, Pro-Phe, Pro-Glu). After up to 120 min incubation at 37°C , 200 μl 7.2 g/l perchloric acid was added, kept at 5°C for 15 min, spun at $2000 \times g$ for 10 min and 50- μl portions used to estimate the liberated amino acid. If required, assays can be kept at this point without hydrolysis of the substrate. Substrate and extract blanks and amino acid standards were run simultaneously. Amino acids were estimated by a method based on that used for tyrosine [10]. The reagent (100 ml) consisting of 3 ml *o*-phthalaldehyde (12 g/l methanol), 3 ml 2-mercaptoethanol (0.6%, v/v, in ethanol) and 94 ml 150 mmol/l Na_2CO_3 - NaHCO_3 buffer, pH 9.5, was freshly prepared, and 850 μl added to a 50- μl portion of the incubation mixture. After a minute for development, the fluorescence was read in a Perkin-Elmer 1000 fluorimeter (excitation 365 nm, emission 455 nm).

For a comparison with the fluorimetric method, the spectrophotometric method for prolinase as given by Powell et al [2] was used. The assay consisted of 50 μl cell extract, 100 μl 200 mmol/l borate buffer, pH 8.0, and 50 μl 24 mmol/l L-prolylglycine (Pro-Gly). After 30 or 60 min incubation at 37°C , 1.3 ml ethanol was added, allowed to stand for 10 min, spun at $2000 \times g$ for 10 min and a 1-ml sample read at 220 nm. The assay was also stopped and analysed as for the fluorimetric assay.

Using the fluorimetric assays various characteristics of prolinase of cultured skin fibroblasts were studied. Subcellular fractionation was carried out as previously described [11]. For heat stability, extracts in water or 10 mmol/l veronal-HCl buffer, pH 7.4, were kept at 45 – 60°C for 5 min or at 52°C for up to 60 min prior to assay. For pH stability, the pH of the extract was adjusted with 0.01 mmol/l HCl or NaOH for 30 min and neutralised prior to assay.

For Sephadex G150-120 (Sigma Ltd.) gel filtration, the extract was dialysed for 60 min against the elution buffer and spun at $100000 \times g$ for 30 min, 2 ml was applied to a 2.5×55 cm column, eluted with 10 mmol/l veronal-HCl pH 7.4 containing 100 mmol/l NaCl at 4 ml/h and 1.25 ml fractions collected. For DEAE-cellulose ion-exchange chromatography, the extract was dialysed for 60 min against the elution buffer and spun at $100000 \times g$ for 30 min, 2 ml was applied to a

1 × 25 cm column, eluted with a 0–250 mmol/l NaCl linear gradient in 10 mmol/l veronal-HCl buffer, pH 7.4, and 1.25-ml fractions collected. Sucrose density isoelectrofocusing was carried out as previously described [11], with the sample being applied without prior dialysis.

Hydrophobic chromatography was carried out by applying a 2-ml sample, dialysed against elution buffer, to a 0.8 × 10 cm column of butyl-agarose (Miles Lab. Ltd.) and eluted with a 0–300 mmol/l NaCl linear gradient in 20 mmol/l maleate buffer, pH 6.5. Affinity chromatography was performed by applying a 2-ml sample, dialysed against elution buffer, to a 0.8 × 9 cm column of concanavalin A-Sepharose (Pharmacia Ltd.) using a 10 mmol/l veronal-HCl buffer, pH 7.4, containing 0.1 mmol/l MnCl₂, 100 mmol/l NaCl and 0.1% (v/v) Triton X-100 (Sigma Ltd.) and eluted with 0–1 mol/l α -methyl-D-glucoside (Sigma Ltd.). Chromatography using wheat-germ lectin-Sepharose (Sigma Ltd.) was undertaken using a 0–1 mol/l *N*-acetyl-D-glucosamine (Sigma Ltd.) linear gradient in 10 mmol/l veronal-HCl buffer, pH 7.4, containing 100 mmol/l NaCl and 0.1% (v/v) Triton X-100.

Cathepsin B [12], α -L-fucosidase and *N*-acetyl- β -D-glucosaminidase [13], β -D-glucuronidase [14], fumarase [15], lactic dehydrogenase [16] and protein [17] were estimated as previously described. Non-specific dipeptidase activity was estimated using a micromethod based upon the assay of Shoaf et al [18]. Each assay contained 25 μ l extract, 50 μ l 100 mmol/l Tris(hydroxymethyl) aminomethane-HCl buffer, pH 8.0, and 25 μ l 150 mmol/l glycyl-L-leucine (Gly-Leu; Sigma Ltd.) or L-phenylalanyl-L-alanine (Phe-Ala; Sigma Ltd.). Following incubation for 15 min at 37°C, 250 μ l L-amino acid oxidase reagent (2.7 g/l L-amino acid oxidase, 2 mg/l horseradish peroxidase and 7.2 g/l *o*-dianisidine) was added. After a further 10 min, 250 μ l 50% H₂SO₄ was added and the colour read at 530 nm.

Results and discussion

The time taken to reach the maximum fluorescence and the rate of decay of this fluorescence depended upon the particular amino acid. Glycine and alanine showed maximum fluorescence after 1 min and had lost 25% of this fluorescence after 8 min. The fluorescence for valine, leucine, phenylalanine and glutamic acid was maximal after 1½ min and remained at this level for at least 5 min. Prolylglycine was used, despite the relatively short-lived fluorescence of the glycine conjugate, in order to compare the present with previous studies. All the amino acids tested gave linear calibration curves up to at least the limits of the fluorimeter with the fluorescence units being 950, 830, 1050, 990, 1050 and 1060 for 10 nmol glycine, alanine, valine, leucine, phenylalanine and glutamic acid, respectively. The reagent blank was less than 20 units when prepared and increased by only a few units after many hours. The fluorimeter was standardized against the stable fluorochrome quinine to give 250 fluorescence units at 1 mg/l.

The fluorescence depended upon the molarity of the reagent buffer, as it has to counteract the low pH consequent to the use of perchloric acid as a protein precipitant. The amount of assay mixture taken to estimate the liberated amino acid can be increased, but the reagent buffer must be increased accordingly. Although

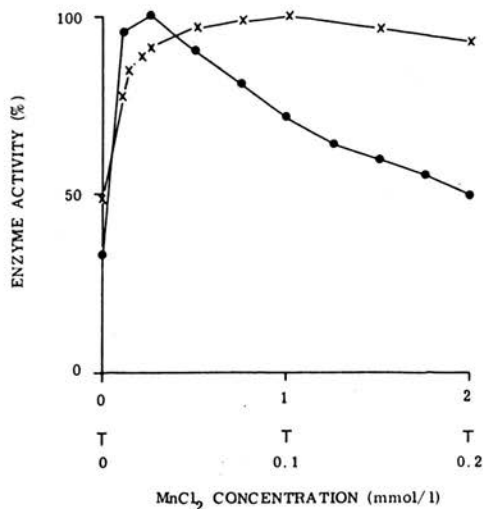


Fig. 1. Effect of manganese on fibroblast prolinase assayed at pH 7.4 (×, 0–2 mmol/l) and pH 9.2 (●, 0–0.2 mmol/l).

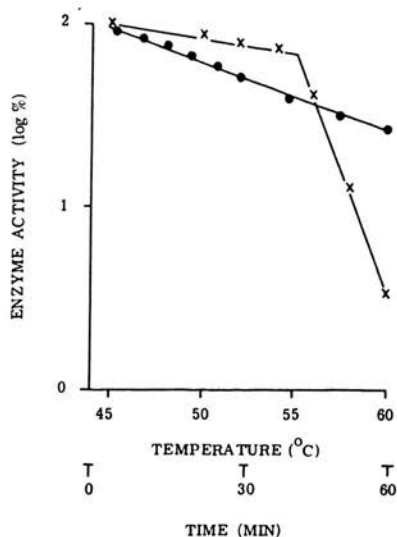


Fig. 2. Effect on fibroblast prolinase activity of (×) heating at various temperatures for 5 min and (●) heating at 52°C for up to 60 min.

100 mmol/l buffer will suffice for 50 μ l assay volume, 150 mmol/l was routinely used to permit either 50 or 100 μ l to be taken, and for 400 μ l, the maximum tested, 1000 mmol/l buffer was needed. At the level of *o*-phthaldialdehyde and 2-mercaptoethanol used, no changes in concentrations were needed for up to 400 μ l assay volume.

The pH profile of prolinase was dependent upon the level of Mn^{2+} present. Without added Mn^{2+} a broad optimum at pH 8.4–9.4 was found, whilst the optimum with 1 mmol/l Mn^{2+} was pH 7.4–7.8 and with 0.025 mmol/l Mn^{2+} pH 9.2, this last condition giving the highest activity. The saturating concentration of Mn^{2+} at pH 7.4 was 1 mmol/l and at pH 9.2, 0.025 mmol/l, with inhibition above those respective concentrations (Fig. 1). The assay was linear with time for at least 3 h at pH 7.4 and pH 9.2, and with protein concentrations up to that hydrolysing 20% substrate. A typical assay using about 10 μ g fibroblast protein hydrolysed less than 4% of the substrate. The time linearity was in contrast to the marked non-linearity after 40 min observed by Powell et al [2]. As analysed by Lineweaver-Burke plots the K_m for L-prolylglycine was 6.8 mmol/l at pH 7.4 and 2.3 mmol/l at pH 9.2.

Dialysis of the enzyme for 60 min against distilled water reduced prolinase activity by 75–85%, but by only 25% against 100 mmol/l NaCl and less than 20% against 10 mmol/l veronal-HCl buffer pH 7.4. The enzyme was thermolabile with a marked transition point at about 55°C (Fig. 2) with a linear fall off at 52°C. The presence of 100 mmol/l NaCl or 0.2 mg bovine serum albumin during the heat

treatment increased the thermolability. The enzyme was stable between pH 6.5–9.5, but the activity was reduced by 70% below pH 6.0.

Prolinase was present in the final soluble fraction on subcellular fractionation, with lactic dehydrogenase, fumarase, α -L-fucosidase and *N*-acetyl- β -D-glucosaminidase showing their expected distribution and latency effect for the last two enzymes.

Using Pro-Gly, cultured skin fibroblasts ($n = 15$) had a prolinase mean specific activity ($\text{nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ protein \pm SD) of 42.4 ± 12.2 (range 26–72) at pH 7.4 and 80.1 ± 24.2 (range 47–134) at pH 9.2, whilst amniotic fluid cells (passage 2 + 3, $n = 19$) had 56.9 ± 30.3 (range 21–124) and 108.5 ± 58.5 (range 41–237), respectively. The omission of the four epithelioid amniotic fluid cell cultures tested, which had high prolinase values, resulted in mean values very similar to those for fibroblasts. The fibroblast values were about three times higher than those previously reported by Arata et al [1], who omitted manganese. Three white blood cell samples (polymorphonuclear cells) had specific activities of 34, 55 and 82 at pH 7.4 and 51, 77 and 122 at pH 9.2, respectively, which compare with a mean value of 24 reported by Powell et al [2] for pH 8.0. Three red blood cell samples had rather low specific activities of 7.3, 8.7 and 8.8 at pH 7.4, and 10.5, 11.8 and 12.9 at pH 9.2, respectively, which were much higher than the value of 0.2 reported by Powell et al [2]. This very low value was probably due to the dialysis step used in their method. Prolinase activity was also estimated in a number of tissues obtained at autopsy

TABLE I

SPECIFIC ACTIVITY ($\text{nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ protein) OF TISSUE PROLINASE, CATHEPSIN B AND HEXOSAMINIDASE

Tissue	Prolinase		Cathepsin B	Hexosaminidase
	pH 7.4	pH 9.2		
Kidney	451	974	8.2	49.5
Thyroid	338	554	94.2	41.9
Thymus	200	324	28.6	82.6
Pancreas	160	317	5.3	29.1
Adrenal	153	273	7.9	52.4
Pituitary	150	270	2.9	26.2
Liver	115	237	6.9	68.4
Choroid plexus	116	234	5.2	31.1
Cerebral cortex	123	231	0.8	5.8
Small bowel	107	211	3.8	69.1
Lung	101	183	2.2	14.4
Skeletal muscle	87	155	1.1	6.5
Skin	80	125	2.6	7.2
Spleen	64	117	2.4	15.9
Cartilage	54	108	4.0	23.4
Placenta	55	89	1.8	9.7
Heart	19	29	2.4	13.8

TABLE II
DIPEPTIDASE ACTIVITY ($\text{nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ protein) OF CULTURED CELLS AT INDICATED pH VALUES *

Cell type	Pro-Gly		Pro-Ala		Pro-Leu		Pro-Phe		Pro-Val		Hydro-Gly	Gly-Leu	Phe-Ala
	7.6	9.2	7.6	9.2	7.6	9.2	7.6	9.2	7.6	9.2			
Fibroblast	27	42	44	66	63	106	57	115	69	81	6	462	218
Amniotic fluid	53	89	77	115	117	189	127	211	129	175	12	1150	68

* Veronal-HCl buffer, pH 7.6, + 1 mmol/l Mn^{2+} , pH 9.2, + 0.025 mmol/l Mn^{2+} , pH 8.8, + 1 mmol/l Mn^{2+} ; Tris-HCl buffer, pH 8.0.

(Table I). Apart from heart muscle, all tissues had moderate activity, with kidney being particularly active.

The hydrolyses of a number of proline dipeptides, hydroxypropylglycine (Hypro-Gly) and two non-specific dipeptidase substrates Gly-Leu and Phe-Ala by skin fibroblast and amniotic fluid cell cultures are given in Table II. The substrate Pro-Gly previously used for studies on human prolinase [1-4] gave a lower specific activity than Pro-Leu, Pro-Phe and Pro-Val, and together with the more unstable fluorescence for glycine this makes any of these three substrates preferable to Pro-Gly. The variable ratios of activities at pH 7.6 to pH 9.2 for the different substrates indicate that there is a single enzyme with variable responses for each substrate or that there is more than one form of prolinase. Pig kidney prolinase was found [19] to hydrolyse Hypro-Gly faster than Pro-Gly, and was claimed to be more specific for prolinase, but Mayer and Nordwig [20] found Hypro-Gly to be hydrolysed at only a third of the rate of Pro-Gly. For the cultured cells, Hypro-Gly was a poor substrate and of little use for the estimation of prolinase. Prolyl-glutamic acid was not hydrolysed by cultured cell prolinase in agreement with the finding for pig kidney enzyme [21]. It is noteworthy that the non-specific dipeptidase activity was higher than the prolinase activity, and the difference between the fibroblast and amniotic fluid cell cultures for the two non-specific substrates deserves further study.

The main advantage of the use of *o*-phthaldialdehyde for the assay of prolinase is that it does not react with proline unless modified and hence substrates of the type proline-X (X = amino acid with one NH₂ group) will not give a fluorescence, whereas ninhydrin reacts with proline, necessitating separation of substrate and products. Furthermore, the estimation of amino acids using ninhydrin is about ten times less sensitive than using *o*-phthaldialdehyde. The method given for prolinase by Powell et al [2], based upon the detection of proline using the ninhydrin method of Troll and Lindsley [22], is time-consuming, used benzene, and required at least 20

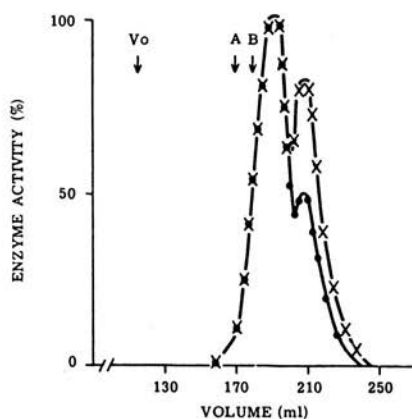


Fig. 3. Sephadex G150-120 gel filtration of fibroblast prolinase assayed at pH 7.4 (×) and pH 9.2 (●). 2.5 × 55 cm column eluted with 10 mmol/l veronal-HCl buffer, pH 7.4, containing 100 mmol/l NaCl. Vo, void volume; A, hexosaminidase; B, α -L-fucosidase.

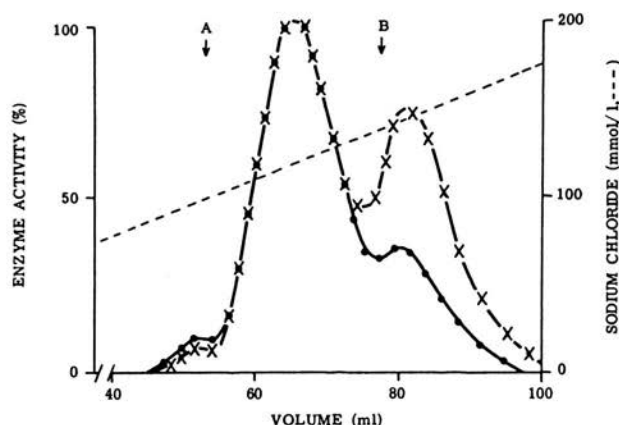


Fig. 4. DEAE-Cellulose column chromatography of fibroblast prolinase assayed at pH 7.4 (\times) and pH 9.2 (\bullet). 1×25 cm column eluted with 0–250 mmol/l NaCl in 10 mmol/l veronal-HCl buffer, pH 7.4. A, hexosaminidase B; B, α -L-fucosidase.

times more enzyme protein in the assay than the present method. The method of Arata et al [1] used an amino acid analyser to separate the products for the substrate, followed by ninhydrin detection, and required at least 20 times more enzyme protein in the assay than the present method. This technique is also not suitable for a large number of samples as generated by kinetic and chromatographic studies. Comparison was made with the method of Josefsson and Lindberg [3] as modified for prolinase by Powell et al [2], which depends upon the fall in absorbance with substrate hydrolysis. However, the low rate of substrate hydrolysis with cultured cells only gave a very small drop in absorbance, which, read against the initial high value, resulted in low sensitivity. The spectrophotometric method was not feasible in the presence of haemoglobin [2], whereas it had no effect in the present fluorimetric method. By taking 400 μ l rather than the usual 50 μ l assay volume for amino acid estimation, the amount of enzyme needed can be reduced to about 1 μ g protein/assay, making the present method about 200 times more sensitive than the other methods.

Chromatographic techniques were used to characterise the enzyme from skin fibroblasts to see if more than one form was present. For assays of prolinase by the fluorescent method after chromatographic procedure, it was not usually necessary to centrifuge following addition of perchloric acid, as the enzyme blank was very low. Gel filtration of prolinase showed a large and a small peak, the latter being more pronounced at pH 7.4 than at pH 9.2 (Fig. 3). Bovine prolinase [23] gave a single peak of molecular mass 75 000, similar to the fibroblast large peak. Ion-exchange chromatography (Fig. 4) also gave a large (120 mmol/l NaCl) and a small peak (145 mmol/l NaCl), with the latter again being more pronounced at pH 7.4. The two peaks gave the same pH profile with 0.025 mmol/l Mn^{2+} , but with 1 mmol/l Mn^{2+} the first peak gave an optimum near pH 8.0, whilst that of the second peak was nearer pH 7.4, and would explain the variable pH profiles for a total extract.

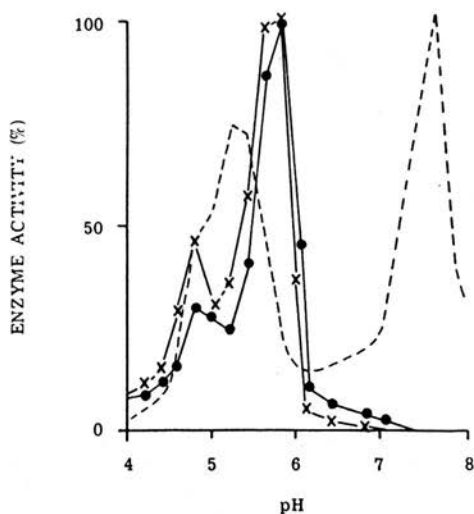


Fig. 5. Sucrose density isoelectrofocusing of fibroblast prolinase assayed at pH 7.4 (×) and pH 9.2 (●). (-----) Hexosaminidase.

Hydrophobic chromatography, using butyl-agarose only gave a single peak for pH 7.4 and pH 9.2 at 90 mmol/l NaCl. Cathepsin B was unbound, β -D-glucuronidase released almost immediately on application of the gradient, whilst *N*-acetyl- β -D-glucosaminidase was tightly bound. For sucrose density isoelectrofocusing it was necessary to dilute the fractions tenfold to reduce the blanks produced by the ampholine. A large peak was obtained at pI 5.75 with a smaller peak at pI 4.8 (Fig. 5). This latter peak was associated with a protein band, although it was not removed by centrifugation and showed a relatively higher activity at pH 7.4. A pI 4.3 was reported [23] for bovine prolinase, markedly different to the present value for the human enzyme. Prolinase did not bind to concanavalin A-Sepharose nor wheat-germ lectin-Sepharose, although *N*-acetyl- β -D-glucosaminidase did, indicating it is not a glycoprotein. It is of interest that the fluorimetric method detected *N*-acetyl-D-glucosamine such that 50 μ l 200 mmol/l gave a reading of 390 after 6 min development time and can be used to easily measure the gradient of the lectin column.

The present study indicates that prolinase exists in two forms. Thus a deficiency of prolinase may be partial, not absolute, and explain why no such deficiency has as yet been found. In this case it will be necessary to estimate the two forms of prolinase separately, which would require an assay at least as sensitive as the present fluorimetric method.

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Substrate Specificity of Manganese-activated Prolidase in Control and Prolidase-deficient Cultured Skin Fibroblasts

J. BUTTERWORTH and D. PRIESTMAN

Department of Pathology, Royal Hospital for Sick Children and University of Edinburgh, Edinburgh EH9 1LF, Scotland

Skin fibroblasts have a single enzyme, Mn^{2+} -activated prolidase, that hydrolyses a range of amino acid-proline dipeptides. Two cases of prolidase deficiency showed a marked loss of activity against glycyl-proline irrespective of Mn^{2+} conditions. However, the abnormal enzyme showed only moderate reductions in activity against phenylalanyl-, alanyl-, and leucyl-proline following preincubation with Mn^{2+} or addition of Mn^{2+} with the substrate. Control prolidase was stable to prolonged preincubation with Mn^{2+} , whereas the abnormal prolidase was progressively inactivated. The findings indicate, for at least the present two cases, that prolidase deficiency results from an altered rather than a marked reduction in the amount of normal enzyme.

Prolidase (EC 3.4.13.9) deficiency (McKusick 26413) is an autosomal recessive disease associated with chronic ulcerative dermatitis, mental retardation and urinary excretion of iminodipeptides (Scriver, 1977). The disease can be diagnosed by assay of prolidase in cultured skin fibroblasts (Arata *et al.*, 1979; Myara *et al.*, 1983; Sheffield *et al.*, 1977) as well as red and white blood cells (Endo and Matsuda, 1981; Ogata *et al.*, 1981; Powell *et al.*, 1974). All reports have utilized glycyl-proline as the substrate and have indicated an almost complete deficiency of the enzyme. The present study shows that human cultured skin fibroblast Mn^{2+} -activated prolidase, as for other sources (Endo *et al.*, 1982; Sjöström *et al.*, 1973), can hydrolyse a range of proline dipeptides and that in two unrelated cases of prolidase deficiency the enzyme is altered, not absent, such that activity against glycyl-proline is very low, but only moderately reduced against other substrates.

MATERIALS AND METHODS

Skin fibroblast cultures were supplied from a male Turkish patient (Case 1) by Dr E. Christensen, Denmark, and from a male Italian patient (Case 2; Sheffield *et al.*, 1977) by Professor Danks, Australia. The patients had iminodipeptiduria and a marked deficiency of prolidase against glycyl-proline. Fibroblasts were grown in bottles (40 cm² surface area) using Ham's F10 (Flow Lab. Ltd.) with 20% (v/v) calf serum (Gibco-Biocult Ltd.) in the presence of 5% CO₂-95% air. Cell extracts were prepared (Butterworth, 1982) using confluent cultures at passages 10–25.

DEAE-cellulose ion-exchange chromatography, Sephadex G-150 gel filtration and sucrose density isoelectric focusing were carried out as described previously (Butterworth and Priestman, 1982). Prolidase was assayed using 25 µl extract, 50 µl 50 mmol/l barbital-HCl buffer pH 8.0 and 25 µl 80 mmol/l glycyl-proline (gly-pro), phenylalanyl-proline

(phe-pro), leucyl-proline (leu-pro) or alanyl-proline (ala-pro; Sigma Ltd.). After incubation at 37 °C for 30 min the liberated proline was estimated by the method of Mayer and Nordwig (1973) using half their volumes. Where appropriate, extracts with the buffer were preincubated at 37 °C with or without MnCl₂ at the indicated concentration or an equivalent final Mn^{2+} concentration was added with the substrate. Prolidase was assayed using prolyl-alanine (Sigma Ltd.) as the substrate (Butterworth and Priestman, 1982). Protein was estimated (Lowry *et al.*, 1951) using bovine serum albumin (Sigma Ltd.) as the standard.

RESULTS

Sephadex gel filtration gave a single peak of prolidase eluting just prior to the first prolinase peak, whilst DEAE-cellulose ion-exchange chromatography gave a single peak of prolidase eluting at 135 mmol/l NaCl, just after the second major peak of prolinase at 130 mmol/l NaCl. As all the prolidase activity was found to be below pH 6.0, isoelectric focusing was carried out using a pH 4–6 gradient. Control fibroblast prolidase showed a peak against gly-pro and phe-pro at pI 4.5 (Figure 1). A prolidase-deficient cell line (Case 1) showed a peak at pI 4.6 for both these substrates. Prolidase activity against leu-pro and ala-pro coincided with these peaks. The minor peak at about pI 5.0 was associated with a large protein band and was probably artefactual. These results indicate that cultured skin fibroblasts have a single enzyme, prolidase, hydrolysing amino acid-proline dipeptides.

The activity of prolidase from control and prolidase-deficient fibroblasts was estimated against gly-pro, phe-pro, leu-pro and ala-pro after preincubation with or without 1.33 mmol/l Mn^{2+} (1 mmol/l in assay) for 5 min (Table 1). Clearly, assaying without Mn^{2+} resulted in a deficiency for all four substrates. However, prolidase is a Mn^{2+} -activated enzyme as seen in Table 1 and when

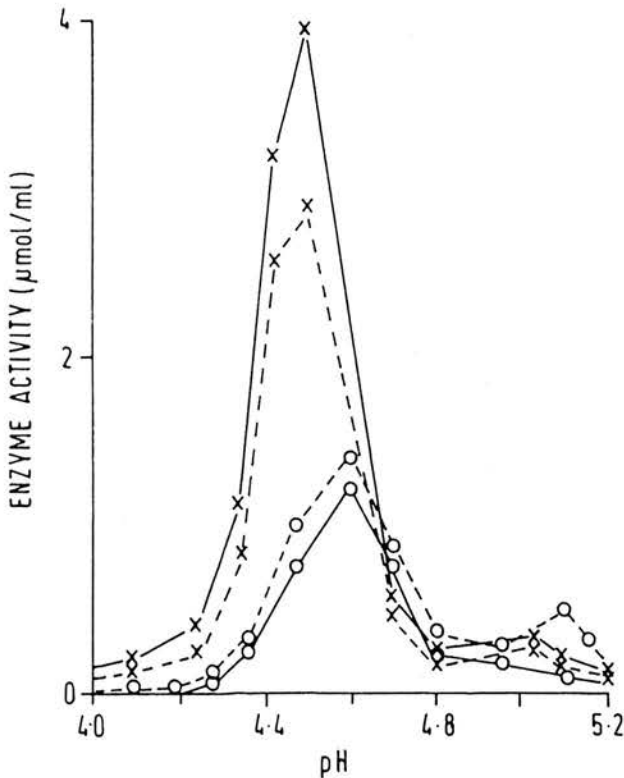


Figure 1 Sucrose-density isoelectric focusing of control (X) and Case 1 (O) fibroblast prolidase assayed with gly-pro (—) or phe-pro (---). Control was assayed for 30 min and Case 1 for 60 min

preincubated with Mn^{2+} the deficiency was still marked for gly-pro (94%), but less so for phe-pro (55%), leu-pro (40%) and ala-pro (70%).

The response of prolidase to Mn^{2+} was analysed further. Control prolidase activity assayed against phen-pro was unaltered by preincubation in buffer. Addition of Mn^{2+} to the enzyme immediately followed by the substrate and addition of Mn^{2+} with the substrate gave the same increase in activity ($\times 3-4$) compared to no Mn^{2+} . Preincubation with Mn^{2+} gave a further increase in activity maximal at 5 min followed by a gradual return to the initial Mn^{2+} value (Figure 2). The abnormal prolidase (Cases 1 and 2) was stable in buffer but was progressively inhibited in the presence of Mn^{2+}

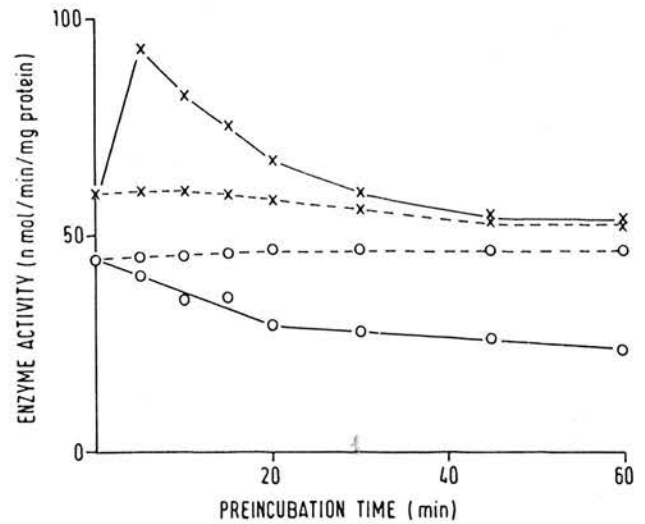


Figure 2 Effect of preincubation of control (X) and Case 1 (O) fibroblast extract for up to 60 min with 1.33 mmol/l Mn^{2+} (—) or without Mn^{2+} (---) on prolidase activity assayed with phe-pro. Mn^{2+} was added to the assay with the substrate or extract preincubated without Mn^{2+}

(Figure 2; Case 1 shown). Similar results were obtained against leu-pro and ala-pro. The data (Figure 2, zero time) illustrate another feature that addition of Mn^{2+} with the substrate resulted in only a small reduction (25%) in activity against phe-pro for both cases. A similar reduction was noted against ala-pro, whilst no reduction was found for leu-pro. The findings for gly-pro were different to those for the above three substrates. Control prolidase activity increased over a 60 min period of preincubation with Mn^{2+} (1.33 mmol/l), such that the activity was increased 4–5 fold after 5 min and 8–10 fold after 60 min compared to the no Mn^{2+} activity. In contrast, the abnormal prolidase (Cases 1 and 2) increased by only 10% after 5 min and 50% after 60 min. Addition of Mn^{2+} with the substrate gly-pro showed a greater reduction (75%) in activity for the abnormal prolidase (Cases 1 and 2) than found for the other three substrates. The deficiency in prolidase activity against the four substrates was in the order: gly-pro $>$ ala-pro \geq phe-pro $>$ leu-pro, whilst the deficiency against all four substrates varied with the

Table 1 Prolidase activity ($nmol\ min^{-1}\ mg\ protein^{-1}$) against different substrates in cultured skin fibroblasts from controls ($n = 10$) and cases

Cells	Gly-pro		Phe-pro		Leu-pro		Ala-pro	
	$-Mn^{2+}$	$+Mn^{2+}$	$-Mn^{2+}$	$+Mn^{2+}$	$-Mn^{2+}$	$+Mn^{2+}$	$-Mn^{2+}$	$+Mn^{2+}$
Controls								
Mean	13.4	87.6	28.1	95.4	21.3	77.9	68.8	135.8
SD	2.8	12	2.7	11	2.3	8.4	3.7	17.6
Case								
1	0.31	5.7	0.8	49.1	1.1	41.5	7.5	39.5
2	0.34	5.3	0.7	38.0	3.1	52.4	7.0	43.5

Extracts were preincubated in buffer without Mn^{2+} ($-Mn^{2+}$) or with 1.33 mmol/l Mn^{2+} ($+Mn^{2+}$) for 5 min at 37°C. No additional Mn^{2+} was added to the assay

assay conditions in the order: no Mn^{2+} > 5 min Mn^{2+} preincubation > 60 min Mn^{2+} preincubation > Mn^{2+} with substrate.

DISCUSSION

The present chromatographic findings show that human skin fibroblasts have only a single enzyme hydrolysing amino acid-proline dipeptides, in agreement with previous work on human erythrocyte prolidase (Endo *et al.*, 1982). Hence an absence of the enzyme should result in the disappearance of activity against all the amino acid-proline substrates, but this was not so in the present two unrelated cases of prolidase deficiency. When activated with Mn^{2+} prolidase showed a marked deficiency against gly-pro, but not against the other substrates tested, indicating the enzyme was altered in specificity rather than absent. Previous studies (Arata *et al.*, 1979; Endo and Matsuda, 1981; Endo *et al.*, 1982; Ogata *et al.*, 1981; Powell *et al.*, 1974; Sheffield *et al.*, 1977) have all used gly-pro with differing levels of Mn^{2+} added to the assay and have shown an almost complete absence of the enzyme. The present study showed that a deficiency in prolidase would be expected with gly-pro irrespective of the conditions of assay. However, using other substrates the detection of the disease would be more difficult requiring a more detailed study of the enzyme. Whether other cases have a similar gene mutation to the present two unrelated cases resulting in an altered ability of Mn^{2+} to stabilize/activate prolidase and a changed substrate specificity or a mutation resulting in a prolidase inactive against all the substrates deserves investigation.

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Brief technical note

Prolidase deficiency: characteristics of human skin fibroblast prolidase using colorimetric and fluorimetric assays

David A. Priestman * and John Butterworth

Department of Pathology, Royal Hospital for Sick Children and University of Edinburgh, Edinburgh EH9 1LF (UK)

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Key words: Colorimetric assay; Enzyme kinetics; Fluorimetric assay; Human skin fibroblasts; Prolidase; Prolidase deficiency

Introduction

Prolidase (EC 3.4.13.9) deficiency (McKusick 26413) is an autosomal recessive disease associated with chronic ulcerative dermatitis, mental retardation and imino-dipeptiduria [1]. A single form of prolidase was found in human erythrocytes [2] and an almost total deficiency of the enzyme against the substrate glycyl-proline has been reported [3-9] in the disease. In a preliminary report on cultured human skin fibroblast prolidase [10] we showed that the disease enzyme activity against other substrates was not as reduced. Characteristics of the normal and abnormal enzyme of skin fibroblasts are described that enable diagnosis of the disease to be made using substrates in addition to glycyl-proline.

Materials and methods

Skin fibroblast cultures were obtained from a Turkish male patient (Case 1) [9] and a male Italian patient (Case 2) [4]. The cells were cultured and extracts prepared as previously described [10,11].

Prolidase was assayed using glycyl-proline (gly-pro), phenylalanyl-proline (phe-pro), alanyl-proline (ala-pro), valyl-proline (val-pro), seryl-proline (ser-pro), methionyl-proline (met-pro) obtained from Sigma Ltd. (St. Louis, MO, USA) and glutamyl-proline (glu-pro) obtained from BaChem Ltd. as substrates and followed by estimating liberated proline [10]. Two other possible methods for phe-pro were

* To whom correspondence should be addressed.

evaluated. Firstly, liberated phenylalanine was estimated spectrophotometrically using a micromethod based upon the nonspecific dipeptidase assay of Shoaf et al [12]. The assay contained 25 μ l extract, 50 μ l 50 mmol/l barbital-HCl buffer pH 8.0 and 25 μ l 80 mmol/l phe-pro and after 30 min incubation at 37°C the reaction was stopped by heating at 100°C for 1 min. Reagent (250 μ l of 25 mmol/l barbital-HCl buffer pH 8.0 containing 0.27 g/l L-amino acid oxidase, Sigma Ltd. type VI; 20 mg/l horseradish peroxidase, Sigma Ltd. type II; and 0.2 g/l *o*-dianisidine · 2 HCl Sigma Ltd.) was added and after 10 min at 37°C 250 μ l 50% (v/v) sulphuric acid added. The colour was read at 530 nm and compared to a phenylalanine standard curve. Secondly, the method of Matsumoto et al [13] for monoamine oxidase was applied to the fluorimetric estimation of phenylalanine. After heating the assay tubes at 100°C for 1 min, 400 μ l reagent (25 mmol/l barbital-HCl buffer pH 8.0 containing 0.1 g/l L-amino acid oxidase, 10 mg/l horseradish peroxidase, 50 mmol/l EDTA and 0.5 μ mol/l homovanillic acid, Sigma Ltd.) was added. After incubation at 37°C for 10 min, 500 μ l 0.5 mol/l NaOH was added and the fluorescence read on a Perkin-Elmer 1000 fluorimeter (excitation 323 nm; emission 426 nm) and related to a phenylalanine standard curve. Addition of EDTA was needed to prevent the precipitation of Mn^{2+} used to activate prolidase. Protein was estimated [14] using bovine serum albumin (Sigma Ltd.) as standard.

The effect of Mn^{2+} on prolidase activity was followed by the addition of increasing amounts of $MnCl_2$ to the assay or by preincubating extract with 1.33 mmol/l Mn^{2+} . Substrate kinetics for enzyme preincubated with 1.33 mmol/l Mn^{2+} for 5 min was calculated by Eadie-Hofstee plots using a Hewlett-Packard HP97 [15] for a number of substrates over the range 1–20 mmol/l. Substrate interaction was also studied for phe-pro by the method of Chrastil and Wilson [16] by following product (phenylalanine) formation with time under limiting substrate level (0.5 mmol/l) for normal and abnormal enzyme at the same protein level. Heat stability of prolidase in the presence (1.33 mmol/l) or absence of Mn^{2+} was investigated by treating extract at 30–60°C for 5 min or at 48°C for up to 60 min. For extracts treated without Mn^{2+} an equivalent amount of Mn^{2+} was added with the substrate. The effect of *p*-hydroxymercuribenzoate (PHMB, Sigma Ltd.) on prolidase activity against phe-pro was studied by the addition of 0.01–0.5 mmol/l PHMB before or after treatment with 1.33 mmol/l Mn^{2+} . Based upon these results 0.05 mmol/l PHMB was added to normal and abnormal prolidase following preincubation with 1.33 mmol/l Mn^{2+} and assayed with a number of substrates.

Results and discussion

Assays for prolidase

Myara et al [8] reported a method using Chinard's ninhydrin reagent optimised for the assay of prolidase. With this method gly-pro was rapidly hydrolysed, such that at 100°C an absorbance of 1.0 was reached after 20 min necessitating the use of a lowered temperature which reduced assay sensitivity. The present method was based on the ninhydrin method of Mayer and Nordwig [17] and at 100°C the absorbance was only 0.09 after 20 min enabling 100°C to be used. The development

of the proline colour and hydrolysis of gly-pro to give the substrate blank were critically dependent upon the temperature. Using heating blocks in which there was a difference of only a degree between blocks gave unreliable readings. This problem was overcome by ensuring a uniform temperature in a boiling waterbath by continuous stirring. All the other substrates were more stable and gave a lower blank than gly-pro. Glycine was found to increase the proline standard reading and its inclusion in the method, as recommended by Mayer and Nordwig [17], eliminated any tendency for the glycine liberated by the enzyme from gly-pro to increase the reading.

The L-amino acid oxidase colorimetric method gave 3.8 times the colour for a given phenylalanine standard (10 nmol = absorbance 0.285) compared to an equivalent proline standard in the Mayer and Nordwig [17] method and a slightly better enzyme reading to blank ratio. The method was linear up to at least an absorbance of 1.0 equivalent to 35 nmol phenylalanine. The L-amino acid oxidase fluorimetric method gave a reading of 900 fluorescent units for 10 nmol phenylalanine with the fluorimeter standardised to 200 U with 1 mg/l quinine and an enzyme reading to blank ratio twice as high as the proline method. Hence, both L-amino acid oxidase methods gave larger readings for a given enzyme level than the proline method and were suited for the rapid analysis of a large number of samples as generated by kinetic and chromatographic studies.

An optimum of pH 8.0 was obtained for normal and abnormal fibroblast prolidase using barbital-HCl buffer. The three enzyme assays were linear with time for at least 60 min and with protein up to at least 40 μg /assay. A range of 5–15 μg protein/assay was used for the L-amino acid oxidase methods and 15–40 μg protein/assay for the proline method. The three methods gave similar prolidase activities for a number of fibroblast cultures.

Effects of manganese

Control prolidase was activated by the addition of Mn^{2+} with the substrate (2.5 mmol/l Mn^{2+} optimal gly-pro; 1 mmol/l Mn^{2+} optimal other substrates), except for glu-pro which did not require Mn^{2+} . Preincubation of the enzyme with Mn^{2+} at 37°C further increased control prolidase activity, with the same levels of Mn^{2+} being optimal (Fig. 1). Preincubation for 60 min for gly-pro and 5 min for the other substrates (phe-pro and leu-pro shown) were required for optimal activation. The increase in activity with preincubation at 37°C did not occur at 48°C. The abnormal enzyme was activated by the addition of Mn^{2+} with the substrate, but the optimal Mn^{2+} for Case 1 prolidase activity against phe-pro was about 0.05 mmol/l Mn^{2+} and Case 2 showed about 90% of maximum activity at this level (Fig. 2). Abnormal prolidase against phe-pro was always reduced by preincubation irrespective of the level of Mn^{2+} . However, both cases still required 60 min preincubation for gly-pro, but maximum prolidase activity only required 0.05 mmol/l Mn^{2+} . Previous studies have used different conditions for Mn^{2+} ranging from no Mn^{2+} [5], 10 mmol/l [4] or 16 mmol/l [6] with no preincubation to 1 mmol/l preincubated for 2 h [3,7] or 24 h [8] or 10 mmol/l for 1 h at 37°C [3,6]. Based upon the present data these variable Mn^{2+} conditions would partly explain the very variable prolidase

levels reported [3-9]. In addition, Mn^{2+} conditions optimised for control prolidase are not necessarily those needed by the abnormal enzyme.

Substrate specificity

Skin fibroblast prolidase activated by preincubation for 5 min at 37°C with 1.33 mmol/l Mn^{2+} showed a tight range of values for all the eight substrates (20

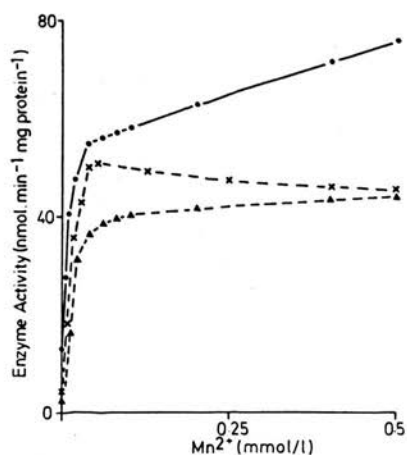


Fig. 1. Effect of preincubation with 1.33 mmol/l Mn^{2+} at 37°C on control prolidase activity against gly-pro (...), phe-pro (-) and leu-pro (- -).

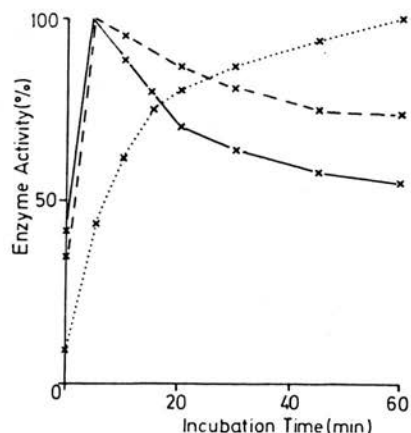


Fig. 2. Effect of preincubation with different Mn^{2+} levels for 5 min at 37°C on control (●), Case 1 (x) and Case 2 (▲) prolidase activity against phe-pro.

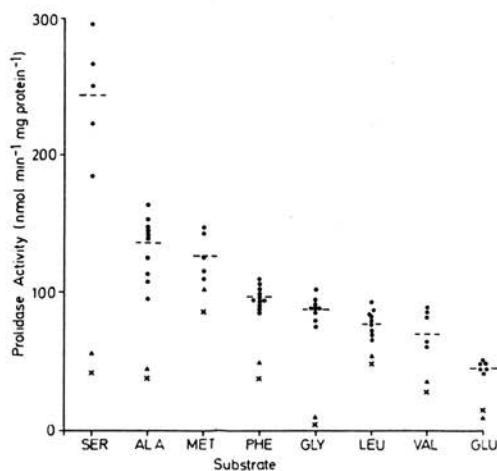


Fig. 3. Activity of control (●), Case 1 (x) and Case 2 (▲) skin fibroblast prolidase (- - -; mean activity) following preincubation with 1.33 mmol/l Mn^{2+} for 5 min at 37°C against 20 mmol/l substrate (x-pro; x = amino acid shown).

mmol/l) used (Fig. 3). The highest specific activity was against ser-pro, whilst the lowest was against glu-pro, the only substrate not requiring Mn^{2+} . Activity against gly-pro, when fully activated by 60 min preincubation with 2.5 mmol/l Mn^{2+} , would be increased about 2.5 times to a mean specific activity of 220 $nmol \cdot min^{-1} \cdot mg^{-1}$ protein. The reported mean specific activity ($nmol \cdot min^{-1} \cdot mg^{-1}$ protein) of skin fibroblast prolidase ranged from 7.2 [5], 20 [4], 50 [8] to 154 [9], all less than the present level, and, in part, reflects the variable Mn^{2+} conditions used. Abnormal prolidase activity against gly-pro has consistently been reported [3–10] to be absent or very low and in the present study activity in the two cases was reduced to 3–6% depending upon the Mn^{2+} conditions. The reduction in activity against the other substrates was not as marked ranging from 27 to 80% (Fig. 3). The enzyme deficiency against substrates with a polar amino acid (gly, ser, glu) was greater than against those with a non-polar amino acid (ala, val, phe, leu, met).

Substrate kinetics

The response of normal and abnormal prolidase (Case 2), following preincubation with 1.33 mmol/l Mn^{2+} for 5 min at 37°C, to a number of substrates was analysed by Eadie–Hofstee plots [15] and the results for corrected K_m and V_{max} given in Table I. The estimate of data variation [SD(E)rad; 15] for all substrates was less than 0.07 indicating the results to be acceptable. The normal enzyme exhibited biphasic kinetics against phe-pro, whilst the abnormal enzyme showed only a single line. The abnormal enzyme had a lowered affinity for all the substrates with the smallest change being for glu-pro and the largest for ser-pro, phe-pro and leu-pro. The maximum activity of the abnormal enzyme, calculated from the Eadie–Hofstee plots was increased for met-pro, unchanged for phe-pro and leu-pro, somewhat reduced for ser-pro and val-pro and most reduced for ala-pro, glu-pro and gly-pro. Owing to the altered K_m values the deficiency in abnormal prolidase activity would be more marked the lower the substrate level used. The substrate (gly-pro) level used in previous work has varied from 1 [4], 3.7 [3,7], 7 [5], 10 [6] to 47 mmol/l [8] which

TABLE I

Substrate kinetics of control and Case 2 prolidase

Substrate	K_m		V_{max}	
	Control	Case	Control	Case
Ser-pro	3.8	36.2	279	142
Ala-pro	2.3	8.1	221	62
Met-pro	4.5	14.4	147	210
Phe-pro	2.8, 6.8	24.9	136	147
Gly-pro	2.8	13.7	150	20
Leu-pro	1.3	10.4	80	72
Val-pro	0.9	3.3	68	40
Glu-pro	4.4	6.1	33	6

1–20 mmol/l substrate analysed by Eadie–Hofstee plots [15]

would differentially affect the level of control and abnormal prolidase. As with Mn^{2+} conditions, the level of substrate optimised for the assay of control prolidase would not be optimal for the measurement of the abnormal enzyme.

Analysis of product formation using phe-pro as substrate by the method of Chrastil and Wilson [16] indicated the abnormal enzyme had an unchanged n (sterical structure constant) value of over 0.9 and p_{∞} (maximum product formation) value of 0.35 mmol/l, but the k (rate constant related to diffusion coefficient) was reduced to 0.0023 as compared to 0.0086 $l \cdot g^{-1} \cdot s^{-1}$ of the normal enzyme. The data indicated no major alteration in enzyme structure or absorption affinity for the substrate, but faulty activation or presence of inactive enzyme.

Heat stability

Both normal and abnormal prolidase activity against phe-pro were thermolabile above 45°C with only about 10% residual activity at 55°C after 5 min (Fig. 4A). The addition of Mn^{2+} increased the heat stability of normal, but decreased that of the abnormal enzyme. Following the loss of activity at 48°C with time showed (Fig. 4B) that 70–90% of control enzyme activity (example shown typical of six tested) was lost after 60 min without Mn^{2+} , but that with 1.33 mmol/l Mn^{2+} only about 25% was lost. The two cases (Case 2 shown) showed a similar pattern of loss without Mn^{2+} as control enzyme, but in the presence of 1.33 mmol/l Mn^{2+} the abnormal enzyme was initially even more labile. These results indicate that normal prolidase consists of two forms, one labile and one stable in the presence of Mn^{2+} and that for the abnormal enzyme the stable form is largely absent.

Effect of *p*-hydroxymercuribenzoate (PHMB)

The effect of PHMB on prolidase activity was tested, as PHMB binds to swine intestinal prolidase [18] and sulphhydryl reagents inhibit swine kidney prolidase [19]. The normal and abnormal prolidase activity against phe-pro was strongly inhibited by treatment with PHMB prior to incubation with Mn^{2+} . However, preincubation with 1.33 mmol/l Mn^{2+} protected the normal enzyme, but not the abnormal enzyme

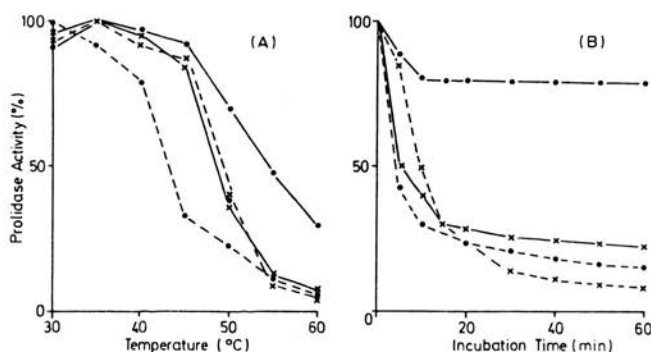


Fig. 4. Heat stability of control (—) and Case 2 (---) prolidase activity against phe-pro without (x) or with (●) 1.33 mmol/l Mn^{2+} . A. Effect of 5 min at 30–60°C. B. Effect of preincubation at 48°C for up to 60 min.

against PHMB inhibition (Fig. 5). Addition of PHMB and Mn^{2+} simultaneously gave an intermediate result for control prolidase with 0.05 mmol/l PHMB plus 1.33 mmol/l Mn^{2+} inhibiting 30% compared to < 5% when PHMB was added after the Mn^{2+} . The abnormal enzyme (Cases 1 and 2) was inhibited > 95% under both these conditions. A level of 0.05 mmol/l PHMB was, therefore, chosen to analyse the effect of PHMB on the normal and abnormal enzyme for a number of substrates (Table II). The addition of Mn^{2+} prior to PHMB protected normal prolidase activity for all the substrates, except for a small reduction with gly-pro and a moderate reduction with met-pro. However, the abnormal enzyme was markedly inhibited by PHMB for all the substrates, except for a moderate reduction of the small residual activity against gly-pro. Inhibition of swine kidney prolidase activity by iodoacetamide has previously been noted [19] to be prevented by Mn^{2+} . Since PHMB and iodoacetamide react with SH groups, it seems likely that Mn^{2+} activates prolidase by binding to SH groups.

TABLE II

Relative activity (%) of prolidase against different substrates following PHMB treatment

Substrate	Controls			Cases	
	1	2	3	1	2
Ser-pro	100	97	100	14	14
Ala-pro	92	98	100	0	4
Met-pro	45	53	56	4	3
Phe-pro	113	108	100	6	3
Gly-pro	76	81	80	58	58
Leu-pro	117	93	75	2	4
Val-pro	131	126	120	4	3

Prolidase activated by preincubation with 1.33 mmol/l Mn^{2+} for 5 min at 37°C and PHMB added with the substrate to give a concentration of 0.05 mmol/l.

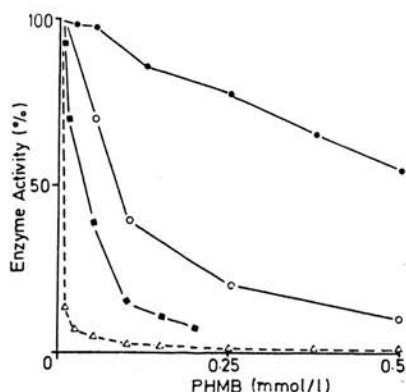


Fig. 5. Effect of PHMB on control (—) and abnormal (---) prolidase activity against phe-pro. ■ = PHMB added before Mn^{2+} ; ○ = PHMB added with Mn^{2+} ; ● = PHMB added after Mn^{2+} ; △ = PHMB added before or after Mn^{2+} .

Diagnostic properties of abnormal prolidase

The present data showed that the following properties can distinguish control and abnormal prolidase: (1) Mn^{2+} : control activated but abnormal inactivated by preincubation with high level of Mn^{2+} ; (2) substrates: abnormal enzyme reduced specific activities and increased K_m values compared to control; (3) heat stability: control more stable, but abnormal enzyme more labile to heat treatment in presence of Mn^{2+} and (4) PHMB: inhibition of control prevented, but abnormal enzyme not protected by Mn^{2+} . Any one of these properties could be used to detect the abnormal prolidase in cultured skin fibroblasts. The results could be explained by either a change in the properties of a single form of prolidase or the presence of two forms of prolidase at least one of which is altered. Although only a single form of prolidase was found in erythrocytes using gly-pro as substrate [2], the present results indicate that a second form of the enzyme would not be able to hydrolyse gly-pro efficiently and would require the use of another substrate for detection. Which of the possibilities is correct will be resolved by the use of chromatographic procedures and detection of prolidase with a number of substrates.

Acknowledgements

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Prolidases of human skin fibroblasts

JOHN BUTTERWORTH and DAVID A. PRIESTMAN

Department of Genetic Pathology, Royal Hospital for Sick Children and University of Edinburgh, Edinburgh EH9 1LF, Scotland, U.K.

Prolidase deficiency (McKusick 26413) is an autosomal recessive disease of humans associated with chronic ulcerative dermatitis, mental retardation and iminodipeptiduria (Scriver, 1977). In the disease, cultured skin fibroblasts have been shown (Sheffield *et al.*, 1977; Arata *et al.*, 1979; Myara *et al.*, 1983) to have little or no prolidase (EC 3.4.13.9) activity against glycyl-L-proline. However, we have found (Butterworth & Priestman, 1984; Priestman & Butterworth, 1984) that the activity against other substrates was only reduced 30–80%, with the reduction being the largest for *N*-terminal polar amino acids.

Control prolidase was activated by the addition of Mn^{2+} with the substrate and even more so after preincubation with Mn^{2+} . However, the abnormal enzyme required a much lower level of Mn^{2+} when added with the substrate and was inactivated by preincubation with the usual level of Mn^{2+} . The affinity of the abnormal enzyme for a number of substrates was reduced with, for example, the K_m for leucyl-L-proline being increased from 1.3 to 10.4 mM. Heat stability (48°C) of normal prolidase activity was increased, but abnormal prolidase activity decreased in the presence of Mn^{2+} . Inhibition of normal prolidase by *p*-hydroxymercuribenzoate (0.05 mM) was largely prevented by preincubation of the enzyme with Mn^{2+} , whereas the abnormal enzyme was almost totally inactivated whether or not Mn^{2+} was present. These results indicate that either a single form of prolidase is altered kinetically or that there are two forms of prolidase, at least one of which is altered in the disease.

To decide between these two alternatives, the separation of fibroblast prolidase into different forms was attempted by using ion-exchange chromatography. Fibroblasts were cultured and cell extracts prepared as before (Butterworth, 1982; Butterworth & Priestman, 1982). DEAE-cellulose ion-exchange chromatography was carried out as before (Butterworth & Priestman, 1982), except the sample was applied without prior dialysis and prolidase assayed with a number of substrates (Butterworth & Priestman, 1984). Two peaks of prolidase activity were obtained eluting at 135 and 220 mM-NaCl (Fig. 1). Peak 1 was very active against glycyl-L-proline compared with phenylalanyl-L-proline, whereas peak 2 was relatively less active against glycyl-L-proline. The activity against phenylalanyl-L-proline was split about 60% peak 1 and 40% peak 2. Both peaks were also active against leucyl-L-proline, alanyl-L-proline, valyl-L-proline and methionyl-L-proline. A previous inability to detect the second peak in fibroblasts (Butterworth & Priestman, 1984) was due to dialysis of the extract before chromatography, as it was found that peak 2 activity was lost by dialysis. Although a sulfhydryl reagent was not needed for peak 2 activity, the inclusion of 0.1 mM-dithiothreitol during dialysis prevented its loss. We found that human erythrocytes contained about 10% peak 2 activity against phenylalanyl-L-proline, and the previous failure (Endo *et al.*, 1982) to demonstrate this peak 2 activity was probably due to the low level measurable with glycyl-L-proline and the preparative procedure used. The fibroblast prolidase activity of two unrelated cases with prolidase-deficiency showed almost a total lack of peak 1 activity against all the substrates, whereas peak 2 showed much reduced activity

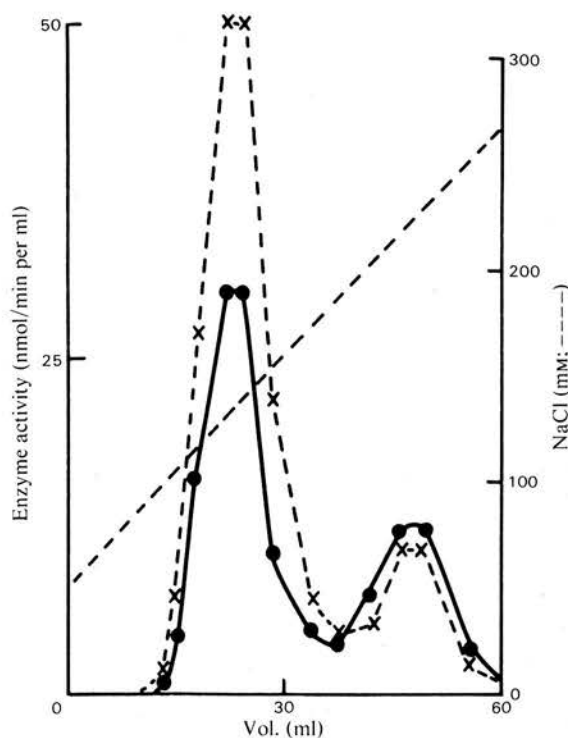


Fig. 1. Cultured skin fibroblast prolidase separated by DEAE-cellulose chromatography

Prolidase was assayed with glycyl-L-proline (×---×) and phenylalanyl-L-proline (●—●).

against glycyl-L-proline, but not the other substrates tested. The abnormal prolidase second peak showed a reduced affinity for phenylalanyl-L-proline and decreased heat stability (48°C), as compared with that of the control. Hence there are two forms of prolidase in cultured skin fibroblasts, both showing changes in the disease indicating them to be inter-related.

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Molecular differences between the human and pig erythrocyte nucleoside transporters

FRANCIS Y. P. KWONG,* YUEN-MIN CHOY,*
SIMON M. JARVIS† and JAMES D. YOUNG*

*Department of Biochemistry, Faculty of Medicine,
The Chinese University of Hong Kong, Shatin, N.T., Hong
Kong, and †Department of Physiology, University of Alberta,
Edmonton, Alberta, Canada T6G 2H7

The entry of nucleosides into mammalian cells occurs largely by nucleoside-specific facilitated diffusion mechanisms which can be selectively inhibited by nanomolar concentrations of NBMPR (Young & Jarvis, 1983). Inhibition of nucleoside transport by NBMPR is associated with reversible high-affinity binding of inhibitor to the carrier mechanism (apparent K_D 0.1–1 nM). This binding is normally reversible. However, exposure of site-bound [3H]NBMPR to high-intensity u.v. light results in the specific covalent radiolabelling of the transporter (Wu *et al.*, 1983; Young *et al.*, 1983; Shi *et al.*, 1984). We report here a comparison of radiolabelled nucleoside transporters from human and pig erythrocytes.

Human and pig erythrocyte membranes were photoaffinity labelled with [3H]NBMPR under equilibrium binding conditions in the absence and in the presence of excess non-radioactive NBTGR as described previously (Wu *et al.*, 1983). The radiolabelled membranes were then subjected to SDS/polyacrylamide-gel electrophoresis by the method of Thompson & Maddy (1982). Gel-associated radioactivity was determined by slicing gels into 2 mm fractions and measuring the 3H content of these slices by liquid scintillation counting (Wu *et al.*, 1983).

Fig. 1 compares the SDS/polyacrylamide-gel 3H profiles of radiolabelled human and pig erythrocyte membranes run on the same slab gel. Membranes from human species gave a major peak of radiolabelling in the band 4.5 region of the gel (M_r 45 000–66 000 [nomenclature of Steck (1974)]). However, the two radiolabelled transporters migrated with significantly different apparent M_r values, the pig protein having the higher apparent M_r . Covalent labelling of these polypeptides was abolished when photolysis was performed in the presence of NBTGR (data not shown). The minor high and low M_r peaks in Fig. 1 represent aggregates of the transporters and degradation products, respectively. Both the human and pig erythrocyte membrane preparations exhibited non-specific labelling in the lipid region of the gel.

The human and pig erythrocyte nucleoside transporters were also found to behave differently during DEAE-cellulose ion-exchange chromatography of n-octyl glucoside membrane extracts. For these experiments, human and pig erythrocyte membranes were depleted of extrinsic membrane proteins and photoaffinity labelled with [3H]NBMPR as before except that photoactivation was performed in the absence of unbound 3H -labelled ligand (non-equilibrium binding conditions) to minimize the possibility of non-specific labelling (e.g. of membrane lipid) (Wu *et al.*, 1983). Under these photolysis conditions, more than 80% of the covalently bound radioactivity represented specific radiolabelling of carrier protein. Samples were solubilized with 46 mM-n-octyl glucoside in 50 mM-Tris/HCl, 2 mM-dithiothreitol (pH 7.4 at 4°C) and the 130 000g supernatant applied to DEAE-cellulose columns equilibrated with the same buffer. Proteins bound to the columns were eluted with buffer containing 1 M-NaCl.

For human erythrocyte membrane extracts, the majority of applied radioactivity (75%) was recovered in the column

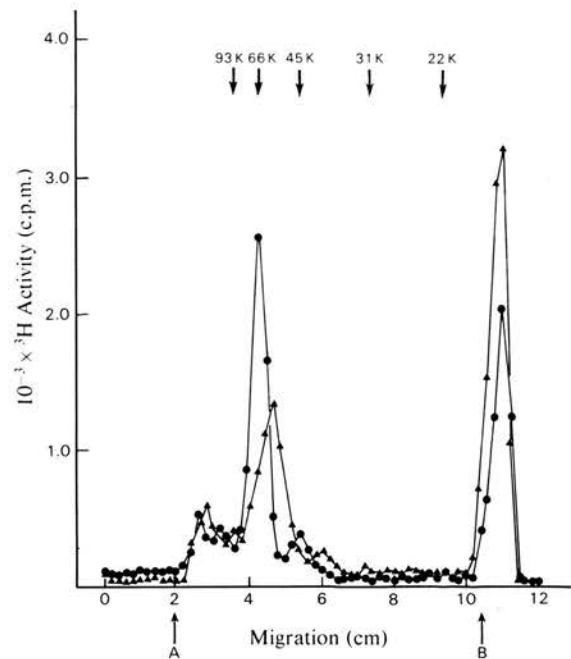


Fig. 1. Comparison of the photoaffinity labelling of human and pig erythrocyte membranes with [3H]NBMPR

Human (\blacktriangle) and pig erythrocyte 'ghosts' (\bullet) were equilibrated with 75 nM-[3H]NBMPR, supplemented with dithiothreitol (final concentration 50 mM), and exposed to high-intensity u.v. light from a 450 W mercury arc lamp (Wu *et al.*, 1983) for 45 s. Unreacted [3H]NBMPR was removed by washing and the washed membrane pellets (100 μ g of protein) electrophoresed in a 12% SDS/polyacrylamide-gel as described in the text. M_r standards are from the same slab gel. The positions of the stacking gel-running gel interface and the tracking dye are indicated by A and B, respectively.

void-volume fractions containing only 9% of the applied protein. The 1 M-NaCl fractions contained large amounts of protein (mostly band 3), but little radioactivity. There was therefore an 8-fold purification of radiolabelled transporter during ion-exchange chromatography, a value similar to that obtained by Jarvis & Young (1981) using an assay based on reversible [3H]NBMPR-binding activity. In contrast, void-volume fractions from pig erythrocyte membrane extracts contained only traces of radioactivity. The majority of applied 3H and protein eluted in the 1 M-NaCl fractions.

We conclude from these experiments that there are significant molecular differences between the human and pig erythrocyte nucleoside transporters. Isolation techniques devised for the partial purification of the human erythrocyte transporter (Jarvis & Young, 1981) are not appropriate for the pig system.

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Abbreviations used: NBMPR, nitrobenzylthioinosine; NBTGR, nitrobenzylthioguanosine; SDS, sodium dodecyl sulphate.

Presence in Human Cells and Tissues of Two Prolidases and their Alteration in Prolidase Deficiency

J. BUTTERWORTH and D. A. PRIESTMAN

Department of Pathology, Royal Hospital for Sick Children and University of Edinburgh, Edinburgh EH9 1LF, UK

Two forms of prolidase can be separated for all the human cells and tissues examined by DEAE-cellulose column chromatography or batch methods. Serum had a very low prolidase activity eluting as a single peak prior to tissue peak I prolidase. Analysis of the two peaks can readily be carried out using white blood cells, cultured skin fibroblasts and amniotic fluid cells. Dialysis inactivated peak II prolidase although the loss can be prevented by the presence of dithiothreitol. The two peaks differed in their response to Mn^{2+} , substrate specificity, heat stability and inhibition by *p*-hydroxymercuribenzoate. In two unrelated cases of prolidase deficiency, fibroblast peak I was markedly reduced, although still detectable, whereas peak II was active against all the substrates, except for a 90% reduction against glycyl-L-proline. The properties of peak II were altered in the disease. The results imply that the two forms of prolidase are structurally related.

Prolidase (EC 3.4.13.9) deficiency (McKusick 26413) is an autosomal recessive disease associated with chronic ulcerative dermatitis, mental retardation and urinary excretion of iminodipeptides (Myara *et al.*, 1984). Although activity against the substrate glycyl-L-proline has often been found to be almost totally deficient in the disease (Butterworth and Priestman, 1984; Myara *et al.*, 1984), activity against other substrates was not so deficient (Butterworth and Priestman, 1984; Priestman and Butterworth, 1984). Properties of residual prolidase in the disease have been shown to be different to control enzyme from cultured skin fibroblasts (Priestman and Butterworth, 1984). The present study shows by ion-exchange chromatography that different tissues have different proportions of two enzymes capable of hydrolysing amino acid-proline substrates and that both enzymes are altered in the disease.

MATERIALS AND METHODS

Skin fibroblasts were obtained from a male Turkish patient (Case 1) (Pedersen *et al.*, 1983) and a male Italian patient (Case 2) (Sheffield *et al.*, 1977) with prolidase deficiency. Fibroblasts and amniotic fluid cells were cultured as previously described (Butterworth, 1982; Butterworth and Priestman, 1984). Red and white blood cells were obtained from venous blood from children by dextran sedimentation (Snyder and Brady, 1969) and washed three times with 155 mmol L^{-1} sodium chloride. Tissues were obtained at autopsy within 48 h of death from children aged less than 1 year old. All cells and tissues were stored at -70°C until required. Cell and tissue extracts were prepared in 10 mmol L^{-1} veronal-HCl buffer pH 7.4 with 50 mmol L^{-1} sodium chloride and centrifuged at $100\,000\text{ g}$ for 60 min (Butterworth and Priestman, 1982), or without sodium chloride and centrifuged at 8800 g for 5 min (Eppendorf 5413 Centrifuge).

The separation of cell and tissue prolidase into two components was achieved by applying 1 ml (10% w/v)

tissue extract, 1 ml red blood cell extract or serum (50 mg protein ml^{-1}) or 1 ml fibroblast extract (5 mg protein ml^{-1}) in 10 mmol L^{-1} veronal-HCl buffer pH 7.4 containing 50 mmol L^{-1} sodium chloride to a $0.8 \times 5\text{ cm}$ DEAE-cellulose column, eluting with a $50\text{--}300\text{ mmol L}^{-1}$ sodium chloride gradient and collecting 1.5 ml fractions. A DEAE-cellulose batch method was developed for the separation and estimation of the two prolidase activities. A mixture of 0.25 ml 50% (v/v) DEAE-cellulose slurry in 10 mmol L^{-1} veronal-HCl buffer pH 7.4, 0.125 ml sodium chloride and 0.125 ml 2% (w/v) extract in 10 mmol L^{-1} veronal-HCl buffer pH 7.4 was mixed for 15 min at 10°C , centrifuged at 1000 g for 10 min and the amount of unbound prolidase estimated against phenylalanyl-L-proline. The addition of 480 mmol L^{-1} sodium chloride released peak I prolidase and 1000 mmol L^{-1} sodium chloride released both peaks and their sequential use allowed the separation of both peaks.

Prolidase activity was measured using a variety of substrates (glycyl-L-proline, Gly-Pro; phenylalanyl-L-proline, Phe-Pro; alanyl-L-proline, Ala-Pro; valyl-L-proline, Val-Pro and leucyl-L-proline, Leu-Pro; Sigma Ltd) by estimating liberated proline or phenylalanine (Mayer and Nordwig, 1973; Butterworth and Priestman, 1984; Priestman and Butterworth, 1984) and related to protein using bovine serum albumin as standard (Lowry *et al.*, 1951). The properties of the two peaks were investigated as previously described (Priestman and Butterworth, 1984).

RESULTS AND DISCUSSION

DEAE-cellulose chromatography

Using DEAE-cellulose column chromatography two peaks of prolidase were eluted at 135 and 210 mmol L^{-1} sodium chloride with a $>70\%$ recovery (Figure 1). Dialysis of a sample against buffer inactivated peak II but the inclusion of 0.1 mmol L^{-1} dithiothreitol during dialysis prevented this loss. This would explain previous

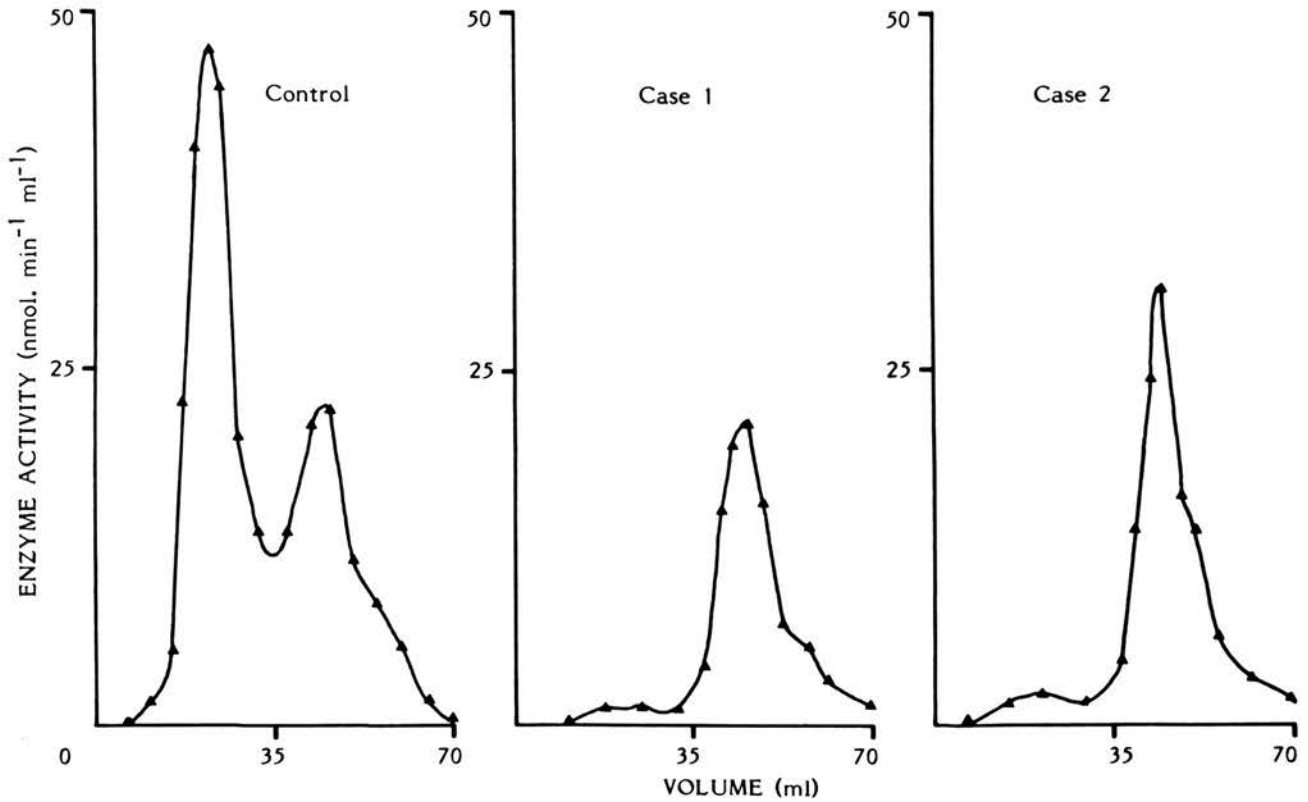


Figure 1 DEAE-cellulose column chromatography of control, Case 1 and Case 2 fibroblast prolidase activity against Phe-Pro. Peaks I and II are named in order of elution. Samples were preincubated at 37°C for 5 min with 1 mmol L⁻¹ Mn²⁺

failures to detect peak II activity (Butterworth and Priestman, 1984; Myara *et al.*, 1984). All the control cells and tissues examined had the two peaks of prolidase, whilst serum with a very low activity (1 nmol min⁻¹ (mg protein⁻¹)) showed a single peak eluting prior to the position of peak I at about 85 mmol L⁻¹ NaCl. A marked reduction in peak I activity, but an unchanged peak II activity, was found in fibroblasts from the two cases of prolidase-deficiency (Figure 1). The first of the two peaks, therefore, corresponds to the prolidase purified from red blood cells by Endo *et al.* (1982).

The DEAE-cellulose batch method gave a recovery of >95% of total activity. The reproducibility of the batch method, as checked by estimating the two peaks ten times for one frontal cortex extract, was excellent with the coefficient of variation for peak I being 2% and for peak II 3%. Hence estimation of the two peaks should be more reliable, besides being quicker and easier, with the batch than the column method. Following separation the two peaks could be mixed together to give the expected activity indicating no interference with each other in the assay. The specific activities against Phe-Pro and the percentage of peak I determined by the batch method for the different cells and tissues are given in Table 1. A 2% (w/v) extract was needed for the tissues, except for kidney and pancreas (1% w/v), and about 1 mg protein for the cells. An assay time of 20 min was required for all the enzyme sources, except for red blood cells (30 min). The percentages of peak I in control cells and tissues determined by the DEAE-cellulose column

method were similar to the values given for the batch method. The high level of peak II prolidase in the pancreas indicates a particularly important role for this enzyme in the pancreas. The percentage of residual peak I activity in the two cases was higher for the batch than the column method. The greater loss of enzyme in the

Table 1 Cell and tissue prolidase activity against Phe-Pro* and percent peak I estimated by the DEAE-cellulose batch method

Sample	Specific activity	% Peak I
Fibroblasts:		
Controls, n = 10	100 ± 8†	53 ± 4
Case 1	42	16
Case 2	57	11
Amniotic fluid cells,		
n = 10	108 ± 15	55 ± 3
Red blood cells	5.7, 6.1, 6.7	68, 73
White blood cells	54, 58, 64, 66	42, 42, 44, 50
Kidney	190, 213, 236, 291	60, 68, 73
Liver	56, 63	77, 77
Spleen	57, 71	68, 74
Pancreas	235, 287	24, 34
Cerebral frontal cortex		
n = 10	108 ± 16	40 ± 4
Heart	64, 77	53, 61
Skeletal muscle	55	50

* nmol min⁻¹ (mg protein⁻¹); enzyme preincubated 5 min with 1 mmol L⁻¹ Mn²⁺

† Mean ± SD

Table 2 Activity, relative % and residual percent of prolidase peaks of control and Case 1 prolidase-deficient fibroblasts separated by the DEAE-cellulose batch method

Substrate	Control				Case 1						
	Peak I		Peak II		Activity	Peak I		Residual %‡	Activity	Peak II	
	Activity*	Relative %†	Activity	Relative %	Activity	Relative %	Relative %‡	Activity	Relative %	Residual %	
Phe-Pro	4.5	100	3.2	100	0.40	100	9	3.6	100	113	
Ala-Pro	3.1	69	2.6	81	0.11	28	4	2.5	69	96	
Ile-Pro	3.3	73	3.0	94	0.26	65	8	3.4	95	113	
Val-Pro	9.4	211	4.3	135	0.16	40	2	4.3	119	100	
Gly-Pro	16.4	365	3.4	106	0.46	115	3	0.34	9	10	

*nmol min⁻¹ ml⁻¹; peak I preincubated with 1 mmol L⁻¹ Mn²⁺ 30 min, except Gly-Pro 60 min; peak II no preincubation, 1 mmol L⁻¹ Mn²⁺ with substrate, except Gly-Pro 60 min preincubation
 †relative %: activity against substrate relative to Phe-Pro
 ‡residual %: case peak activity relative to control peak activity

column method possibly resulted in an underestimate of abnormal peak I. The specific activity and percentage of the two peaks for cultured skin fibroblasts and amniotic fluid cells were similar indicating that a prolidase abnormality detected in skin fibroblasts should also be present in amniotic fluid cells.

Properties

Scaling up the DEAE-cellular batch method allowed the rapid preparation of the two peaks with excellent recovery permitting analysis of both prolidase peaks from control and prolidase-deficient fibroblasts.

Substrates (Table 2)

Control peak I prolidase hydrolysed Ala-Pro, and particularly Gly-Pro, at a faster rate than peak II. As shown for Case 1, peak I residual activity against all five substrates was low compared with the control. Peak II relative and residual activities were similar to those of the control, except that both of these parameters were reduced to about 10% for Gly-Pro. The previous finding (Priestman and Butterworth, 1984) that the enzyme in

prolidase-deficient fibroblast extracts showed changes in the rate of hydrolysis of a number of substrates can be explained by the changed rate at which both peaks of abnormal prolidase hydrolyse the various substrates. Indeed the much reduced ability of abnormal peak II to hydrolyse Gly-Pro as compared with the other substrates explains why only activity against Gly-Pro showed a marked deficiency in an abnormal fibroblast extract (Priestman and Butterworth, 1984). Reports (Gray *et al.*, 1983; Pedersen *et al.*, 1983; Butterworth and Priestman, 1984; Naughton *et al.*, 1984; Priestman and Butterworth, 1984) showing residual activity against Gly-Pro in prolidase deficiency can be attributed to the use of assay conditions capable of detecting peak II activity.

Mn²⁺ Response (Figure 2)

Control peak I activity towards Phe-Pro was optimal following 10–20 min preincubation at 37°C with Mn²⁺, but was reduced by preincubation without Mn²⁺ (Figure 2(A), (B)). In contrast, control peak II was inactivated by preincubation with Mn²⁺ (Figure 2(A))

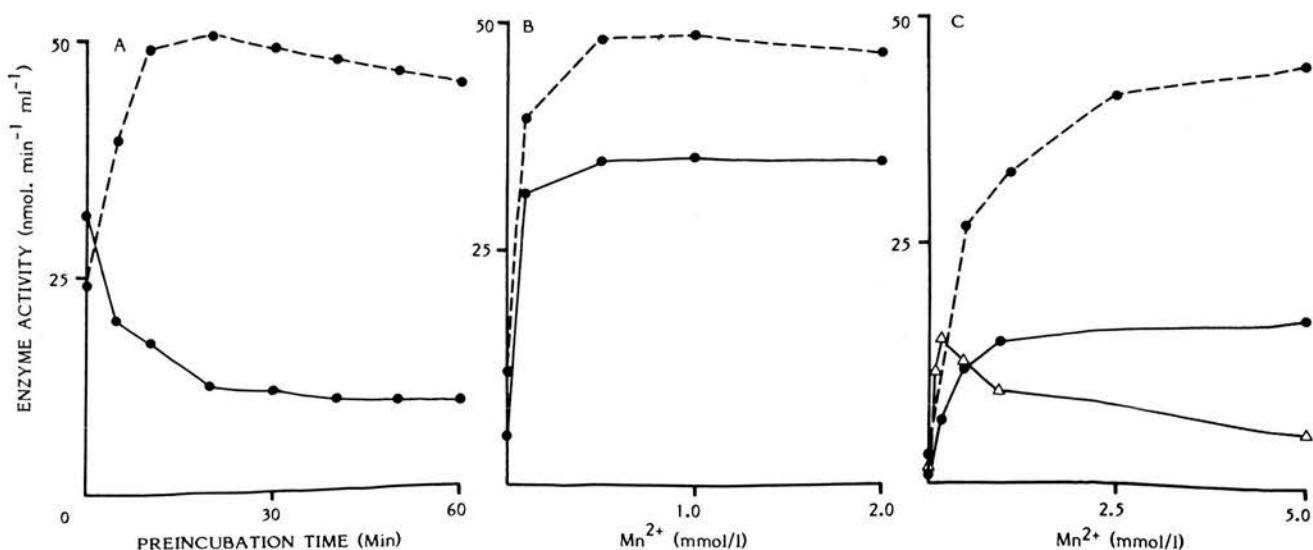


Figure 2 Effect of Mn²⁺ on control (●) and abnormal (△) peak I (---) and peak II (—) fibroblast prolidase activity. (A) Phe-Pro: preincubated with 1 mmol L⁻¹ Mn²⁺ for up to 60 min. (B) Phe-Pro: peak I preincubated 20 min with Mn²⁺; peak II Mn²⁺ added with substrate. (C) Gly-Pro: preincubated 60 min with Mn²⁺

Table 3 K_m values of control and abnormal fibroblast prolidase

Substrate	Source	Preincubation (min)	Mn^{2+} (mmol L ⁻¹)	K_m
Gly-Pro	Control extract	5	5	2.5
	Control extract	60	5	9.0
	Control peak I	60	5	17
	Control peak II	60	5	7
	Case 2 peak II	60	5	33
Phe-Pro	Control peak I	30	1	0.5
	Control peak II	0	1	8
	Case 1 peak II	0	1	16
	Case 2 peak II	0	1	36

Enzyme was preincubated with Mn^{2+} or Mn^{2+} added with substrate (0–20 mmol L⁻¹)

and optimal activity was obtained by the addition of 0.5–1 mmol L⁻¹ Mn^{2+} with the substrate (Figure 2(B)). The two abnormal peaks had the same responses, except that peak II was even more unstable in the presence of Mn^{2+} . Full activity against Gly-Pro for both control peaks required preincubation with 5 mmol L⁻¹ Mn^{2+} for 60 min (Figure 2(C)). The small amount of peak I in the cases also needed 5 mmol L⁻¹ Mn^{2+} , but peak II only required 0.1 mmol L⁻¹ Mn^{2+} (Figure 2(C)). Hence the failure of prolidase-deficient fibroblast activity against Phe-Pro to be increased by Mn^{2+} preincubation (Butterworth and Priestman, 1984; Priestman and Butterworth, 1984) can be explained by the loss of Mn^{2+} -activated peak I in the disease.

Substrate K_m (Table 3)

The response of normal and abnormal prolidase to a number of substrates was analysed by Eadie–Hofstee plots and the K_m values are given in Table 3. The

estimate of data variation (SD(E)Rad; Zivin and Waud, 1982) for both substrates was less than 0.07, indicating the results to be acceptable. There was insufficient abnormal peak I for the reliable estimation of K_m values. The results indicate that peak II prolidase of the cases had decreased affinities for both Gly-Pro and Phe-Pro. Decreased affinities for eight substrates have previously been reported (Priestman and Butterworth, 1984) for abnormal fibroblast prolidase. The present results for Gly-Pro and Phe-Pro would indicate that this finding was due to the loss of peak I and the increase in the K_m values for peak II in the disease.

Heat stability (Figure 3)

At 48°C loss of control peak I activity against Phe-Pro was completely prevented by the presence of 1 mmol L⁻¹ Mn^{2+} , whereas control peak II activity was only partially protected. However, abnormal peak II was very labile in Case 1 whether or not Mn^{2+} was

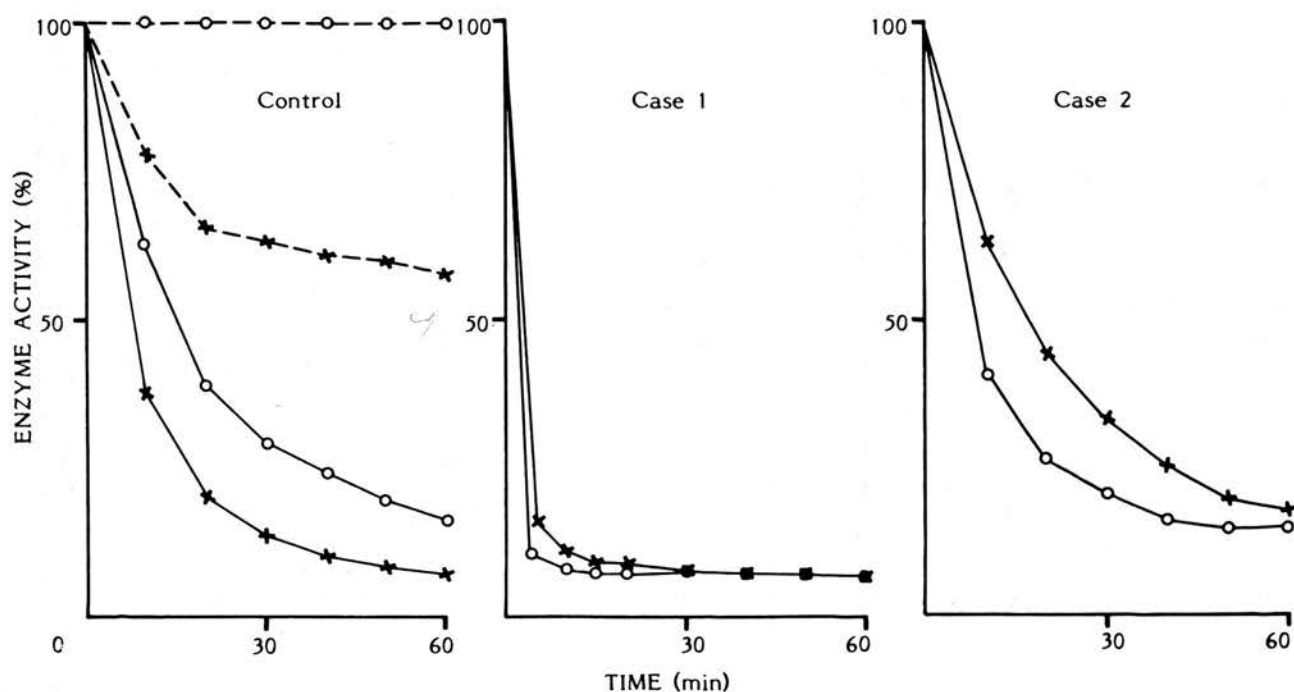


Figure 3 Effect of heat (48°C) on control, Case 1 and Case 2 fibroblast prolidase peak I (---) and peak II (—) in the presence (○) or absence (×) of 1 mmol L⁻¹ Mn^{2+} and assayed with Phe-Pro

present, whilst in Case 2 the activity was more stable in the absence of Mn^{2+} and more labile in the presence of Mn^{2+} than control peak II. Similar, but smaller, changes were observed at 37°C. Loss of Mn^{2+} stabilized peak I and decreased stability of peak II explains the reduced heat stability of prolidase-deficient fibroblast activity (Priestman and Butterworth, 1984).

Para-hydroxymercuribenzoate (PHMB) inhibition

Control peak I preincubated with 1 mmol L⁻¹ Mn^{2+} for 30 min lost about 20% of activity against Phe-Pro when treated with 0.05 mmol L⁻¹ PHMB. Control peak II activity towards Phe-Pro, which does not require Mn^{2+} preincubation, was inhibited 90% by 0.05 mmol L⁻¹ PHMB, whilst the activity against Gly-Pro, which does require Mn^{2+} preincubation, was only inhibited 50% following preincubation with 1 mmol L⁻¹ Mn^{2+} for 60 min. However, irrespective of conditions of treatment peak II activity of both cases against Phe-Pro and Gly-Pro was completely abolished by 0.05 mmol L⁻¹ PHMB. Protection of human fibroblast peak I prolidase activity by Mn^{2+} against PHMB inhibition suggests that Mn^{2+} binds to prolidase via sulphhydryl groups, as found for swine kidney prolidase (Davis and Smith, 1953). The inability of Mn^{2+} to protect peak II activity and the need to add Mn^{2+} with the substrate indicates that at least part of the Mn^{2+} effect on peak II activity involves interaction with the substrate. The loss of peak I explains the failure of Mn^{2+} to protect prolidase-deficient fibroblast activity against PHMB (Priestman and Butterworth, 1984).

Implications

The finding of a second form of prolidase can explain the presence of a moderate prolidase activity in prolidase deficiency cells (Butterworth and Priestman, 1984; Priestman and Butterworth, 1984). The differences in the properties of the two prolidases and their changes in the disease mean that particular attention has to be paid to their estimation. This can most easily be achieved by the use of the DEAE-cellulose batch method followed by appropriate assay conditions. Alteration of both prolidases in prolidase-deficiency indicates a structural relationship. As prolidase (peak I) is known (Endo *et al.*, 1982) to consist of two subunits, the most likely explanation is a common subunit, which is changed in the disease such as to alter substrate hydrolysis, but further work on the purified enzymes will be needed to answer this point.

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Intracellular Activity of HPRT_{Cape Town}: Purine Uptake and Growth of Cultured Cells in Selective Media

L. M. STEYN¹ and E. H. HARLEY²

Department of Chemical Pathology, University of Cape Town Medical School, Observatory, 7925, South Africa

The low activity of the human variant HPRT_{Cape Town} is associated with substrate inhibition by hypoxanthine and guanine *in vitro*. The intracellular activity of this variant was investigated by studying the relative uptake of radiolabelled purine nucleotide precursors and the growth in selective media of EBvirus-transformed lymphoblasts prepared from the proband (TK). These cells incorporated less than 10% of the [¹⁴C]hypoxanthine and [¹⁴C]guanine of the control cells; while their purine *de novo* synthesis was accelerated 8-fold. In selective media the HPRT_{Cape Town} cells grew in a similar manner to HPRT-ve cells. These results indicate that if substrate inhibition is responsible for the low intracellular activity of HPRT_{Cape Town}, the concentration of either hypoxanthine or guanine in the vicinity of the active site of the enzyme must be greater than the $K_{i(\text{app})}$ for these substrates, 118 and 28 $\mu\text{mol L}^{-1}$ respectively. Evidence is presented that the intracellular concentration of guanine, but not hypoxanthine, is well in excess of the $K_{i(\text{app})}$ in cultured lymphoblasts.

Hypoxanthine–guanine phosphoribosyltransferase (EC 2.4.2.8) (HPRT) catalyses the salvage of the purine bases hypoxanthine and guanine to form the product nucleotides IMP and GMP with 5-phosphorylribose-1-pyrophosphate, PP-ribose-P, acting as the phosphoribosyl donor. In man, hereditary deficiency of this enzyme activity is associated with purine overproduction and gout. The Lesch–Nyhan syndrome, with its characteristic neurological features of choreoathetosis, mental retardation and compulsive self-mutilation, is found in patients with the lowest intracellular enzyme activities (Page *et al.*, 1981).

Several low activity variants of human HPRT have been described and changes in various kinetic parameters and physical properties of these variants have been characterized. These include altered Michaelis constants for both purine base and phosphoribosyl pyrophosphate substrates (McDonald and Kelley, 1971; Benke *et al.*, 1973; Henderson *et al.*, 1976; Gutensohn and Jahn, 1979), increased intracellular enzyme lability (Balis *et al.*, 1974), increased susceptibility to product inhibition (Sperling *et al.*, 1974; Gutensohn and Jahn, 1979), a reduction in immunologically detectable HPRT protein (Upchurch *et al.*, 1975; Ghangas and Milman, 1975), and altered electrophoretic mobility (Bakay and Nyhan, 1972; Steyn and Harley, 1981; Wilson *et al.*, 1982). The molecular defect in HPRT_{London} has been defined as an amino acid substitution (Ser → Leu) at position 109 (Wilson *et al.*, 1983).

We have recently studied the low activity variant, HPRT_{Cape Town}, which is associated with purine overproduction and gout in the proband, TK (Cassidy *et al.*,

1980). Kinetic studies on the isolated enzyme have shown that the decreased activity is not caused by a change in the V_{max} or K_{m} of the enzyme for any of its substrates, but is associated with substrate inhibition by the purine bases, hypoxanthine and guanine (Steyn and Harley, 1984). The acquisition of substrate inhibition represents a most unusual association with a low enzyme activity in an inherited metabolic disorder. In HPRT_{Cape Town} it is not certain whether this property is the consequence of a mutation affecting the primary amino acid sequence of the enzyme, a mutation resulting in altered post-translational modification, or even the absence or alteration of a factor influencing normal HPRT kinetics.

In order to help resolve these questions the intracellular activity of HPRT_{Cape Town} was investigated in transformed lymphocytes by assessing the uptake of radioactive purines by these cells and by determining whether there were any secondary biochemical changes that could be attributed to disruption of the purine metabolic pathways. The incorporation of radioactive purine bases into these cells by the salvage pathway was found to be less than 10% of that of the control cell lines. In contrast the flux through the *de novo* purine synthetic pathway was eight times higher than in the normal cells and was similar to that found in HPRT-ve cells, obtained from a patient with the Lesch–Nyhan syndrome. Furthermore, the HPRT_{Cape Town} cells behaved like the HPRT-ve cells when they were cultured in various selective media.

MATERIALS

The following radioactive substrates were obtained from The Radiochemical Centre, Amersham, UK: [⁸⁻¹⁴C]hypoxanthine (55 mCi mmol⁻¹); [⁸⁻¹⁴C]guanine sulphate (51 mCi mmol⁻¹); [^{G-3}H]hypoxanthine (1.2 Ci mmol⁻¹); [^{G-3}H]guanine sulphate (7 Ci mmol⁻¹); [¹⁴C]formic acid, sodium salt

¹ Present address: Department of Medical Microbiology, University of Cape Town Medical School, Observatory, 7925, South Africa.

² Author to whom correspondence should be addressed.

Prolinase and non-specific dipeptidase of human kidney

David A. PRIESTMAN and John BUTTERWORTH

Department of Pathology, Royal Hospital for Sick Children and University of Edinburgh, Edinburgh EH9 1LF, Scotland, U.K.

Human kidney prolinase, assayed with Pro-Ala, and non-specific dipeptidase, assayed with Gly-Leu, were purified by using DEAE-cellulose, gel-filtration, metal-ion-chelate, hydrophobic and adsorption chromatography and chromatofocusing. Both enzymes gave single peaks of activity that were congruent and the ratio of their activities was constant throughout the purification. Gel filtration indicated an M_r of 100000 and chromatofocusing a pI of 5.4. Ni^{2+} -chelate chromatography demonstrated the presence of exposed histidine residues on the enzyme and was an effective separative procedure. Polyacrylamide-gel electrophoresis of the final preparation showed the two enzyme activities to be coincident. Both enzyme activities decayed at the same rate at 53 °C and were inhibited to the same extent by *p*-hydroxymercuribenzoate. Of six non-specific dipeptidase substrates tested Gly-Leu gave the highest activity, and of six prolinase substrates Pro-Leu had the highest activity. Gly-Leu was hydrolysed at double the rate of Pro-Leu. Pro-Ala was a competitive inhibitor of activity towards Gly-Leu, and Gly-Leu was a competitive inhibitor of activity towards Pro-Ala. Mixed-substrate studies strongly suggested that Gly-Leu and Pro-Ala were hydrolysed at a common active site. The data are consistent with prolinase and non-specific dipeptidase activity in human kidney being due to a single enzyme.

INTRODUCTION

Prolinase (EC 3.4.13.8) has been purified from a number of animal tissues (Sarid *et al.*, 1962; Mayer & Nordwig, 1973; Akrawi & Bailey, 1976; Reith & Neidle, 1979), and in all cases the final preparations had activity against non-specific-dipeptidase (EC 3.4.13.11) substrates, which was attributed to a contaminating enzyme. However, it has been proposed that the two activities may be due to a single enzyme (Hayman & Patterson, 1971). As kidney has been widely used in studies on dipeptidases from animals (Sarid *et al.*, 1962; Mayer & Nordwig, 1973; Akrawi & Bailey, 1976) and had the highest prolinase activity of the human organs tested (Butterworth & Priestman, 1982), prolinase was purified from human kidney. Increasing lability of dipeptidases with purification appears to preclude purification to homogeneity (Hayman & Patterson, 1971; Mayer & Nordwig, 1973). To ascertain whether human kidney prolinase and non-specific dipeptidase were the same enzyme, we studied properties of the enzyme, including substrate kinetics (Dixon & Webb, 1964, pp. 84–87), after purification by the use of a wide range of separative techniques.

MATERIALS AND METHODS

Chemicals

DEAE-cellulose was obtained from Whatman, Ultrogel AcA-44 was from LKB, butyl-agarose from Miles Laboratories, spheroidal hydroxyapatite was from BDH Chemicals, 4-methylumbelliferyl 2-acetamido-2-deoxy- β -D-glucopyranoside and 4-methylumbelliferyl α -D-mannopyranoside were from Koch-Light Laboratories, and all other chemicals were from Sigma Chemical Co.

Enzyme assays

Prolinase was assayed in 25 mM-barbital/HCl buffer, pH 9.2, containing 12.5 μ M-MnCl₂ and 10 mM-prolyl-L-

alanine (Pro-Ala), prolyl-L-glycine (Pro-Gly), prolyl-L-leucine (Pro-Leu), prolyl-L-phenylalanine (Pro-Phe), prolyl-L-valine (Pro-Val) or hydroxyprolylglycine (Hyp-Gly), and the liberated amino acid was determined fluorimetrically by using *o*-phthaldialdehyde (Butterworth & Priestman, 1982). Non-specific dipeptidase was assayed in 25 mM-barbital/HCl buffer, pH 8.0, containing 10 mM-glycyl-L-leucine (Gly-Leu), L-alanyl-L-leucine (Ala-Leu), L-valyl-L-leucine (Val-Leu), L-seryl-L-leucine (Ser-Leu), L-phenylalanyl-glycine (Phe-Gly) or L-leucyl-glycyl-glycine (Leu-Gly-Gly), and the liberated L-leucine or L-phenylalanine was determined spectrophotometrically by the L-amino acid oxidase/peroxidase-coupled oxidation of *o*-dianisidine (Butterworth & Priestman, 1982). Prolidase (EC 3.4.13.9) was assayed against glycyl-L-proline (Gly-Pro) by determination of liberated proline and against L-phenylalanyl-L-proline (Phe-Pro) by determination of liberated L-phenylalanine (Priestman & Butterworth, 1984). Proline iminopeptidase (EC 3.4.11.5) activity was determined by determination of liberated proline from prolylglycyl-glycine (Pro-Gly-Gly) (Butterworth & Priestman, 1984).

Column fractions were diluted appropriately before enzyme assay with bovine serum albumin (0.25 mg/ml) in 10 mM-barbital/HCl buffer, pH 7.4, containing 50 mM-NaCl. Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. One unit of enzyme activity was defined as the formation of 1 μ mol of product/min at 37 °C.

Extract preparation

Human kidney was obtained at autopsy 24 h after death and stored at –70 °C. A 20% (w/v) extract was prepared by homogenizing in 10 mM-barbital/HCl buffer, pH 7.4, containing 50 mM-NaCl (buffer A). The homogenate was sonicated at 8–12 μ m (MSE mark II ultrasonic disintegrator) and centrifuged at 100000 *g* for 60 min.

Chromatographic procedures

All procedures were carried out at 4 °C unless otherwise stated.

DEAE-cellulose. Kidney extract (50 ml) was applied to a 3.5 cm × 45 cm DEAE-cellulose column equilibrated with buffer A. After a washing with 200 ml of buffer A, the column was eluted with 500 ml of a 50–300 mM-NaCl linear gradient, and 10 ml fractions were collected.

Gel filtration. The DEAE-cellulose peak (95 ml) was concentrated 10-fold by using a PM10 ultrafiltration membrane system (Diaflo), applied to a 2.5 cm × 75 cm Ultrogel AcA-44 column, eluted with buffer A at 20 ml/h and 10 ml fractions were collected.

Metal-ion-chelate chromatography. Iminodiacetic acid was coupled to epoxy-activated Sepharose 6B (Porath *et al.*, 1975) and converted into a metal-ion-chelate gel by the passage of 5 ml of 50 mM-NiCl₂ or 50 mM-MnCl₂ in water through 2.5 ml of gel in a 1 cm × 3.5 cm column. The Ni²⁺-chelate gel was equilibrated with buffer A, and 40 ml of Ultrogel prolinase peak was applied to the column. After washing with 20 ml of 10 mM-barbital/HCl buffer, pH 7.4, containing 10 mM-NaCl (buffer B), the column was eluted with 60 ml of a 0–100 mM-imidazole linear gradient (in buffer B), and 2 ml fractions were collected.

Hydrophobic chromatography. The prolinase peak (16 ml) from the metal-ion-chelate chromatography was applied to a 1.4 cm × 25 cm butyl-agarose column equilibrated with buffer B. The column was washed with 75 ml of buffer B, eluted with 100 ml of a 10–300 mM-NaCl linear gradient, and 4 ml fractions were collected.

Hydroxyapatite chromatography. The prolinase peak (30 ml) from the hydrophobic chromatography was applied to a 1.3 cm × 6.5 cm hydroxyapatite column equilibrated with buffer A. The column was washed with 50 ml of buffer A, eluted with 100 ml of a 0–150 mM-sodium phosphate linear gradient in buffer A, and 5 ml fractions were collected.

Chromatofocusing. The prolinase peak (15 ml) from the hydroxyapatite chromatography was concentrated 10-fold by ultrafiltration, diluted (1+1) with 25 mM-imidazole/HCl buffer, pH 7.4, and applied to a 1.3 cm × 5 cm PBE 94 column equilibrated with this buffer. Elution was carried out with 60 ml of Polybuffer 74 titrated with HCl to pH 4.0, and 2 ml fractions were collected.

M_r determination

A 1% (w/v) kidney extract (10 ml) in buffer A was applied to a 2.5 cm × 75 cm Ultrogel AcA-44 column, eluted with buffer A at 20 ml/h, and 10 ml fractions were collected. The elution volumes of prolinase, *N*-acetyl-β-D-glucosaminidase (Butterworth *et al.*, 1976) and α-D-mannosidase (Butterworth, 1980) were determined. A mixture (10 ml) containing 1 ml of the enzyme peak from hydrophobic chromatography and five protein standards was applied to the same column, and 3 ml fractions were collected. The elution volumes of the protein standards were determined after detection at 280 nm.

Polyacrylamide-gel electrophoresis

Discontinuous electrophoresis (4.5% stacking, 7.5% separating gel, 0.75 mm thick) was performed with a Bio-Rad Protean slab electrophoresis cell (Davis, 1964). Concentrated samples (10 μl) of the original kidney extract and the hydroxyapatite peak were applied and electrophoresed at 14 mA for 4.5 h. The gel lanes were cut into 0.25 cm strips and bisected, one half for prolinase assay and the other half for non-specific-dipeptidase assay. For prolinase each gel strip was put into 100 μl of 50 mM-barbital/HCl buffer, pH 9.2, containing 0.025 mM-MnCl₂, incubated for 60 min after the addition of 100 μl of 10 mM-Pro-Phe, and liberated phenylalanine was determined (Butterworth & Priestman, 1982). For non-specific dipeptidase each gel strip was put into 100 μl of 50 mM-barbital/HCl buffer, pH 8.0, incubated for 60 min after the addition of 100 μl of 25 mM-Gly-Leu, and liberated leucine was determined (Butterworth & Priestman, 1982). Protein bands were detected by using the silver staining method of Merrill & Goldman (1984).

Enzyme characteristics

Studies were performed on the dipeptidase peak eluted after hydrophobic chromatography and after chromatofocusing following equilibration with buffer A by ultrafiltration.

Heat-lability. Enzyme samples were incubated at 53 °C for up to 30 min before the assay of prolinase (Pro-Ala) and non-specific dipeptidase (Gly-Leu).

p-Hydroxymercuribenzoate treatment. Enzyme samples were treated with 1–50 μM-p-hydroxymercuribenzoate for 5 min at 37 °C before the assay of prolinase (Pro-Ala) and non-specific dipeptidase (Gly-Leu).

Substrate specificity. The enzyme preparation after chromatofocusing was tested against a number of potential substrates (see above under 'Enzyme assays').

Substrate kinetics. The type of inhibition and *K_i* values were determined (Dixon & Webb, 1964, pp. 327–329) by studying the hydrolysis of Pro-Ala (2 or 5 mM) at pH 9.2 in the presence of Gly-Leu (0.5–10 and 1–25 mM respectively) and of Gly-Leu (0.5 or 2 mM) at pH 8.0 in the presence of Pro-Ala (0.1–2 and 1–10 mM respectively). As a test of the activities being due to a single enzyme (Dixon & Webb, 1964, pp. 84–87), Pro-Ala (5 mM) hydrolysis at pH 9.2 with or without Gly-Leu (5 mM) and Gly-Leu (5 mM) hydrolysis at pH 9.2 with or without Pro-Ala (5 mM) were monitored, and the *K_i* value for Pro-Ala at pH 9.2 was determined.

RESULTS

The purification of prolinase and non-specific dipeptidase is summarized in Table 1. To assay the two enzyme activities in the original kidney extract, it was necessary to dilute to a low protein concentration (< 0.01 mg/ml), at which the enzyme was unstable. Addition of bovine serum albumin (0.25 mg/ml) stabilized the enzyme to dilution. During the purification procedure the protein concentration of the active fractions up to and including the hydroxyapatite column chromatography was sufficient to maintain stability, but addition of bovine serum

Table 1. Purification of human kidney prolinase and non-specific dipeptidase

For experimental details see the text.

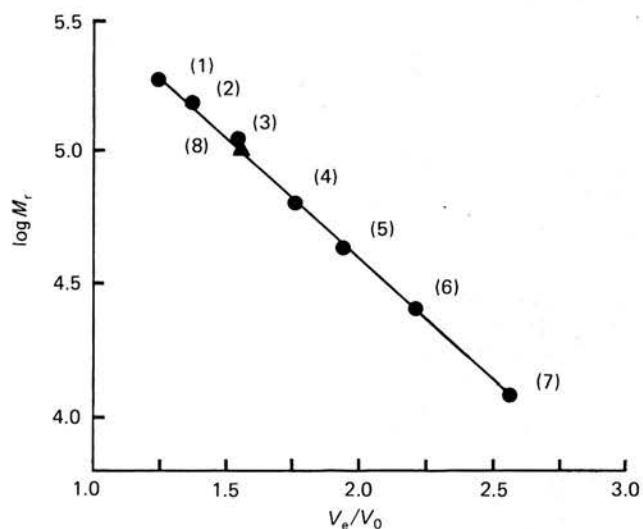
	Prolinase				Non-specific dipeptidase		
	Protein (mg)	Activity (units)	Specific activity (units/mg)	Yield (%)	Activity (units)	Specific activity (units/mg)	Yield (%)
Crude extract	948	631	0.657	100	4888	5.16	100
100000 g supernatant	354	573	1.62	91	4444	12.6	91
DEAE-cellulose fraction	56.2	319	5.68	50	2333	41.5	48
Ultrogel AcA-44 fraction	15.6	283	18.1	45	2151	138	44
Ni ²⁺ -chelated-Sepharose fraction	3.7	148	40.0	23	1194	323	24
Butyl-agarose fraction	1.27	127	100	20	1050	827	21
Hydroxyapatite fraction	0.27	87	319	14	700	2564	14
Chromatofocusing fraction	—	17	—	2.6	116	—	2.4

albumin was required for dilution of the column fractions before assay. A preparation retained after hydrophobic chromatography was stable for 9 months at 4 °C in the presence of bovine serum albumin. Co-elution of both of the enzyme activities as a single peak was observed for all the separative procedures, either performed singly with a crude kidney extract or during the sequential purification. The ratio of the two activities was similar after each of the separative procedures.

During the sequential purification procedure the two enzyme activities were co-eluted with the highest activity at 110 mM-NaCl for the DEAE-cellulose column, 30 mM-imidazole for the Ni²⁺-chelate column, 100 mM-NaCl for the butyl-agarose column and 30 mM-phosphate for the hydroxyapatite column. A Mn²⁺-chelate gel did not retain either enzyme activity. The relative elution volumes (V_e/V_0) of *N*-acetyl- β -D-glucosaminidase and α -D-mannosidase lay on the gel-filtration M_r calibration curve and give M_r values of 110000 and 180000 respectively. The position of the enzyme, as detected by its prolinase and non-specific dipeptidase activity, indicated an M_r of 100000 (Fig. 1). Elution from the chromatofocusing column indicated a pI of 5.3 for the enzyme. Silver staining of the polyacrylamide gel after electrophoresis of the pooled enzyme activity from the hydroxyapatite column revealed the presence of two major and three minor protein bands (Fig. 2a). Band I (Fig. 2a) was the marker Bromophenol Blue and band II was a staining artifact. The gel showed a coincident band of prolinase and non-specific dipeptidase activity (Fig. 2b). A crude kidney extract gave the same position for the two enzyme activities.

As recovery of enzyme activity was poor from the chromatofocusing column and involved exposure to acidic pH, properties of the enzyme were studied after the hydrophobic-chromatography as well as the chromatofocusing procedures. However, the results were the same for both enzyme preparations. Heat treatment of the enzyme at 53 °C resulted in a similar rate of loss of activity against Gly-Leu and Pro-Ala, with a 50% loss of activity after about 12 min (Fig. 3). The thiol-blocking reagent *p*-hydroxymercuribenzoate was markedly inhibitory of enzyme activity against both substrates with 50% inhibition at about 14 μ M (Fig. 4).

The activity of the final enzyme preparation against a range of potential substrates (10 mM) is given in Table 2.

**Fig. 1. Calibration of Ultrogel column for M_r determination**

The relationship between the relative elution volumes (V_e , elution volume; V_0 , void volume) and the logarithms of the M_r values of certain proteins is shown for Ultrogel AcA-44. For experimental details see the Materials and methods section. Key: (1) α -D-mannosidase; (2) alcohol dehydrogenase; (3) *N*-acetyl- β -D-glucosaminidase; (4) haemoglobin; (5) ovalbumin; (6) chymotrypsinogen A; (7) cytochrome c; (8) prolinase and non-specific dipeptidase.

Of the non-specific dipeptidase substrates the highest activity was against Gly-Leu; Phe-Gly was not hydrolysed. Three of the prolinase substrates (Pro-Leu, Pro-Phe and Pro-Val) gave activities that were higher than the activity towards Pro-Ala, the substrate used to monitor the enzyme purification. The enzyme had no activity towards Pro-Gly-Gly, the substrate for proline iminopeptidase, and towards Gly-Pro and Phe-Pro, substrates for prolidase. Analysis of substrate interactions by Dixon (1953) plots showed that Gly-Leu was a competitive inhibitor of enzyme activity towards Pro-Ala with a K_i of 1.1 mM at pH 9.2 (Fig. 5), and Pro-Ala was a competitive inhibitor of enzyme activity towards Gly-Leu with a K_i of 0.22 mM at pH 8.0 (Fig. 6) and 2.2 mM at pH

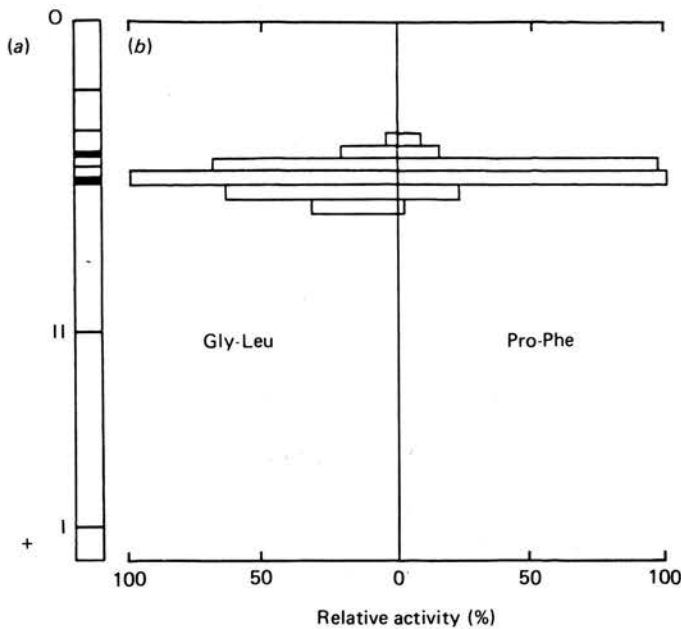


Fig. 2. Polyacrylamide-gel electrophoresis of human kidney dipeptidase

The gel was prepared, run and stained or the enzyme activities were determined for the hydroxyapatite peak as described in the Materials and methods section. (a) Silver-staining for protein in the separating gel (10 cm). O, Origin; +, anode end of gel. Band I was the tracking dye Bromophenol Blue and Band II was a staining artifact. (b) Dipeptidase relative activity in gel slices (0.25 cm) against Gly-Leu and Pro-Phe.

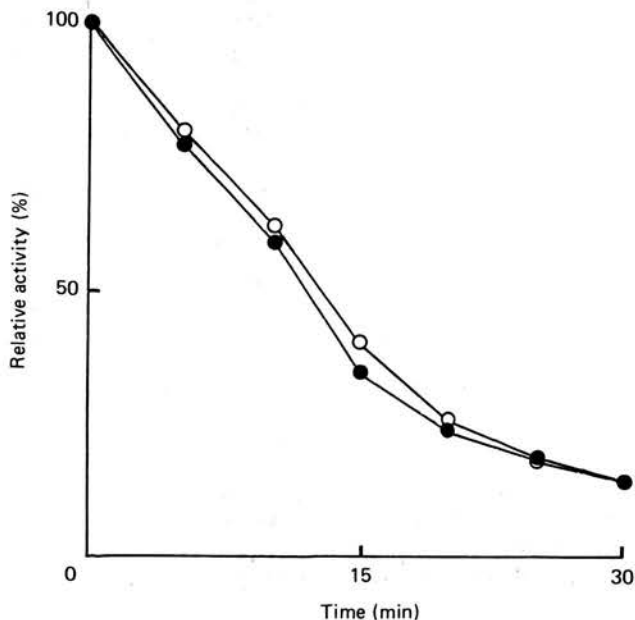


Fig. 3. Heat treatment of human kidney dipeptidase

The enzyme peak after chromatofocusing was equilibrated with 10 mM-barbital/HCl buffer, pH 7.4, containing 50 mM-NaCl, heated at 53°C for up to 30 min and remaining prolinase (●) and non-specific dipeptidase (○) activities were determined as described in the Materials and methods section.

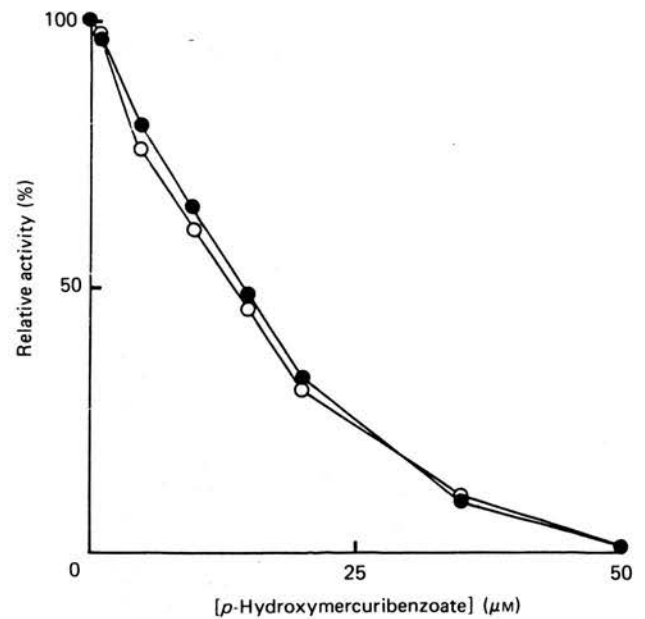


Fig. 4. Inhibition of human kidney dipeptidase by *p*-hydroxymercuribenzoate

The enzyme peak after chromatofocusing was equilibrated with 10 mM-barbital/HCl buffer, pH 7.4, containing 50 mM-NaCl, treated with 1–50 μM-*p*-hydroxymercuribenzoate and remaining prolinase (●) and non-specific dipeptidase (○) were determined as described in the Materials and methods section.

Table 2. Hydrolysis of dipeptides and tripeptides by the purified human kidney enzyme

The activity of the enzyme preparation after chromatofocusing was determined against potential substrates (10 mM) as described in the Materials and methods section.

Substrate	Enzyme activity (units/ml)	Relative activity (%)
Gly-Leu	3.23	100
Ser-Leu	1.29	40
Val-Leu	0.96	30
Ala-Leu	0.89	28
Phe-Ala	0.32	10
Phe-Gly	0	0
Leu-Gly-Gly	0.12	4
Pro-Leu	1.55	48
Pro-Phe	1.42	44
Pro-Val	1.30	40
Pro-Ala	0.43	13
Pro-Gly	0.29	9
Hyp-Gly	0.12	4
Pro-Gly-Gly	0	0
Gly-Pro	0	0
Phe-Pro	0	0

9.2. The K_m for the enzyme against Pro-Ala was 0.47 mM at pH 9.2, and the K_m against Gly-Leu was 0.79 mM at pH 8.0 and pH 9.2. The expected hydrolysis of equimolar concentrations of two substrates by (a) one or (b) two enzymes in an extract can be calculated by using experimentally determined values of V_{max} , K_m and K_i for

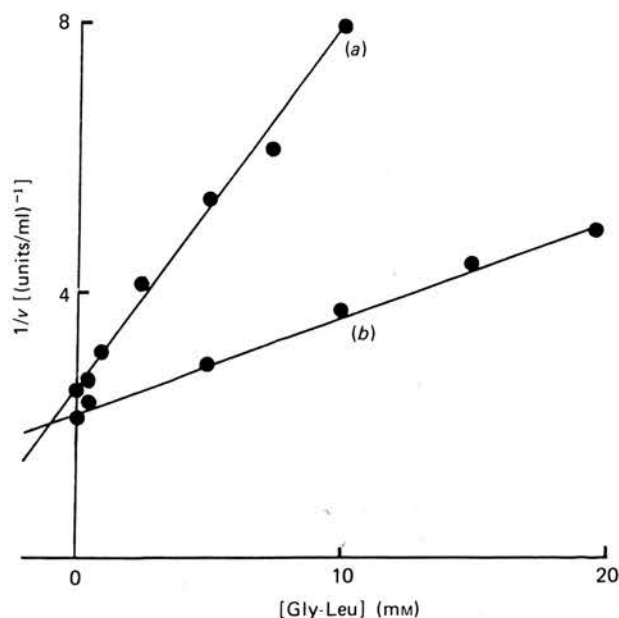


Fig. 5. Determination of K_i for Gly-Leu inhibition of prolinase activity against Pro-Ala

The hydrolysis of (a) 2 mM- and (b) 5 mM-Pro-Ala in the presence of a series of concentrations of Gly-Leu was determined. The K_i was determined by the method of Dixon (1953).

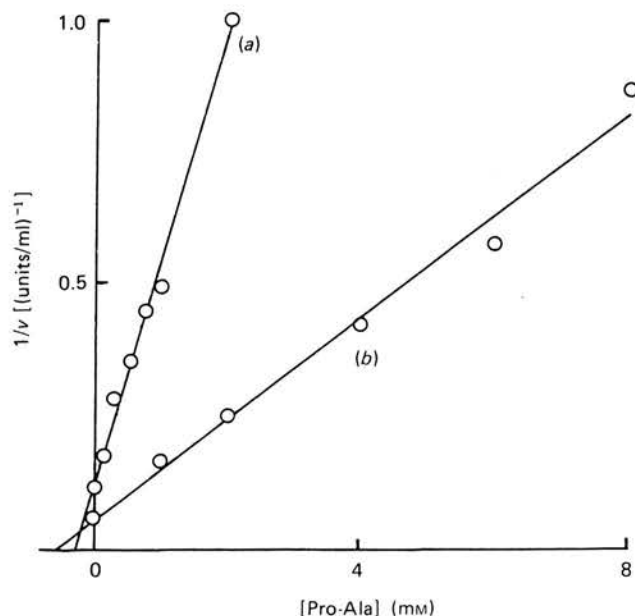


Fig. 6. Determination of K_i for Pro-Ala inhibition of non-specific dipeptidase activity against Gly-Leu

The hydrolysis of (a) 0.5 mM- and (b) 2 mM-Gly-Leu in the presence of a series of concentrations of Pro-Ala was determined. The K_i was determined by the method of Dixon (1953).

the two substrates (Dixon & Webb, 1964, pp. 84–87). Whether the activity against both substrates is due to one or two enzymes can be assessed by comparison of the two calculated rates of hydrolysis with the observed rate. The present data gave a calculated total activity of 8.9

units/ml for one enzyme and 16.6 units/ml for two enzymes working on equimolar concentrations (5 mM) of the two substrates. As the observed total activity was 9.2 units/ml, it seems highly probable that the preparation contained a single enzyme hydrolysing both substrates.

DISCUSSION

Non-specific dipeptidases purified from pig and guinea-pig intestinal mucosa (Noren *et al.*, 1973; Piggott & Fottrell, 1975) and mouse ascites tumour (Hayman & Patterson, 1971) hydrolysed prolinase substrates. Conversely, prolinases purified from pig kidney (Sarid *et al.*, 1962; Mayer & Nordwig, 1973), bovine kidney (Akrawi & Bailey, 1976) and mouse brain (Reith & Neidle, 1979) hydrolysed non-specific dipeptidase substrates. Also, the major peak of human skin-fibroblast prolinase coincided with a peak of non-specific dipeptidase on DEAE-cellulose chromatography (Butterworth & Priestman, 1984). Generally, the two activities had been considered to be due to at least two enzymes, and whether there is an enzyme capable of hydrolysing both prolinase and non-specific-dipeptidase substrates is undecided, particularly for human tissues.

Prolinase and non-specific dipeptidase from human kidney were soluble, in agreement with results for human skin fibroblasts (Butterworth & Priestman, 1982) and pig kidney (Mayer & Nordwig, 1973). The elution position of the human kidney enzyme on DEAE-cellulose chromatography corresponded to that of the major peak of human skin-fibroblast prolinase (Butterworth & Priestman, 1982, 1984). The accuracy of the M_r calibration curve was demonstrated by obtaining the expected M_r values for α -D-mannosidase and *N*-acetyl- β -D-glucosaminidase (Marinkovic & Marinkovic, 1976a, b). The determined M_r value of 100 000 for the human kidney dipeptidase was similar to the value reported for bovine kidney prolinase (Akrawi & Bailey, 1976), but was lower than the value of 300 000 estimated for pig kidney prolinase (Mayer & Nordwig, 1973).

Metal-ion-chelate chromatography separates proteins on the basis of their affinity for heavy-metal ions (Porath *et al.*, 1975; Porath & Olin, 1983). Histidine and cysteine form complexes with Zn^{2+} , Co^{2+} , Hg^{2+} and Ni^{2+} , and proteins containing these amino acids on their surface can potentially bind to metal-ion-chelated Sepharose (Porath *et al.*, 1975). Although Mn^{2+} activates prolinase (Butterworth & Priestman, 1982) and a number of other enzymes (Dixon & Webb, 1964, pp. 672–785), the weak capacity of gel-immobilized Mn^{2+} for protein binding (Porath *et al.*, 1975) could explain why the Mn^{2+} -chelate gel did not adsorb prolinase. Affinity elution of human kidney dipeptidase from a Ni^{2+} -chelate gel by imidazole strongly suggests the presence of histidine residues on the surface of the enzyme (Porath & Olin, 1983). The results obtained with human kidney dipeptidase demonstrate the usefulness of the inclusion of a metal-ion-chelate-chromatography step in enzyme purification.

The binding of human skin-fibroblast prolinase to butyl-agarose (Butterworth & Priestman, 1982) and of pig intestinal non-specific dipeptidase to hydroxyapatite (Noren *et al.*, 1973) was the same as that of the human kidney enzyme. Although bovine (Akrawi & Bailey, 1976) and pig (Mayer & Nordwig, 1973) kidney prolinase gave a pI of 4.3 on isoelectric focusing, the pI value of 5.3 for

the human kidney preparation was within the range 5.05–5.3 obtained by chromatofocusing of preparations of human skin fibroblasts, liver and kidney (Butterworth & Priestman, 1984). The marked loss of human kidney enzyme activity on chromatofocusing was probably due to exposure to acidic pH, which is known to inactivate the enzyme (Butterworth & Priestman, 1982). The presence of multiple bands of activity, probably due to separation into subunits and re-aggregation, on polyacrylamide-gel electrophoresis of purified pig kidney prolinase (Mayer & Nordwig, 1973) was not a feature of the human kidney enzyme.

The inhibition of human kidney dipeptidase activity by *p*-hydroxymercuribenzoate indicates the presence of a thiol group at the active site, as previously suggested for monkey intestinal (Das & Radhakrishnan, 1972) and pig kidney enzyme (Sarid *et al.*, 1962). Although Gly-Leu was the best of the substrates tested for human kidney dipeptidase, the hydrolysis of non-specific-dipeptidase substrates varies widely, depending on conditions of assay and source of enzyme (Hayman & Patterson, 1971; Mayer & Nordwig, 1973; Noren *et al.*, 1973). The ability of the human kidney enzyme to hydrolyse prolinase substrates was similar to that of human skin-fibroblast prolinase (Butterworth & Priestman, 1982). In contrast with the human enzyme, pig kidney (Mayer & Nordwig, 1973) and guinea-pig intestinal-mucosal (Piggott & Fottrell, 1975) prolinase had very little activity towards Pro-Leu and Pro-Phe. The failure of the human kidney enzyme to hydrolyse Pro-Gly-Gly, the substrate for proline iminopeptidase and Gly-Pro and Phe-Pro, substrates for prolidase, agrees with the previous findings for pig and bovine kidney prolinase (Sarid *et al.*, 1962; Mayer & Nordwig, 1973; Akrawi & Bailey, 1976).

Throughout the purification procedures the human kidney prolinase and non-specific dipeptidase activities could not be separated. The enzyme peaks were congruent and the ratio of their activities was constant after each of the separative procedures. In addition, the responses of the two activities to *p*-hydroxymercuribenzoate and to heat treatment were the same. These data, together with the competitive inhibition of prolinase and non-specific dipeptidase activity by Gly-Leu and Pro-Ala respectively and the analysis of substrate interaction (Dixon & Webb, 1964, pp. 84–87), provide firm evidence

for the existence of an enzyme hydrolysing both prolinase and non-specific dipeptidase substrates in human kidney.

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