PURIFICATION AND SOME PROPERTIES OF AN ALKALINE PROTEASE FROM RAT SKELETAL MUSCLE

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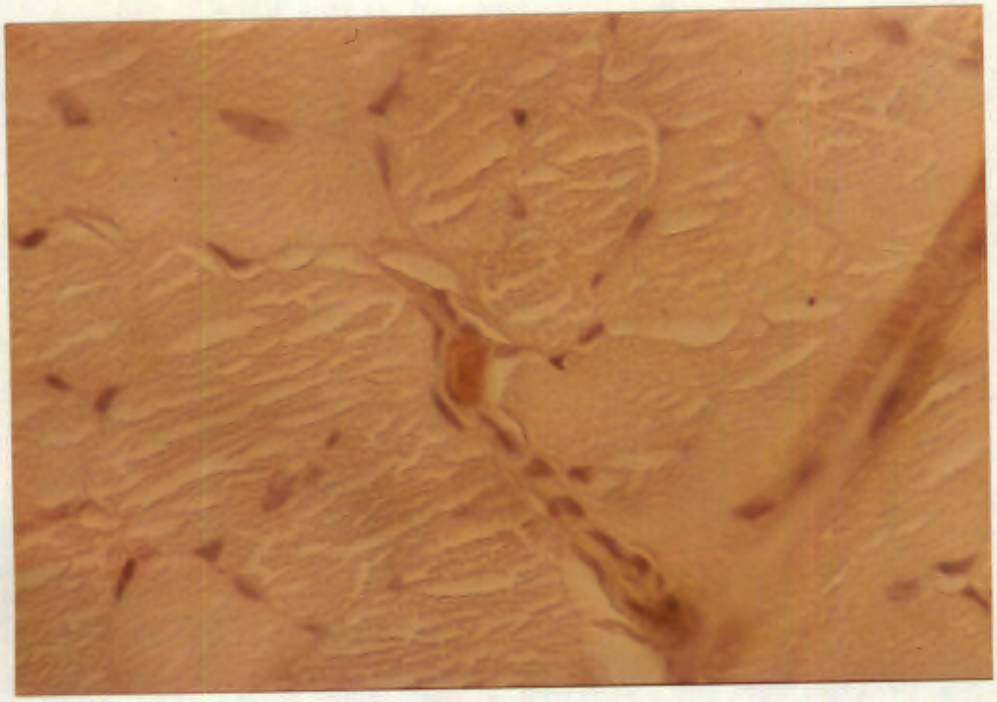

Normal rat skeletal muscle (Bismarck Brown stain) showing muscle fibres containing nuclei, with connective tissue bands containing mast cell(s) between the muscle fibres.

ABSTRACT

Various alkaline proteases derived from skeletal muscle have been described by a number of researchers and have been purified to varying degrees. Such alkaline proteases may play an important role in the metabolism of myofibrillar and other muscle proteins and as such deserve to be fully characterised.

In this study, a major myofibrillar alkaline protease was purified from rat skeletal muscle. The enzyme degraded both denatured casein and azocasein and had a pH optimum of 9,0. The molecular mass was 32 250 ± 650. The presence of a second, minor alkaline protease was demonstrated using three different separation techniques as well as by inhibitor studies.

The major protease was insensitive to inhibition by pepstatin and leupeptin, whilst 90% of the activity was expressed in the presence of 2 mM EGTA. A moderate degree of inhibition was observed in the presence of soybean trypsin inhibitor and the protease was markedly sensitive to chymostatin. A similar alkaline protease was partially purified from rat cardiac muscle using the same purification procedure.

Incubation of washed myofibrils in the presence of sodium pyrophosphate released a factor into the supernatant, the removal of which facilitated the separation of myofibrillar alkaline protease from the myofibrils. The factor appeared to be necessary for binding of the alkaline protease to the myofibrillar proteins but its removal did not disrupt the binding of proteolytic activity already attached to the myofibrillar proteins.

An inhibitor of myofibrillar alakline protease was demonstrated which is, in principle, capable of playing an important regulatory role in controlling the activity of these enzymes and thereby of myofibrillar protein catabolism.

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ABBREVIATIONS

A	- absorbance
Ac	- acetyl
Ala	- alanine
Arg	- arginine
Bz	- benzyl
C	- Celsius
CM	- carboxymethyl
cm	- centimetre
cpm	- counts per minute
DEAE	- diethylaminoethyl
DMSO	- dimethylsulphoxide
DNA	- deoxyribonucleic acid
DTT	- dithiothreitol
EDTA	- ethylenediamine tetra-acetic acid
EGTA	- ethyleneglycol-bis-(β-amino-ethyl ether) N,N'-tetraacetic acid
em	- emission
Enz.	- enzyme
ex	- excitation
g	- gravitational force
h	- hour
LSB	- low salt buffer
M	- molar
mA	- milliamps
MAP	- myofibrillar alkaline protease
mCi	- millicurie ($3,7 \times 10^7$ disintegrations/second)
mg	- milligram
min.	- minute

Mit/lys - mitochondrial-lysosomal

ml - millilitre

ml/g - millilitre per gram

mM - millimolar

mm - millimetre

m/m - mass by mass

mRNA - messenger ribonucleic acid

m/v - mass by volume

MW - molecular weight

nm - nanometre

nmole - nanomole

OEt - ethoxy

OMe - methoxy

PAGE - polyacrylamide gel electrophoresis

Phe - phenylalanine

Pp - pyrophosphate

SDS - sodium dodecyl sulphate

TCA - trichloroacetic acid

Tos - tosyl

Tris - trishydroxymethyl aminomethane

Trp - tryptophan

Tyr - tyrosine

µg - microgram

µl - microlitre

U - unit (enzyme)

V - volt

v/m - volume by mass

vol. - volume

v/v - volume by volume

%	- percent
<	- less than
λ	- wavelength
[ ]	- concentration

CONTENTS

CHAPTER 1

INTRODUCTION

1.1. GENERAL PROTEIN DEGRADATION

Proteins are the most prevalent "building blocks of the cell" and generally constitute about 75% of dry cell mass. The majority of these macromolecules are not, however, purely structural components and most of them play important functional roles in cellular metabolism. Proteins are thus enzymes, regulatory elements, contractile systems, and membrane carriers, to name just a few. Generally, steady-state nett amounts of each of these proteins in a particular cell are a function of the balance between protein synthesis and degradation.

The anabolic pathway has been actively studied for a number of years and the biosynthesis of proteins is thus quite well understood, from the informational role of DNA to the synthesis of polypeptide chains by ribosomes. The rates of synthesis of various proteins, in intact-cell systems, and the effects of various stress conditions, have also been measured and assessed. In contrast, the importance of protein degradation has only been established during the last decade as a result of the appreciation that the control of protein degradation may be important in determining the levels of a number of specific proteins in the cell (Katunuma, 1977). Thus the cellular concentrations of specific functional proteins may be controlled by the activities of various specific proteases, and these latter enzymes may also mediate protein degradation in general.

Intracellular proteases may not only play an important physiological role but may also be involved in pathological processes. Elevated levels of muscle proteases have been observed in various pathological states, such

as cachexia (Mayer et al, 1976), denervation (Goldspink, 1976), myopathy (Katunuma et al, 1977), diabetes (Rithig et al, 1975, 1978) and starvation (Mayer et al, 1974). The degradation of proteins in these states can be seen to be "controlled" (as in starvation to provide energy) or may be "uncontrolled" (as in cachexia). Proteases also act as controllers/ activators in many physiological reactions outside cells, e.g. blood coagulation-fibrinolysis, blood pressure control by the renin-angiotensin system and complement activations, while known intracellular processes involving proteolysis are hormone and enzyme processing and intracellular digestion of endocytosed proteins.

Many proteases in these systems act specifically. In order to understand the regulation of protein degradation in healthy and/or diseased tissues, the various proteases involved have to be fully characterised so that the precise contribution of each protease in complex processes can be assessed.

Lindestrom-Lang (1952) defined two types of proteolysis - the "one-by-one" and the "zipper" types of reaction. Zipper-attack is characteristic of zymogen activation and limited proteolysis, in which a stable derivative is formed. In general it is likely that the initial cleavage of native proteins during intracellular protein degradation occurs by limited proteolysis. This need not be the first "step" in the process, however. Thus, protein degradation may consist of three major steps: the initial and possibly rate-limiting stage involves the transformation of the protein into a form susceptible to proteases. This may depend on changes in the nature of the peptide bonds which are exposed, conformational changes induced by such factors as the absence of co-enzymes, substrates, and allosteric effectors, by covalent modifications and by the extent to which proteins are in an assembled or dissociated state. These structural

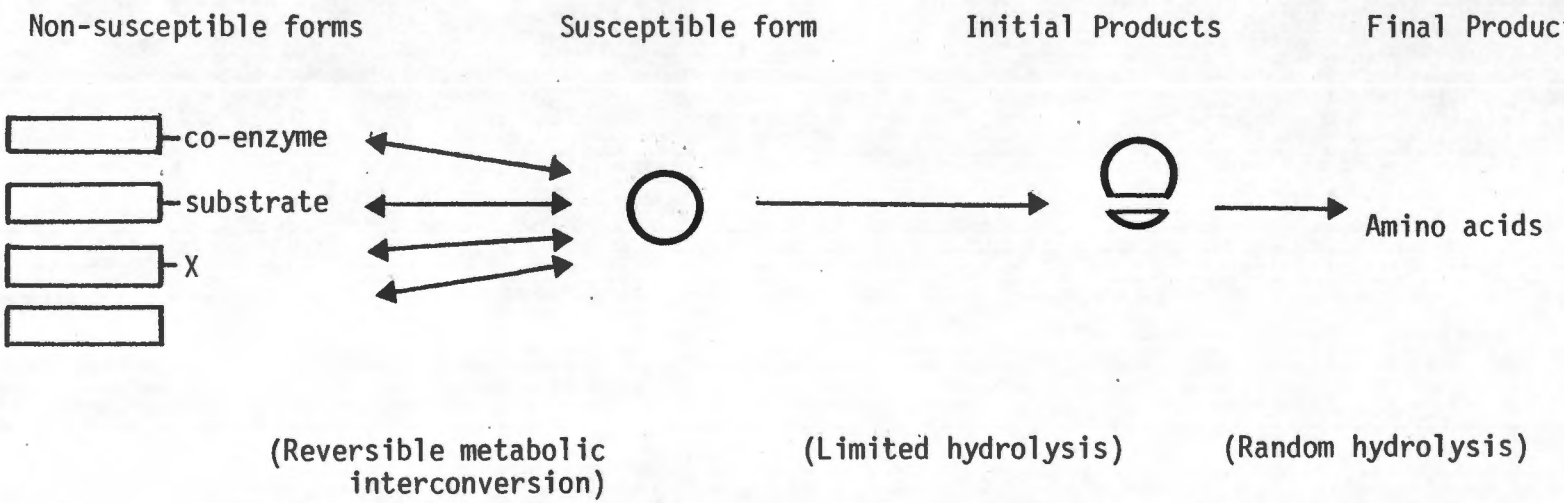


Fig. 1.1. Schematic representation of Zipper-model of proteolysis

factors which greatly influence the catabolic rates of enzymes have been examined by many researchers (Goldberg and Dice, 1974; Schimke, 1975; Kominami et al, 1975; Katunuma, 1975 and Katunuma et al, 1976). For example, the conversion from the holo- to apo-form in the case of ornithine aminotransferase was shown by Katunuma to be accompanied by a conformational change in the molecule, which probably renders the enzyme more susceptible to group-specific proteases.

The second stage of protein degradation may involve limited proteolysis related to the selective specificity of certain groups of enzymes. Such a mechanism may involve a variety of group-specific proteases for all the proteins in the cell. However, although a few group-specific proteases have been reported, they are still not numerous (Bonsignore et al, 1968; Katunuma et al, 1971). An additional possible mechanism for the second step was implied by Prouty et al (1975), who found that the presence of amino acid analogues in proteins during the degradation of abnormal proteins in Escherischia coli, decreased their solubility, so that intracellular inclusions were formed by the spontaneous precipitation of the abnormal proteins. They proposed that the spontaneous aggregation of abnormal proteins serves to promote their recognition by the degradative system. Such a mechanism was also proposed by Ballard and Hopgood (1974) for the degradation of phosphoenolpyruvate carboxykinase in vitro and in vivo; they suggested that the enzyme may be denatured and precipitated before being attacked by the protease (Haider and Segal, 1972).

The third stage of degradation presumably involves the further degradation of the intermediates formed by limited proteolysis since the final outcome seems to be the formation in the cell of free amino acids. Less specific proteases may be active in this process, possibly lysosomal

cathepsins. This would require the translocation of the intermediate products into lysosomes. However, it is not yet clear whether proteins or fragments are translocated into intact lysosomes.

Lysosomes are a major focus of proteases in the cell. Neely and Mortimore (1974) found most of the proteolytic activity to reside in the lysosomal-mitochondrial fraction upon assaying the endogenous proteolytic activity of the sub-cellular fractions of perfused rat liver. De Duve and Wattiaux (1966) reported the presence of lysosomes in virtually all cells. Numerous acidic hydrolases (or cathepsins) are contained in the lysosomes and inactivate or digest several native enzymes or proteins under acidic conditions. Alanine aminotransferase (Segal et al, 1969; Haider and Segal, 1972) and tyrosine aminotransferase (Auricchio et al, 1972) are inactivated at pH 5 by the mitochondrial-lysosomal fraction from rat liver. Johnson and Velich (1972) reported that glyceraldehyde-3-phosphate dehydrogenase and aldolase are inactivated at pH 6,5 by a mitochondrial-lysosomal fraction from rabbit liver. Huisman et al (1973) showed that thiol-cathepsins hydrolyse native proteins,including cytochrome C, ribonuclease and horse-radish peroxidase,at pH 5. However, in all these cases it was necessary to rupture the lysosomes to allow the cathepsins to react with the proteins. It would thus appear that a certain amount of initial protein degradation has to occur in the cytosol before the enzyme/protein is able to enter lysosomes, where the cathepsins can degrade them to completion. Numerous other intracellular proteases have now also been found (for example from rat liver, intestine, heart and skeletal muscle) which are active at neutral to alkaline pH and which cleave protein substrates by limited or complete proteolysis (Kominami et al, 1972; Katunuma, 1973 a,b; Katunuma et al, 1973, 1975).

1.2. MYOFIBRILLAR AND OTHER NON-LYSOSOMAL PROTEASES IN MUSCLE CELLS

Some of these neutral and alkaline proteases are associated in fractionated tissue preparations with the contractile proteins which they can degrade *in vitro* (Bhan et al, 1978; Rothig et al, 1975; Sanada et al, 1978; Yasogawa et al, 1978). Others have been found in both the "organellar" and "soluble" fractions of the cell (see below).

The soluble proteases comprise at least three separable enzyme types:

(a) a Ca^{+2}-activated protease, which removes Z-lines from myofibrils, irreversibly activates muscle phosphorylase kinase, is widely distributed in other tissues and is eluted from a DEAE-cellulose column by 0,25 M NaCl (Busch et al, 1972; Dayton et al, 1976; Ishiura et al, 1978);

(b) an alkaline protease, which has been partially purified from rat skeletal muscle, hydrolyses endogenous muscle protein and N-acetyl-L-tyrosine ethyl ester and is inhibited by metal ions, including Ca^{+2} (Koszalka and Miller, 1960); and

(c) a sulphydryl-dependent neutral protease that degrades insulin but not pro-insulin (Duckworth et al, 1972).

The particulate proteases include:

(a) a "myofibrillar" protease (MAP) from heart and skeletal muscle (Bhan et al, 1978; Griffin and Wildenthal, 1978; Mayer et al, 1974); and

(b) serine proteases from heart and skeletal muscle that hydrolyse substrates of chymotrypsin and are inhibited by soybean trypsin inhibitor (Sanada et al, 1978, 1978; Hasogawa et al, 1978).

It has been suggested that these enzymes specifically inactivate the apoproteins of many pyridoxal-requiring enzymes ("group-specific protease").

1.3. THE PROBLEMS OF THE NATURE AND IDENTITY OF MYOFIBRILLAR ALKALINE PROTEASES (MAP)

The alkaline particulate or "myofibril-associated" proteases have properties that are rather similar to those of the alkaline serine protease(s) of peritoneal mast cells (Noguchi and Kandatsu, 1976) and the activity of the "skeletal muscle enzyme" is markedly reduced by the treatment of rats with Compound 48/80, a mast cell degranulator. Using fluorescent antibodies, a serine protease of skeletal muscle has also been localised to mast cells and shown to be structurally identical to the chymotrypsin-like enzymes of mast cells (Woodbury et al, 1978a,b). In regard to the particulate alkaline proteases (pH 7,8) of rat hearts, that will degrade protein to free amino acids, at least 90% of this activity appears to be localised to mast cells (McKee et al, 1979). These findings cast a serious doubt on the putative role of the proteases which are found associated with myofibrils, presumably artefactually in skeletal muscle preparations.

Katunuma and Kominami (1977) found that the group-specific alkaline proteases catalyse a limited proteolysis, with considerable specificity; this specificity differed for various substrates. Incubation of the apo-form of ornithine aminotransferase with crystalline protease from the muscle layer of the small intestine indicated that the inactivating reaction was associated with limited proteolysis, as only a few peptides and a small amount of ninhydrin-reactive material was released, even

with a total loss of aminotransferase activity. The high molecular weight product was found to have a molecular mass very similar to that of the native enzyme on PAGE. The protease split each subunit to form polypeptide chains of molecular weight 27 000 (Fragment A) and 13 000 (Fragment B). Fragments A and B eluted together on a Sephadex G-100 column, suggesting that the cleavage products remain associated unless the protein is cleaved. On Ouchterlony double-diffusion analysis, the binding affinity for pyridoxal phosphate (1/dimer unit) and the spectral characteristics (A_{420}/A_{280}) were the same between apo-enzyme and product. However, the product had completely lost enzymic activity and the capacity to polymerise an addition of pyridoxal phosphate. Binding affinity for co-enzyme and antibodies, the exposure of thiol groups, tyrosine and tryptophan residues, the apparent tertiary structure of the product (compared with native apo-ornithine transaminase) were all not changed by limited proteolysis (Kominami et al, 1975). In contrast, after denaturation of the apo-ornithine transaminase in 8 M urea, a random proteolysis by group-specific proteases was observed.

Rat liver cystathionase catalyses several different enzymic reactions (Pascal et al, 1972) and is composed of eight apparently identical subunits. Incubation of the apo-form of crystallised cystathionase from rat liver with group-specific proteases from the muscle layer of small intestine at $0^{o}C$ resulted in the splitting of each subunit to form an enzymatically inactive polypeptide chain of molecular weight 38 000. Upon continued incubation, the protein band of molecular weight 38 000, which accumulated during the initial stages, disappeared completely. The 38 000 molecular weight product was completely devoid of enzymic activity. SDS-PAGE indicated that limited proteolysis was

accompanied by dissociation of the four subunits which constitute the native cystathionase enzyme. The initial proteolytic product thus appears to be more susceptible to proteolysis than the native apo-cystathionase.

Two important differences are apparent between the hydrolysis of ornithine aminotransferase and rat liver cystathionase, namely -

(1) limited proteolysis was accompanied by dissociation of the subunits in the one case and not in the other; and

(2) the stability of the peptide fragments formed by limited proteolysis differed.

The reason for these differences in the course of proteolysis is thought to rest with differences in their conformation, as Kominami et al (1975) showed that a conformational contribution is also important in the difference in susceptibility of the holo- and apo-forms of pyridoxal enzymes to group-specific proteases. Matsuda and Fischer (1975) reported that a group-specific protease split each subunit of muscle phosphorylase b (MW 100 000) by limited proteolysis, forming two fragments of molecular weight 60 000 and 36 000. Thus alkaline proteases have the potential to cause specific and selective degradation of proteins to produce products which may be more susceptible to general proteolysis. It can be concluded that alkaline proteases are potentially capable of playing an important regulatory role in protein degradation in vivo and warrant further detailed investigation in order to evaluate the role played by such enzymes in the protein metabolism of muscle cells.

Numerous proteolytic enzymes present in muscle tissue, each with optimum activity in the alkaline pH range, have been described to date, but they have generally been poorly defined and their possible identity or non-identity has not been established. Koszalka and Miller (1960a,b) have described a cytoplasmic alkaline protease in rat skeletal muscle, which has been partially purified by ammonium sulphate precipitation. These authors suggested that their enzyme is involved in the turnover of myofibrils, and this was also proposed for the chymotrypsin-like enzyme purified by Noguchi and Kandatsu (1971). A similar enzyme has been described by Holmes et al (1971) and more recently by Drabikowski et al (1977). It is likely that all these activities originate in mast cells and are released by homogenisation (Park et al, 1973; Noguchi and Kandatsu, 1976; Drabikowski et al, 1977). In an attempt to localise the alkaline proteolytic activity, Pennington and co-workers subjected muscle homogenates to differential and density-gradient centrifugation. They found that "the enzyme is either attached to a cell fragment which sediments with the myofibrils or has become adsorbed on the latter" (Park et al, 1973). Mayer et al (1974) have described some properties and adaptive changes of the rat myofibrillar protease under varying conditions affecting the rates of muscle protein degradation, although it is still not clear whether the alkaline proteolytic activity measured arose from one or several distinctive enzymes (Pennington, 1977). The proteases have generally not been completely purified but have been compared in terms of similarities and differences between properties of the enzymes. Some of these differences may be attributable to the presence of contaminants which may mask certain properties of the enzymes. Additional experiments are clearly needed to show whether the observed differences result from the presence of various impurities or whether in rat skeletal muscle there exist discrete

classes of alkaline proteases (Dahlmann and Reinauer, 1978).

Dahlmann and Reinauer (1978) purified an alkaline protease from rat skeletal muscle. The muscle was homogenised in a buffer at pH 8,5 containing 1 M KCl and was stirred for 12 hours. After centrifuging for 30 min, the enzyme was present in the 15 000 g supernatant. Ammonium sulphate was then added to the supernatant to give a solution 33% saturated with respect to the salt, and after centrifuging (15 000 g; 15 min), the protease was recovered in the precipitate. This precipitate was soluble only in buffer containing at least 0,6 M KCl, which strongly suggests that proteins of the actin-myosin complex constitute the main part of this sediment. The enzyme could not be separated from the myofibrillar proteins by mechanical stirring nor by treatment with Triton X-100, but it was invariably co-solubilised at high concentrations of KCl. The viscosity of the solution was lowered by incubating it with trypsin coupled to Sepharose-4B ($37^{o}C$ for 30 minutes) and then removing the coupled trypsin-Sepharose-4B by centrifugation and filtration. The enzyme was further purified by chromatography on soybean trypsin inhibitor-Sepharose, followed successively by DEAE-Sephadex A-50 and Sephadex G-75. The purified myofibrillar protease migrated as a single protein band on polyacrylamide-gel micro-disc electrophoresis at acid pH and had a molecular mass of 30 800 $\pm$ 330 on a calibrated Sephadex G-75 column. The protease had a pH optimum of 9,4 and 9,6 using azocasein and $|^{14}C|$-haemoglobin as substrates, respectively. A further "cytosolic" protease (Reinauer and Dahlmann, 1979) was purified from the supernatant of the 33% saturation step. This cytosolic protease had a molecular mass of 25 000 on a calibrated Sephadex G-75 column. The cytosolic protease had properties similar to those of the chymotrypsin-like protease from mast cells, whilst the

properties of the myofibrillar protease was more trypsin-like. The cytosolic protease was only soluble in buffers containing 1 M KCl and had a pH optimum similar to that of the myofibrillar protease, in attacking azocasein and haemoglobin. The myofibrillar protease was 93% inhibited by equimolar soybean trypsin inhibitor and only 5% inhibited by 0,1 μg/ml chymostatin. Leupeptin at 10 μg/ml caused a 27% inhibition. Clearly this protease is different from the alkaline protease described by Koszalka and Miller (1960a,b), because the latter enzyme is only precipitated at 40-50% ammonium sulphate saturation in crude muscle homogenates, whilst the enzyme of Dahlmann and Reinauer (1978) was precipitated at 33% saturation. The Dahlmann and Reinauer (1978) enzyme also differed from the insulin-specific protease described by Duckworth et al (1972), which was obtained from a 100 000 g supernatant of muscle homogenate. The protease of Dahlmann and Reinauer (1978) had a molecular mass of 31 000 compared with 25 000 for a chymotrypsin-like protease (Noguchi and Kandatsu, 1976) which again was similar to that described by Park et al (1973). The Dahlmann and Reinauer (1978) protease, however, was not of the "serine-type" but it appeared to have a thiol group at the catalytic site. Furthermore, the Dahlmann and Reinauer (1978) protease was also not inhibited by prior exposure to 60% laevadosin, whilst that of Mayer et al (1974) was completely inhibited.

Kuo and Bhan (1980) have purified a myosin-cleaving protease from cardiac myofibrils of dystrophic hamsters. Steadily increasing levels of the enzyme correlated with the development of the cardiomyopathy. Bhan et al (1978) also showed, using genetically dystrophic hamsters, that a reduction or complete loss of the 18 000 dalton light chain (L_2) occurred during the purification of cardiac myosin and that this was due to elevated levels of a protease co-purifying with myosin. This enzyme

was similar to that purified from rats by Murakami and Uchida (1978): both had a molecular mass of 26 000 daltons, a pH optimum at 9 with casein as substrate, insensitivity towards metal-chelating agents and sulphydryl-binding reagents, association with myofibrils, and the ability to degrade both light (L_2) and heavy chains of myosin. There were, however, differences, such as sensitivity to 1 M KCl, relative degradative activities toward the light and heavy chains of myosin and the effects of 5 mM Ca^{+2} or Mg^{+2}. Although a role of the enzyme in cardiomyopathy has not been established definitively, there are strong reasons to believe that it may be an important one. The initial tissue damage may primarily be due to the myosin-cleaving protease, whereas the action of the lysosomes may be involved in the later tissue destruction.

Mayer et al (1976) have studied changes in the proteolytic activities in skeletal muscle of dystrophic mice, in tumour-bearing rats and in glucocorticoid-treated rats. These three conditions are associated with muscle protein loss. In mice with inherited muscular dystrophy, the progressive weakness and loss of active muscle mass is due to an increased rate of protein catabolism rather than to a defect in protein synthesis (Simon et al, 1962). Similarly, enhanced catabolism of muscle proteins is known to occur in tumour-bearing animals and the ability of the growing tumour to mobilise host protein is a prominent feature of malignancy (Begg, 1958; Henderson and Le Page, 1959). In experimentally induced glucocorticoid atrophy, enhanced degradation of muscle proteins is associated with increased protease activity (Mayer et al, 1974a). Earlier reports (Thomson, 1964; Beckman, 1972) that a mixture of nucleotides and nucleosides (laevadosin) produced improvements in patients with muscular dystrophy and the observation of Mayer et al (1976) that laevadosin inhibits myofibrillar protease activity

both *in vitro* and *in vivo*, have been encouraging and provide an approach towards getting a better understanding of muscle wasting diseases.

Park et al (1973) studied the alkaline protease purified by Holmes et al (1971). Holmes et al reported on an enzyme present in the homogenate sediment following centrifugation at 100 000 g for 30 minutes. This protease had a pH optimum of 11, while soybean trypsin inhibitor (1 µg/0,3 ml) caused a 77% inhibition of activity. The supernatant did not cause any inhibition and the enzyme was thus different in this respect from the protease of Noguchi and Kandatsu (1969, 1970). This protease resembled in many respects the chymotrypsin-like protease found in peritoneal mast cells (Lagunoff and Benditt, 1963) and recently purified (Kawiak et al, 1971). Skeletal muscle contains small numbers of mast cells situated in the connective tissue (Selye, 1965). Park et al (1973) used an increasing daily dose of compound 48/80, a polymer of N-methylhomoanisylamine and formaldehyde, which was injected into rats by the method of Pastan and Almqvist (1966), to investigate whether the protease was situated in the mast cells. Treatment with 48/80 caused loss of most of the alkaline protease activity of the homogenates, and the activity was therefore considered to originate from the mast cells. The drug added *in vitro* did not inhibit the protease activities nor did the muscle homogenate from the drug-treated rats inhibit the autolytic activity of the homogenate from the untreated rats. Goldspink et al (1971a) observed increased alkaline protease activity in denervated skeletal muscle of rats, expressed per mg of non-collagen protein, but the activity per whole muscle was not significantly changed. It is thus possible that this may be due to a preferential maintenance of the original total number of mast cells in the atrophied muscle. Increasing autolytic activity has been found in muscle preparations from vitamin E-

deficient rats and hamsters (Koszalka et al, 1961), mice with hereditary muscular dystrophy (Berlinguet and Srivistava, 1966), rats after denervation (Kohn, 1965) and rats fed on a protein-free diet (Millward, 1971). The contribution of mast cell proteolytic activity may be important in relation to these changes. A higher concentration of mast cells is seen in muscle of genetically dystrophic mice (Bois, 1964).

Griffin and Wildenthal (1978) found an alkaline protease in rat cardiac muscle using the method of Noguchi et al (1974) and by Mayer (1976). The cardiac protease appeared to be similar to the skeletal protease. Cardiac MAP activity was found to increase with age. It increased even more during the development of myocardial atrophy induced by starvation or diabetes mellitus, and was decreased during the development of thyroxine-induced hypertrophy. In these responses it was similar to cathepsin D (Wildenthal and Mueller, 1974; Wildenthal et al, 1975; Wildenthal et al, 1977), which is known to be entirely different from MAP in cellular distribution, class (carboxyl versus trypsin-like) and pH optimum. Griffin and Wildenthal (1978) have further claimed that the failure of the MAP activity to become elevated in tissue that was undergoing a marked increase in the number and type of non-myocytic cells, provided further evidence that the enzyme(s) in question was of specific myocytic origin.

Murakami and Uchida (1977, 1978) have purified an endogenous protease which is capable of degrading myosin, from the myofibrillar fraction of rat hearts. The purified enzyme was unstable at neutral and alkaline pH, in the absence of Ca^{+2}. Ca^{+2} appeared to protect the enzyme from autolysis. These authors found an inhibitor in the supernatant fraction of heart muscle homogenates. The enzyme itself was chymotrypsin-like

and had a molecular mass of about 26 000. Murakami and Uchida (1979) showed that the protease digested myosin, M-protein, L-protein and troponin, but not actin, tropomyosin or α-actinin. They therefore conclude that the protease is directly responsible for the initial degradation of myosin during myofibrillar turnover in vitro. It has been reported that myofibrillar proteins, such as myosin, actin, troponin and tropomyosin, have different rates of turnover and do not degrade as a unit (Koizumi, 1974). The difference in susceptibilities of myofibrillar proteins to this protease might help to explain the heterogeneity in turnover rates of these proteins. Murakami and Uchida (1980) showed that the protease was active in the presence of EDTA and did not require Ca^{+2} for activity. They showed that the protease destroys three structures: Z-lines, M-lines and C-protein, which help to form bridges between neighbouring thick filaments.

Noguchi and Kandatsu (1971) purified an alkaline protease with a pH optimum of 10. They proposed that this protease was responsible for the activity of autolytic breakdown of muscle proteins in the alkaline pH range. The enzyme was purified from the 5 000 g precipitate of rat skeletal muscle homogenates and was thus presumed to be different from that reported by Koszalka et al (1960), which was prepared from the 10 000 g supernatant of homogenates. Koszalka's protease showed a sharp pH optimum whilst that of Noguchi and Kandatsu had a broad pH optimum.

Katunuma et al (1975) purified his enzyme from pellets obtained by centrifugation at 27 000 g for 20 min, of homogenates of skeletal muscle of rats. An acetone powder was then prepared and further purified. Katunuma's pure group-specific protease had relatively high concentrations

of lysine and arginine, indicating that the enzyme is a basic protein. The molecular weights of the four proteases from skeletal muscle, liver and the muscle and mucosal layers of the small intestine were 17 000, 13 000, 24 000 and 21 000 respectively. From the amino acid analyses and the apparent molecular mass of 24 000 determined in the case of the muscle-layer protease of small intestine by SDS-gel electrophoresis, it could be concluded that the enzyme consisted of a single polypeptide chain. The primary structure showed 37% and 38% identity with bovine chymotrypsin A and dogfish trypsin, respectively, according to incomplete ($\sim$ 80%) sequencing data. Katunuma's group-specific protease hydrolysed chymotrypsin substrates (e.g. Ac-Tyr-OEt, Ac-Phe-OEt and Ac-Trp-OEt), whereas trypsin-kallikrein substrates (Tos-Arg-OMe, Bz-Arg-OEt) were not hydrolysed. The muscle layer protease of the small intestine cleaved an elastase substrate (Ac-Ala-Ala-Ala-OMe) to a significant degree, although the rate was considerably lower than that of Ac-Tyr-OEt. A protease from human bone marrow cells differed from the rat proteases in that neither trypsin nor chymotrypsin substrates were hydrolysed, whilst the enzyme cleaved ester substrates of elastase but not amide substrates.

The enzyme activities of these rat proteases were inhibited by chymostatin and a synthetic inhibitor of chymotrypsin. Leupeptin (an inhibitor of trypsin, papain and cathepsin B), antipain (an inhibitor of papain, trypsin and cathepsins A and B) and pepstatin (an inhibitor of pepsin and cathepsin D) were without effect on the protease activities. Elastinal (a specific inhibitor of elastase) inhibited the protease from human bone marrow cells. Both these rat and human proteases showed specificity for the pyridoxal-group of enzymes. Both diisopropylphosphorofluoridate and phenylmethane sulphonyl fluoride were effective inhibitors of the rat

and human proteases which indicated that these were typical serine proteases. Chemical modifications of the enzymes showed that serine, histidine and perhaps tyrosine were essential for activity. Usually, in the case of serine proteases, the serine hydroxyl and histidine labile protons play essential roles in the splitting of the peptide bond.

The four rat and human proteases are thus apparently clearly distinguishable from each other. The rat enzymes are chymotrypsin-like, while the human protease is elastase-like. The protease from the muscle layer of the small intestine of the rat cleaves one of the elastase substrates whereas the others do not. The liver and skeletal muscle enzymes differ in that the molecular mass of the liver protease is lower (13 000 compared to 17 000) and the liver protease is eluted from a CM-cellulose column by 0,02 M phosphate buffer. These group-specific proteases differ from lysosomal proteases in the following points:

(1) Optimum pH is in the alkaline range whereas cathepsins act best in acidic solutions,

(2) the proteases are serine proteases whereas most of the lysosomal proteases are thiol or carboxyl enzymes, and

(3) the proteases have chymotrypsin-like properties.

Chymotrypsin-like proteases have also been found in human seminal plasma (Syner and Moghissi, 1972), the isolated granules of mast cells from the peritoneal cavity of the rat (Kawiak et al, 1971; Vensel et al, 1971) and human neutrophil granulocytes (Rinder- Ludwig and Braunsteiner, 1975). All these proteases are active on tyrosine esters. The plasma from

human seminal fluid is, however, unaffected by diisopropylphosphofluoridate. Tos-Phe-CH_2Cl inhibited the latter two enzymes. The protease from the muscle layer of the small intestine is unaffected by Tos-Phe-CH_2Cl but it is difficult, as yet, to distinguish this enzyme from the mast cell and granulocyte proteases.

Skeletal muscle contains neutral proteases, in addition to cathepsins, making discrimination between these and the chymotrypsin-like protease important. The molecular mass of the protease reported by Huston and Krebs (1968) is 110 000, which is much larger than that of any group-specific protease. The protease of Mayer et al (1974) was unaffected by Ca^{+2}, that of Okitani et al (1974) was activated by Ca^{+2}, whilst the group-specific skeletal muscle protease was inhibited by Ca^{+2}. Neutral proteases have also been reported in liver mitochondria (Fitzpatrick and Pennington, 1969; Gear et al, 1974) and rat liver peroxisomal fraction (Gray et al, 1970), but these enzymes have not been purified and characterised.

The amounts of Katunuma's serine protease in various types of muscles differ greatly; activity being higher in red muscle than in white, whereas none could be detected in cardiac muscle. High activity was observed in immature erythroblasts and granulocytes among bone marrow cell components, but low activity was observed in mature erythrocytes, granulocytes and lymphocytes. The rat liver group-specific protease is associated with the mitochondrial inner membrane. Regenerated and neo-natal liver show very low protease activities (Banno et al, 1975). Activity reached a minimum (< 30%) about 72 hours after hepatectomy relative to sham-operated or normal animals.

Possible mechanisms for the regulation of protease activities under

different physiological conditions include:

(1) de novo synthesis of the protease,

(2) translocation within the cell, and

(3) activation/inhibition of the proteases.

Katunuma has isolated a peptide inhibitor in the same sub-cellular fraction as the protease.

Thus it can be seen that there have been reports of a number of chymotrypsin-like proteases, especially during the last decade, which can be detected in muscle homogenates incubated at alkaline pH. These are summarised in Table 1.1 (Bird et al, 1980). Due to their optimum activity at alkaline pH, the proteases have generally been referred to as "alkaline proteases". Most of these enzymes have been reported to be inhibited by diisopropyl phosphorofluoridate (Dip-F) or phenylmethansulfonylfluoride (Pms-F) and therefore they are classified as "serine proteases". However, clarity as to the precise nature of these enzymes has not yet been achieved.

Investigators	Year	Common name
Koszalka and Miller	1960	Alkaline protease
Noguchi and Kandatsu	1966	Myofibrillar protease
Holmes et al	1971	Alkaline protease
Kar and Pearson	1972	Alkaline cathepsin
Park et al	1973	Mast cell protease
Mayer et al	1974	Myofibrillar protease
Smith and Bird	1975	Alkaline protease
Katunuma et al	1975	Group-specific protease
Mueller et al	1977	Myofibrillar protease
Drabikowsky et al	1977	Alkaline protease
Woodbury et al	1978	Serine protease
McKee et al	1979	Neutral protease

Table 1.1. Muscle serine proteases "alkaline proteases".

1.4. OBJECTIVES OF STUDY

Protein turnover is an important factor in the metabolism of cells and can broadly be divided into two phases. The first phase involves the synthesis of the proteins and has been subjected to intense scrutiny in the past. The second phase involves the degradation of these proteins and has not to date been nearly as well characterised. However, the importance of protein catabolism is now being realised and the process has begun to be studied in more detail. These studies have shown the presence of new groups of proteases - one of these being those proteases with optimal activity in the alkaline pH range. These proteases appear to form a new group which is potentially capable of playing an important role in the catabolism of proteins.

As already reviewed, some of these alkaline proteases have been characterised to varying degrees in the past. One of the major problems has been the separation of the myofibrils from the alkaline protease. This has been achieved in the present investigation by means of a pyrophosphate-wash which removed a factor which appears to assist in the binding of the protease to the myofibrillar proteins. Furthermore, I have attempted to show that the alkaline proteases are not a single entity but that there are a number of different alkaline proteases. The presence of a physiological MAP inhibitor, which is capable of playing an important role in regulating the activity of the alkaline protease, has been verified. An alkaline protease similar to the principal enzyme studied was also demonstrated and partially purified from rat cardiac muscle.

CHAPTER 2

PURIFICATION OF A "MYOFIBRILLAR ALKALINE PROTEASE" FROM RAT SKELETAL MUSCLE

2.1. INTRODUCTION

A major problem in the study of the myofibrillar alkaline protease has been the great difficulty experienced in separating the alkaline protease from myofibrillar components. Clearly, if this separation is achieved, the enzyme can be studied in greater detail, since the reaction conditions can be more sensitively controlled in the absence of a large excess of myofibrillar proteins. In order to establish that the enzyme is not associated with particulate fractions in skeletal muscle homogenates (and therefore in the intact tissue) other than myofibrils, I initially purified "washed myofibrils" by the standard technique and then proceeded to separate the alkaline protease from the myofibrils. This showed that the enzyme was not derived from mitochondria or nuclei, etc. but that it was a true myofibrillar alkaline protease.

2.2. RESULTS

2.2.1. Standardisation of MAP assays

2.2.1.1. Methods of assay

Four methods were tested to establish an assay which was convenient and sensitive for the detection of alkaline protease activity. All four of these methods involved the use of either denatured casein or azocasein as a substrate for the protease. The individual assay procedures used are described in Chapter 4 (4.2). The following substrates and methods of assay were used:

(a) Denatured casein as substrate; tyrosine release measured.

(b) Denatured casein as substrate; A_{280} of the TCA-supernatant measured.

(c) $|^{125}I|$-labelled casein as substrate; release of TCA-soluble radioactivity measured.

(d) Azocasein as substrate; A_{340} of TCA-supernatant measured.

Figs. 2.1 to 2.4 show a typical reaction profile for each of these four assay methods, using the same enzyme concentration, over a 60 minute incubation period. The standard curve for the fluorimetric detection of tyrosine was linear up to 20 nmoles tyrosine (Fig. 2.5).

The tyrosine-release method was linear for an hour and was very sensitive. The A_{280} method showed only a very small change in absorbance over the incubation period and the procedure was abandoned because of its insensitivity. The measurement of $|^{125}I|$-tyrosine release from labelled substrate proteins was very sensitive, and the time course was linear over the full incubation period. However, this method was also abandoned because of the laborious preparation of the $|^{125}I|$-labelled substrate and because a rapid rise in background levels occurred during storage of the material, thus making this method cumbersome for rapid enzyme assays. The azocasein method was ultimately preferred because it was an economical, rapid and sensitive method of determining the alkaline protease activity. This method was also highly reproducible.

A unit of MAP activity was defined as that amount of enzyme, in the standard assay mixture, which would cause an increase in the absorbance of the TCA-supernatant of 0,001 at 340 nm in 60 minutes under the standard assay conditions described in the "Methods" section (4.2.3.).

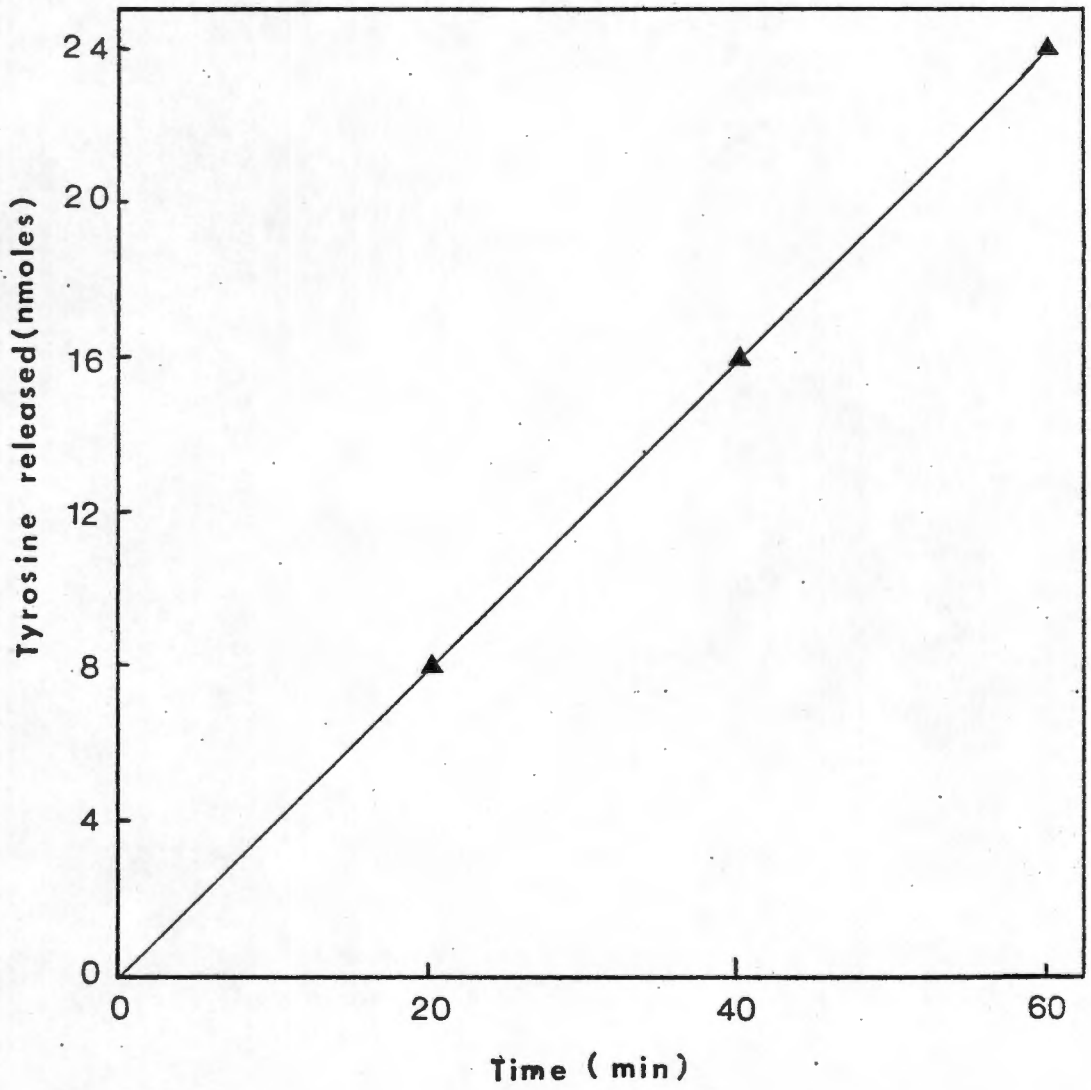


Fig. 2.1. MAP activity using denatured casein as substrate. MAP was prepared as described in 4.4.1 - 4.4.2, and protease activity was measured as in 4.2.1.3.

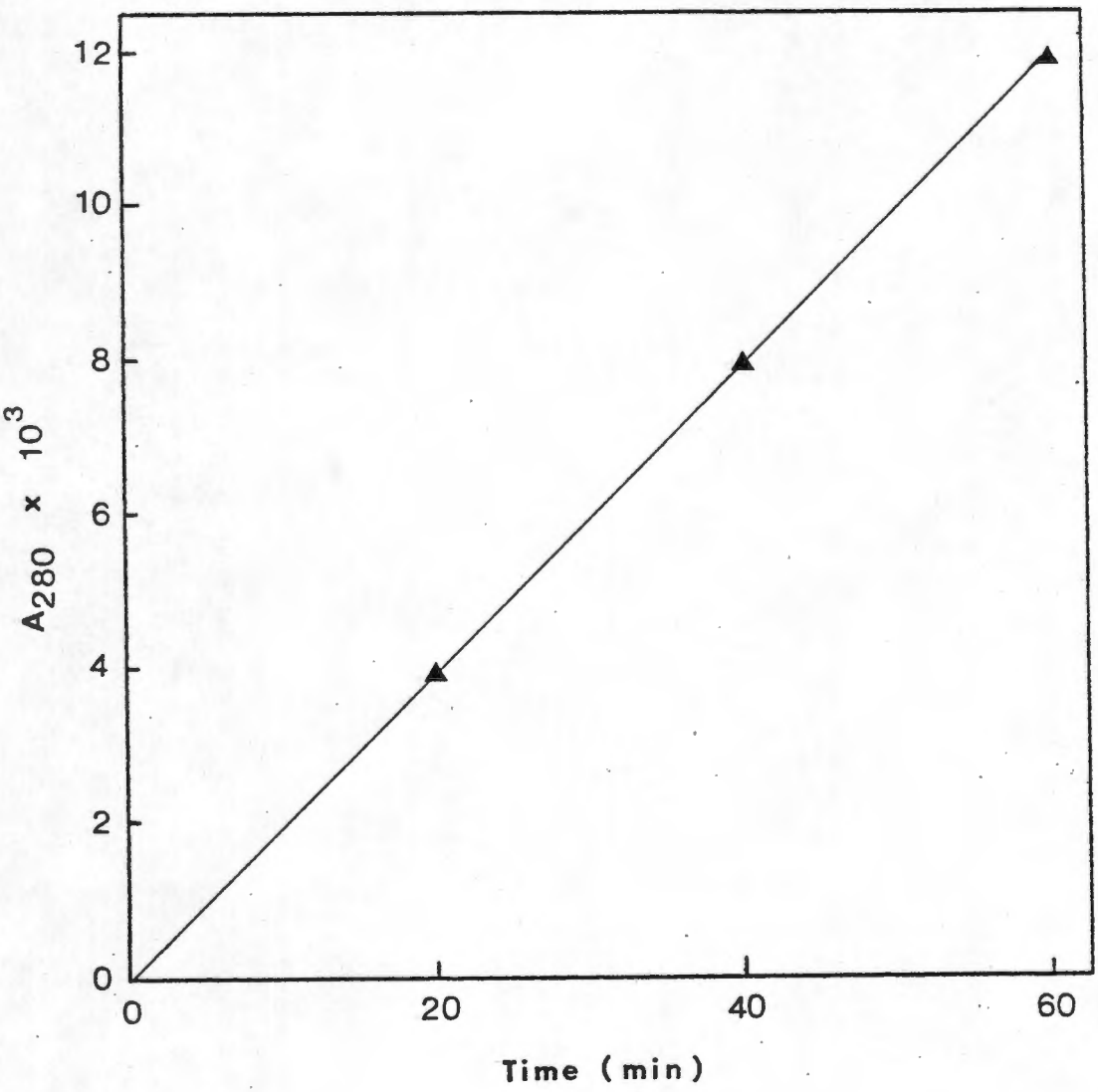


Fig. 2.2. MAP activity using denatured casein as substrate. MAP was prepared as described in 4.4.1 - 4.4.2, and protease activity was measured as in 4.2.1.4.

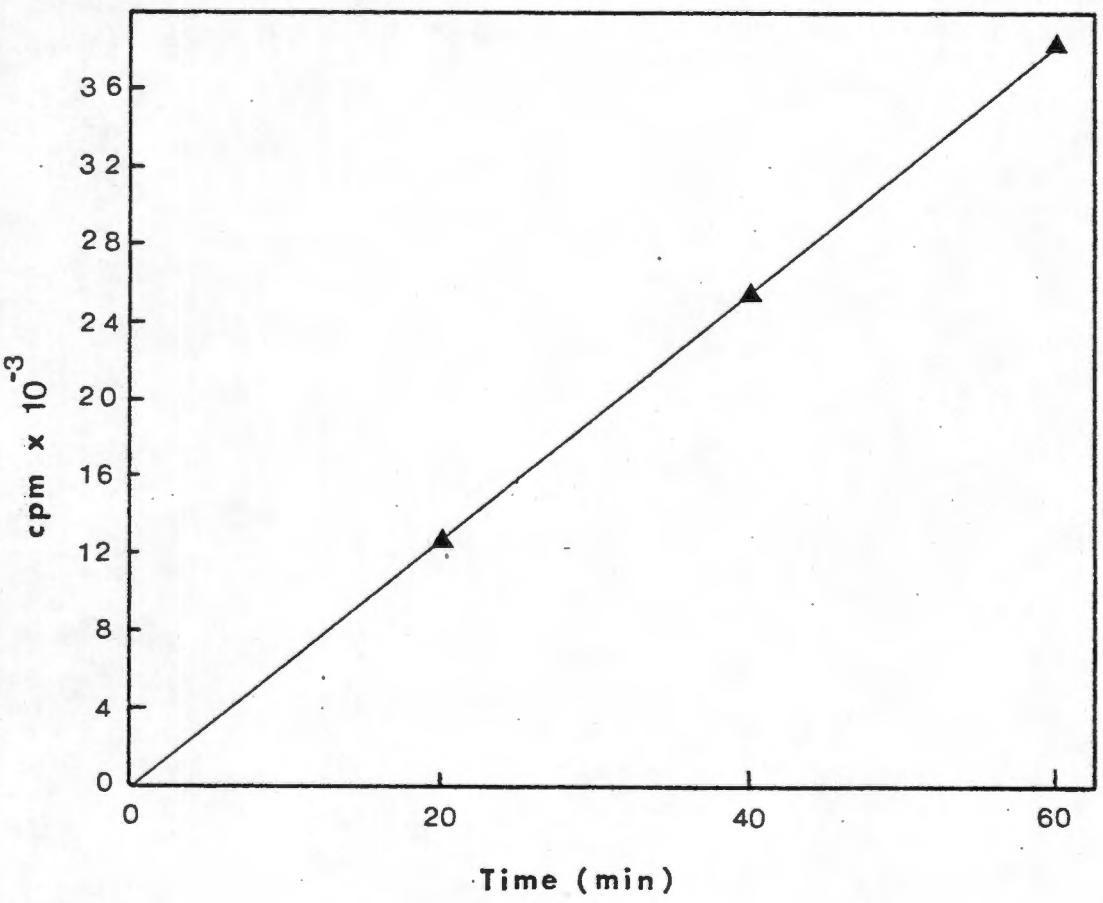


Fig. 2.3. MAP activity using $|^{125}I|$-casein as substrate. MAP was prepared as described in 4.4.1 - 4.4.2, and proteolytic activity was measured as in 4.2.2.3.

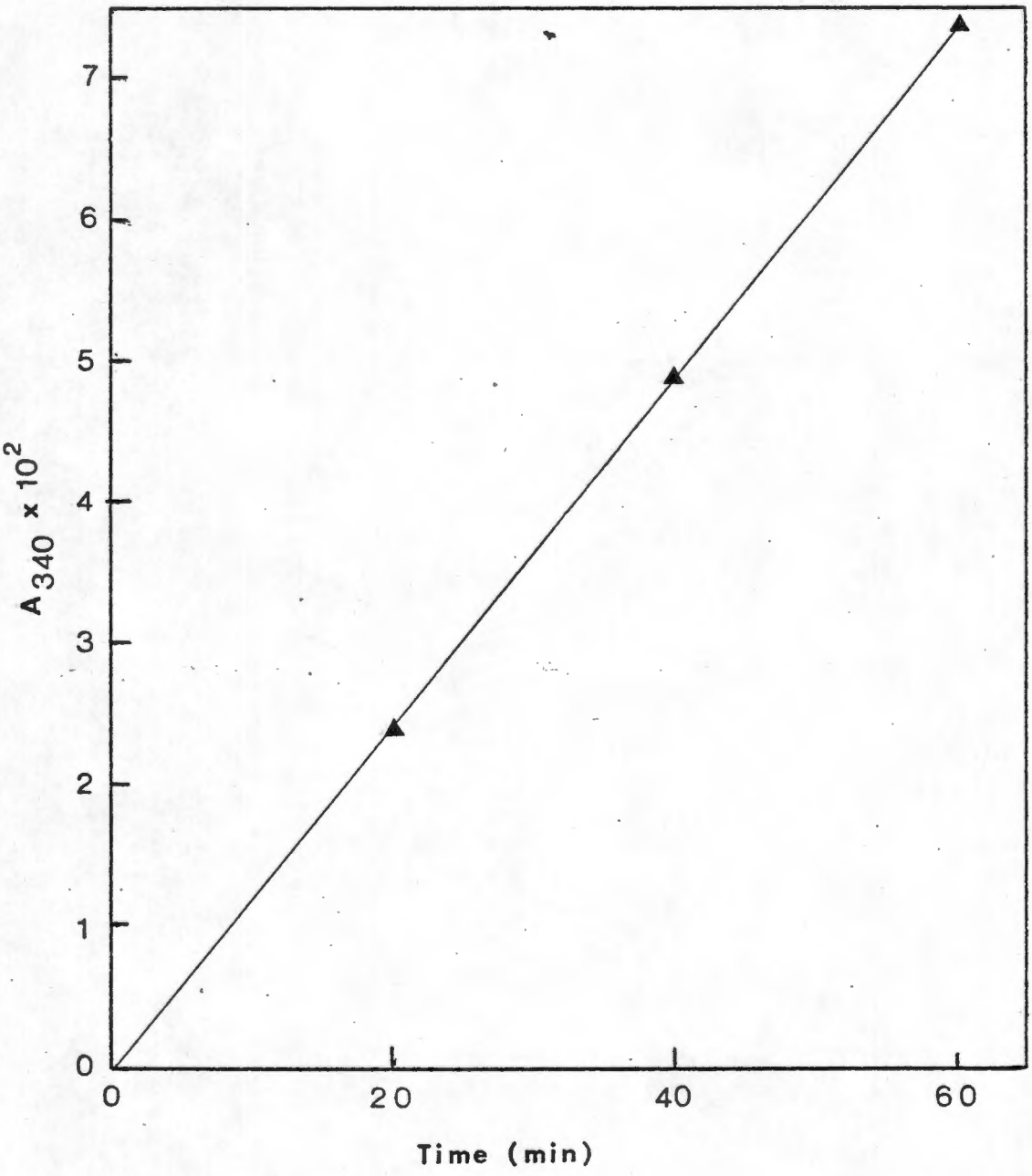


Fig. 2.4. MAP activity using azocasein as substrate. MAP was prepared as described in 4.4.1 - 4.4.2, and proteolytic activity was measured as in 4.2.3.

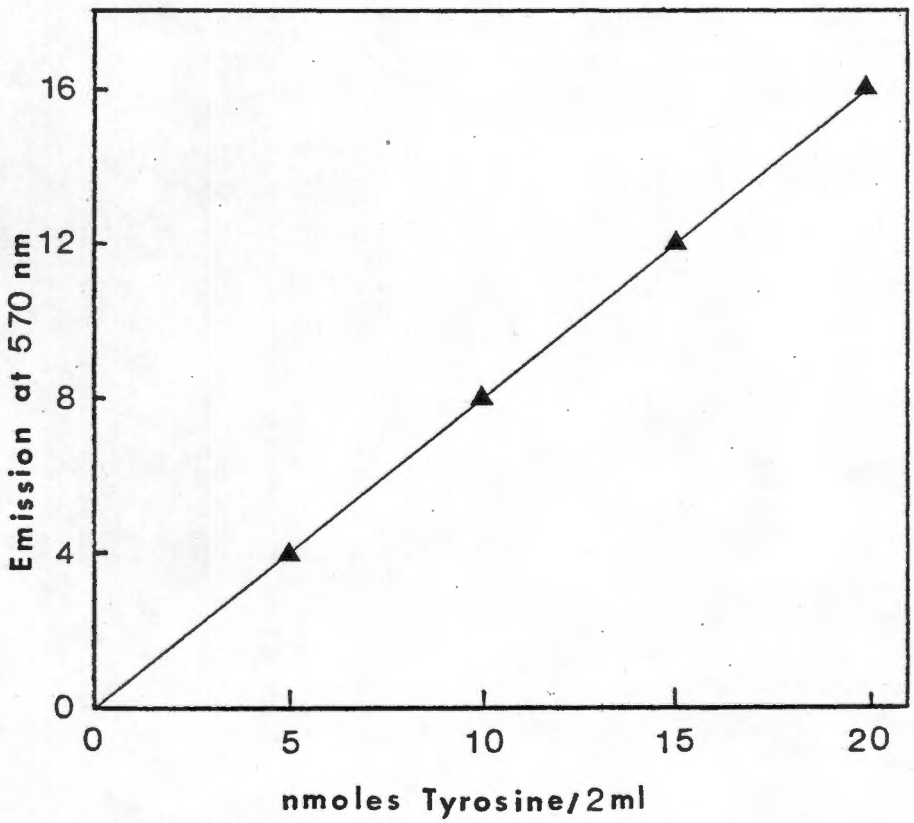


Fig. 2.5. Tyrosine standard curve. Tyrosine concentrations were measured fluorimetrically as described in 4.2.1.3.

2.2.1.2. Time dependence of MAP reaction

The time dependence of MAP activity under standard reaction conditions is shown in Fig. 2.4. The alkaline protease activity was found to be linear over a 60 minute incubation period, in the presence of excess substrate. An incubation period of 45 minutes was used during the routine enzyme activity determinations.

2.2.1.3. pH-dependence of MAP reaction

The pH dependence of MAP activity under standard conditions is shown in Fig. 2.6. This assay was performed using a solubilised washed myofibrillar precipitate which may have contained a mixture of "myofibrillar alkaline proteases". The enzyme was in any case routinely assayed at pH 9,0 during the purification procedure to ensure that only alkaline proteases and not acid- nor neutral proteases were measured.

2.2.2. Isolation and purification of MAP (Description of a typical preparation).

2.2.2.1. Sample preparation

Rat hind leg muscles (237 gram) were trimmed of excess fat and connective tissue, cut into small pieces and then homogenised in 10 vols (ml/g) of cooled pyrophosphate-enriched low salt buffer pH 7,1, as described in the Methods (4.4.1.).

2.2.2.2. Washed myofibril preparations

The homogenate prepared above (2 745 ml) was centrifuged at 800 g for 10 minutes at $4^{o}C$. The supernatant was discarded and the precipitate was re-suspended in 5 vol. of the pyrophosphate-enriched low-salt

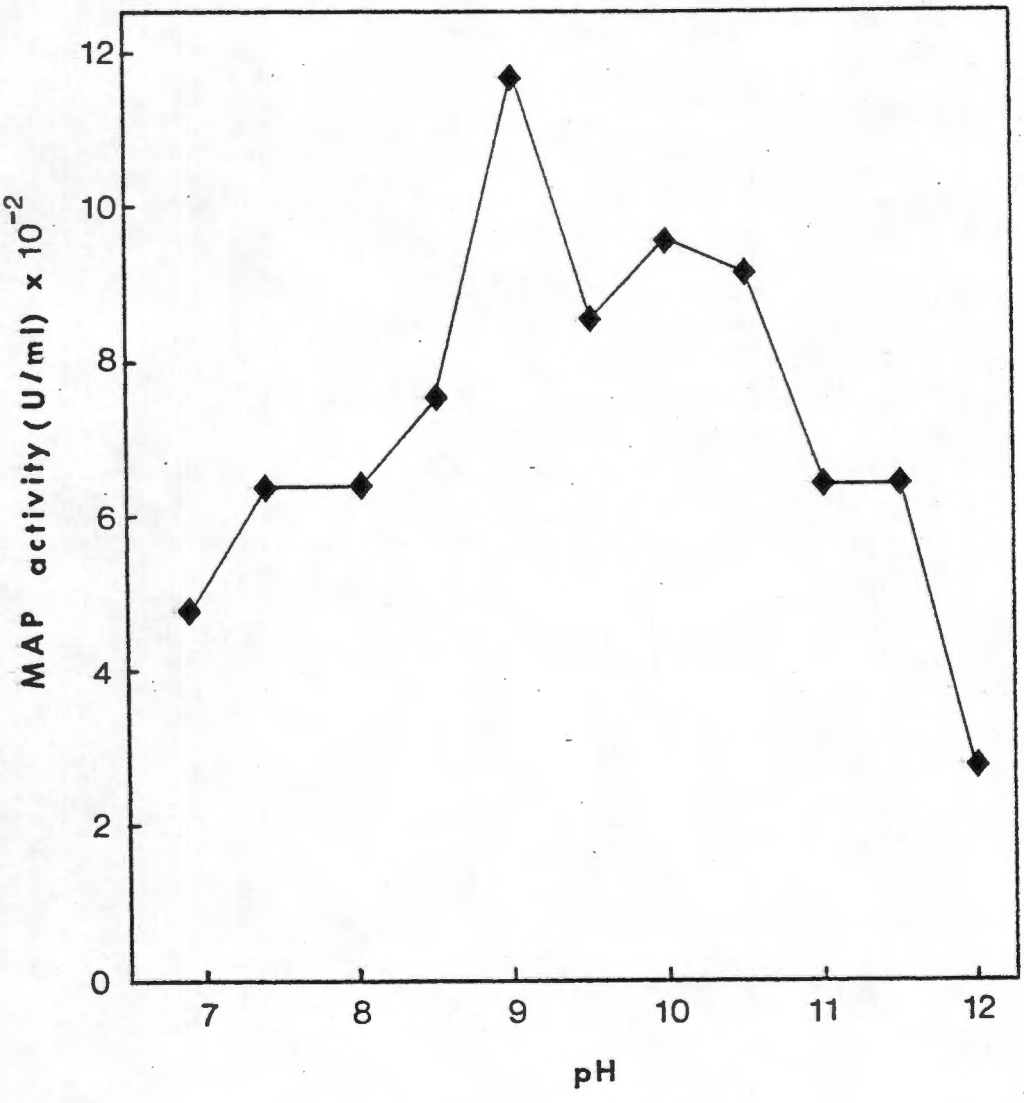


Fig. 2.6. Effect of pH on MAP activity. MAP was prepared from skeletal muscle as in 4.4.1 - 4.4.2. MAP activity was assayed as described in 4.2.3.

buffer pH 7,1. The suspension so produced was called suspension A (1 440 ml). These washes were repeated a further three times. On the fourth rinse, the buffer included Triton X-100 (1%) in addition to the normal constituents. The precipitate was then washed a further two times with the normal pyrophosphate-enriched low-salt buffer and the resulting final precipitate was called the washed myofibril precipitate.

2.2.2.3. Wash at a high concentration of pyrophosphate

The washed myofibril precipitate was stirred in 10 vols of fresh 20 mM tetra-sodium pyrophosphate (pH > 10) for 1 hour at 4°C. The resulting suspension was centrifuged at 8 000 g for 30 minutes at 4°C. The resulting supernatant was discarded, whilst the precipitate was called the "pyrophosphate-wash precipitate".

2.2.2.4. Solubilisation at high salt concentrations

The "pyrophosphate-wash precipitate" was stirred for 1 hour at 4°C in 10 vols of 0,6 M KCl. Thereafter, the mixture was centrifuged at 48 000 g for 30 minutes at 4°C. The supernatant was carefully decanted and retained: the "KCl-supernatant" (880 ml).

2.2.2.5. Dialysis of the "KCl-supernatant"

The "KCl-supernatant" solution was dialysed overnight at 4°C against 10 vols of a 5 mM potassium phosphate buffer containing 0,1 mM DTT (pH 8,5). The equilibrated dialysate was then centrifuged at 4°C for 15 minutes at 48 000 g. The supernatant was retained and called the "dialysed supernatant" (816 ml).

2.2.2.6. CM-Sephadex C-50 batch step

The "dialysed supernatant" was stirred for 30 minutes at 4°C in the presence of pre-equilibrated CM-Sephadex C-50 (prepared as described in 4.4.6), using 1 gram dry CM-Sephadex C-50 per 100 mg protein. The suspension (0 M NaCl) was centrifuged for 1 minute at 1 500 g at 4°C. The CM-Sephadex C-50 sediment was then stirred for 30 minutes at 4°C in 0,7 M NaCl - 10 mM Tris-HCl, pH 6,0. The suspension (0,7 M NaCl) was again centrifuged as before. The sediment was stirred for 30 minutes at 4°C in a 1,5 M NaCl - 10 mM Tris-HCl, pH 6,0 solution. The suspension (1,5 M NaCl) was similarly centrifuged. The final supernatant was decanted and retained: the "CM-1,5 M supernatant" (379 ml). The "CM-1,5 M supernatant" was immediately taken and dialysed overnight at 4°C against 10 vols of 5 mM potassium phosphate buffer and 0,1 mM DTT at pH 8,5.

2.2.2.7. Ultrafiltration

The "dialysed CM-1,5 M supernatant" was filtered at 4°C through an Amicon membrane filter, using a PM-10 disc at a pressure of 4,5 bar, compression being achieved by using nitrogen. The filtration was continued until an approximately 3-fold concentration had been achieved.

2.2.2.8. CM-Sephadex C-50 column chromatography

The concentrated CM-1,5 M solution (120 ml) was applied to an equilibrated CM-Sephadex C-50 column (1,4 x 5,5 cm) and the sample was eluted using a linear gradient of NaCl (0 - 0,7 M) in a 10 mM Tris-HCl, pH 6,0 buffer (See Methods 4.4.8). All fractions were assayed for MAP activity by the standard azocasein-linked spectrophotometric method.

The protein elution profile was obtained using the Bio-Rad colour reagent; the standard curve for protein using this reagent was performed using bovine serum albumin and was found to be linear up to 1 mg protein/ml (Fig. 2.7).

The "bound protein" was found to elute primarily at 0,48 M NaCl, while the "bound enzyme" was found to elute at 0,66 M NaCl (Fig. 2.8). The active peak tubes were pooled (28 ml) and dialysed overnight at $4^{o}C$ against 10 vols 5 mM potassium phosphate buffer and 0,1 mM DTT (pH 8,5). The dialysate was then concentrated by vacuum dialysis at $4^{o}C$ to a final volume of 3 ml.

2.2.2.9. Sephadex G-75 chromatography

The concentrated material was now applied to a 1,6 x 90 cm Sephadex G-75 column at $4^{o}C$. The sample was eluted using a 0,6 M KCl - 10 mM Tris-HCl, pH 7,0 solution (4.4.9). The fractions collected were assayed for protein content, and enzyme activity (Fig. 2.9): the MAP activity eluted as a peak distinct from the protein peak.

The specific activity of MAP was found to increase from 59,4 units/mg in the homogenate to a final specific activity of 602 676 units/mg in the pooled Sephadex-G-75 enzyme peak. This represents a purification of 10 215-fold (Table 2.1).

2.2.3. Determination of the molecular mass of MAP

A molecular weight calibration curve for the Sephadex G-75 column is shown in Fig. 2.10. The molecular weight of the partially purified MAP, as determined by gel filtration of the concentrated enzyme, was about 31 600 daltons.

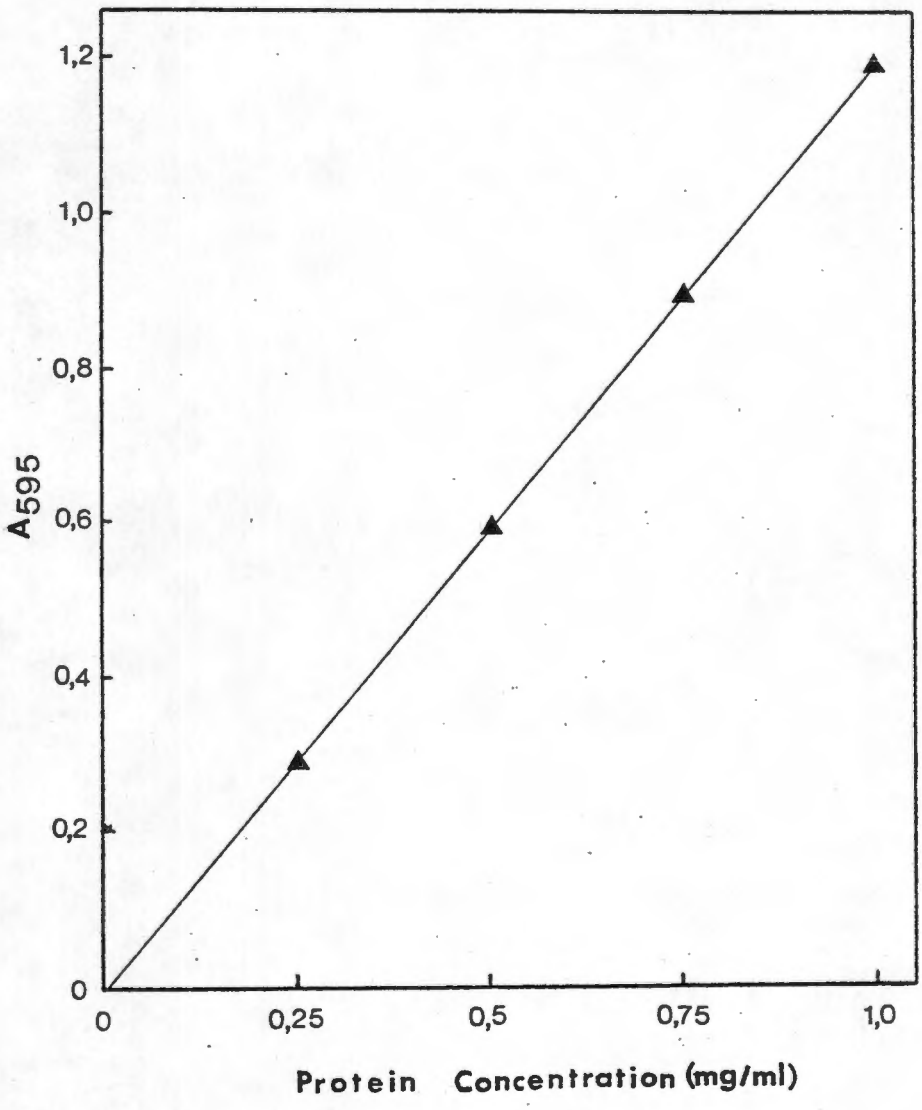


Fig. 2.7. Protein standard curve: Bovine serum albumin (fraction V) was used as standard, and the measurements were made as described in 4.3.

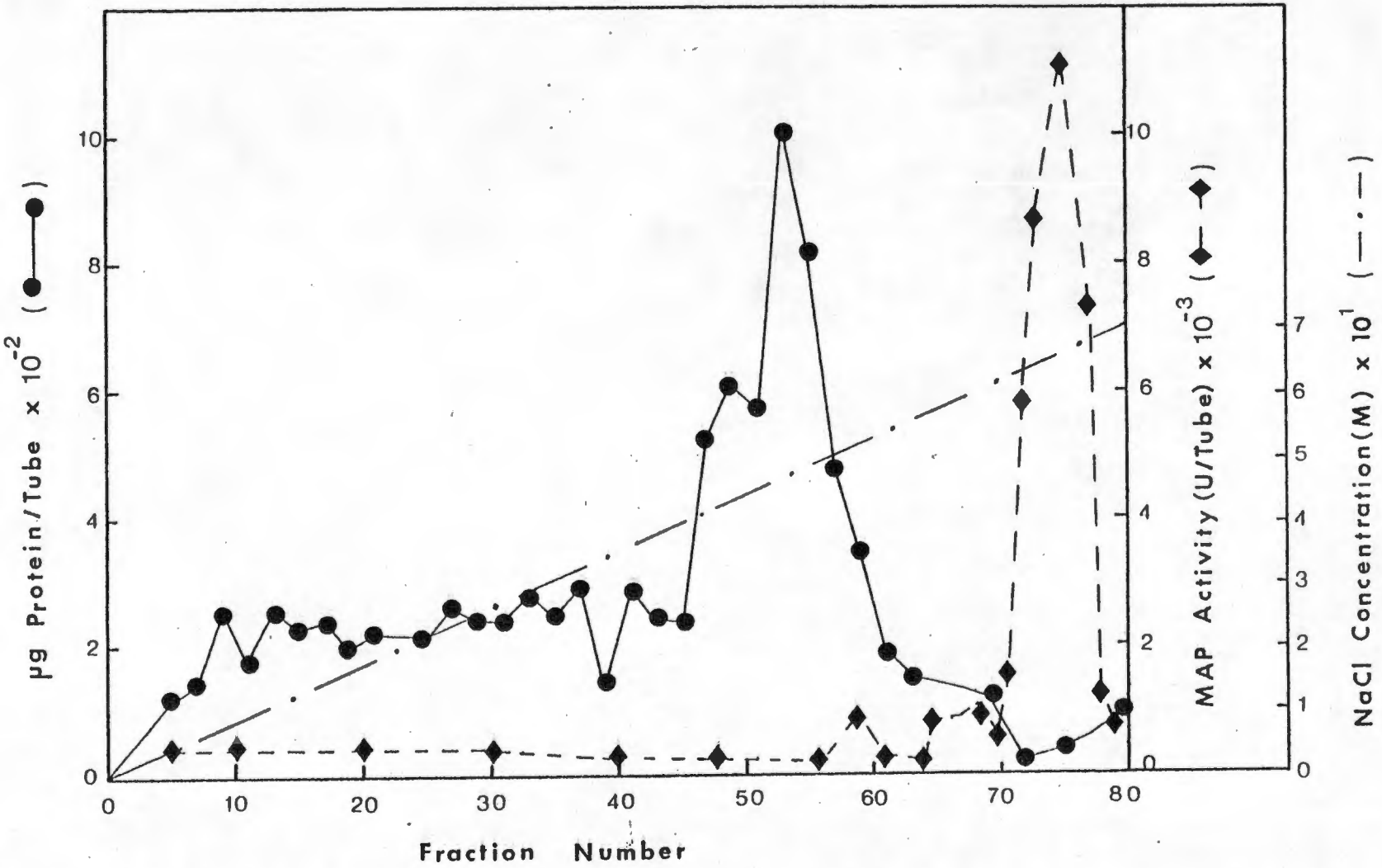


Fig. 2.8. CM-Sephadex C-50 chromatography of MAP from rat skeletal muscle. MAP was prepared as described in 4.4.1 to 4.4.7. A sample of 120 ml (20 mg protein) was applied to the column and elution was performed with a linear NaCl gradient (250 ml) from 0 M to 0,7 M NaCl. The flow rate was 10 ml/hr and each fraction contained 3,125 ml. Aliquots were assayed for proteolytic activity as described in 4.2.3, and protein concentration was determined as described in 4.3.

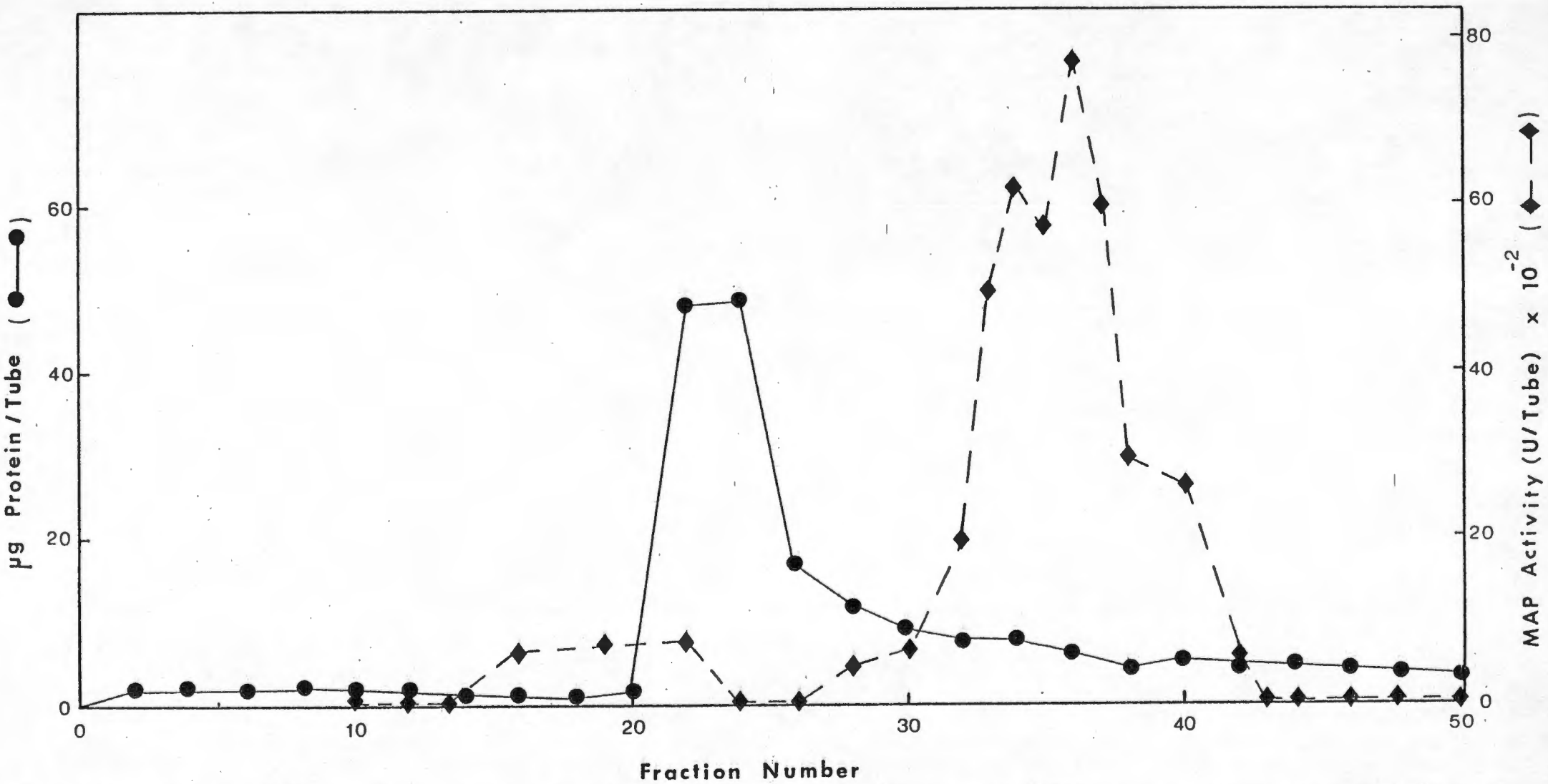


Fig. 2.9. Sephadex G-75 chromatography of material prepared from rat skeletal muscle. MAP was prepared as described in 4.4.1 to 4.4.8. A sample of 3 ml (535 µg protein) was applied to the column and eluted with 0,6 M KCl - 10 mM Tris-HCl, pH 7,0. Elution fractions of 2,5 ml were collected at a flow rate of 10 ml/hr. Aliquots were assayed for proteolytic activity as described in 4.2.3. Protein concentrations were determined at A_{220} relative to bovine serum albumin standards. Elution fractions 30-42 were pooled.

TABLE 2.1.

PURIFICATION OF MAP FROM RAT SKELETAL MUSCLE

Step	Solution	Vol. (ml)	µg Prot./ml	Tot. Protein (mg)	Enzyme Act. (U/ml)	Tot. Enz. Act. (U)	Spec. Act. (U/mg)	% Act.	Purific. fold
1	Homogenate	2 745	5 186	14 236	308	846 147	59	100	-
2	Suspension A	1 440	5 182	7 462	888	1 278 366	171	151	3
3	KCl-supernatant	880	2 141	1 884	955	840 507	446	99	8
4	Dialysed supn.	816	918	749	605	493 961	659	58	11
5	CM-1,5 M supn.	379	65	25	584	221 184	8 847	26	149
6	Conc. CM-1,5 M	120	167	20	1 627	195 200	9 760	23	164
7	CM pooled enzyme	28	19	0,535	2 025	56 700	105 981	7	1 784
8	G75 pooled enzyme	37,5	1,9	0,071	1 141	42 790	602 676	5	10 215

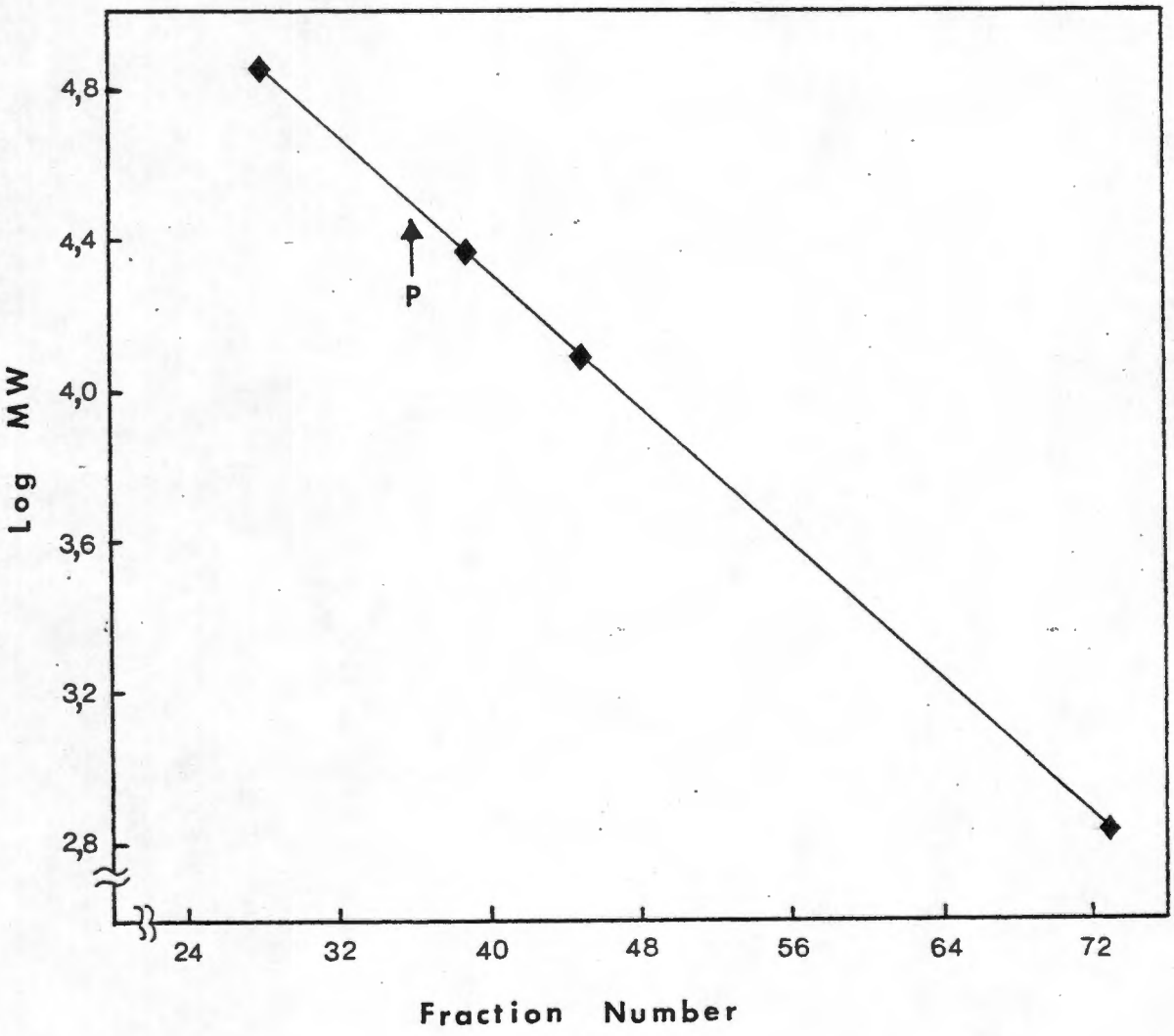


Fig. 2.10. Molecular weight calibration curve for Sephadex G-75 column (1,6 x 90 cm), equilibrated in 10 mM Tris - 0,6 M KCl-HCl, pH 7,0. The standards and their molecular weights were: NAD^+ (664); cytochrome C (12 500); α-chymotrypsin (25 000) and bovine serum albumin (68 000).
"P" indicates the fraction in which MAP eluted.

2.3. DISCUSSION

The present study describes the purification of an alkaline protease from rat skeletal muscle. A 10 215-fold purification of the alkaline protease was obtained at a nett yield of 5% of the total original alkaline protease activity. However, the actual yield of this specific alkaline protease was actually higher as the original homogenate contained a mixture of alkaline proteases. The alkaline protease which was isolated could not be separated from the myofibrils by 1% Triton X-100, stirring or differential centrifugation. The protease was found to be solubilised only in solutions containing a salt concentration of at least 0,6 M KCl, up to the washed myofibrillar precipitate stage. Indeed, upon repeated solubilisation by 0,6 M KCl and sequential dialysis of the washed myofibrillar precipitate, the protease was on all occasions found to be present in the same fraction as the myofibrils. As the alakline protease could be separated from the myofibrils and was then soluble in a buffer free of KCl, it can be concluded that the protease was attached either to organelles which sedimented with the myofibrils or that it had become absorbed onto the myofibrils. The latter would appear to be the true explanation since the organelles were separated from myofibrils by differential centrifugation and by Triton X-100 treatment, and in addition the only particles visible in the washed myofibril precipitate when examined by light microscopy were myofibrils. I have therefore called this purified enzyme a myofibril-associated alakline protease, and it fulfils the requirements of a "myofibrillar alkaline protease" as described by Pennington et al (1973).

The protease was active at 37^{o}C and was capable of utilising both denatured casein and azocasein as substrates. Enzyme activity was

measured by the rate of increase of TCA-soluble material as measured spectrophotometrically at 340 nm. This method was found to be the most rapid and reproducible as well as the most convenient of the enzyme assay procedures tested. The protease activity was found to be linear for the first 60 min in the presence of an excess of substrate, but only the first 45 min were utilised during the routine enzyme assay.

The proteases in the washed myofibrillar precipitate were active over the pH range of 6,9 to 12,0. The highest protease activity was expressed at a pH of 9.0. The protease activity at pH 6,9 and 12,0 were 41% and 23%, respectively, of that observed at pH 9,0. At pH 7,4, the proteolytic activity was 54% of the optimal proteolytic activity. However, the washed myofibrils contained more than one alkaline protease (cf. 3.2.1) with activity at pH 9,0, but by measuring the enzyme activity and consistently checking it's chymostatin sensitivity (cf. 3.2.4.4), the protease of molecular mass 31 600 could be separated from the rest. An increased sensitivity to chymostatin was observed with increasing purification of the alkaline protease (cf. 3.2.1.3).

The alkaline protease was separated from the myofibrils by means of a prior exposure to an alkaline solution of 20 mM sodium pyrophosphate, followed by solubilisation of the myofibrils. Upon subsequent dialysis the protease activity remained in the supernatant fraction whilst the myofibrils were precipitated. This method is less severe than that of Dahlmann and Reinauer (1978) who used bovine trypsin coupled to Sepharose 4B to "lower the viscosity" of the solution. Their solubilisation step showed a 3-fold increase in total proteolytic activity which was shown to be due to leakage of free trypsin from the Sepharose conjugate. Their treatment of the homogenate solubilised in 1 M KCl for the preparation of "myofibrils" was also less specific as they classified everything

which precipitated at 33% saturation with respect to ammonium sulphate as myofibrillar proteins.

The alkaline protease purified in this work was unstable at a pH lower than 7,0. A decrease in protease activity of about 33% was observed if the enzyme solution was left in a buffer at pH 6,0 overnight, whilst at pH 8,5 there was less than a 1% decrease in protease activity over a one-week period in the presence of excess subtrate. Thus the enzyme was assayed immediately following the steps involving buffers at pH 6,0 (CM-Sephadex C-50 batch and column steps) and thereafter the enzyme was immediately dialysed against a 10-fold excess of buffer at pH 8,5.

The myofibrillar alkaline protease which has been purified here is clearly different from the insulin-specific protease described by Duckworth et al (1972) as their protease was obtained from a 100 000 g supernatant of muscle homogenate. Similarly, the protease is different from that of Koszalka et al (1960) which was purified from the 10 000 g supernatant of rat skeletal muscle.

The apparent molecular mass of 31 600 determined by gel filtration on Sephadex G-75 compares favourably with the value of 30 800 obtained by Dahlmann and Reinauer (1978) using gel filtration. Beynon and Kay (1978) purified a neutral protease from intestinal muscle of rats which had a molecular mass of 33 000. Murakami and Uchida (1978) purified an alkaline protease from rat heart and Kuo and Bhan (1980) from hamster heart, both proteases having a molecular mass of 26 000. Bhan et al (1978) purified a myofibril-associated alkaline protease which had a molecular mass of about 14 000 by gel filtration. The molecular mass obtained for the purified protease appears to be different from that

obtained in the case of a chymotrypsin-like protease (Noguchi and Kandatsu, 1976) of 25 000 daltons, which was similar to that described by Park et al (1973) and Holmes et al (1971). The purified protease clearly differs from the group-specific protease from rat skeletal muscle purified by Katunuma et al (1975) which had a molecular mass of 13 000. The purified protease also differs from the alkaline Ca^{2+}-dependent protease purified by Huston and Krebs (1968), which had a molecular mass by gel filtration of 110 000, as well as from the protease purified from liver (Burgher et al, 1972) which had a molecular mass of 80 000 by gel filtration. The serine protease purified by Sanada et al (1978) was found to have a molecular mass of approximately 23 000 by sedimentation equilibrium studies. Woodbury et al (1978) found that both the mast cell protease and a skeletal muscle protease had a molecular mass of 29 000 by PAGE in the presence of SDS and DTT and that this apparent molecular mass decreased in both cases to 26 000 in the presence of 8 M urea.

CHAPTER 3

CHARACTERISATION OF MAP ENZYME SYSTEM

3.1. INTRODUCTION

The confusion still existing concerning the identity of MAP enzyme(s) in muscle is largely due to the heterogeneity of the systems that have been studied and assays conducted on impure preparations. Substrates and enzyme preparations have also differed markedly in the systems used by different researchers.

One area where marked differences have been reported is that of the molecular mass of the MAP. Values ranging between 13 000 (Katunuma et al, 1975) and 110 000 (Huston and Krebs, 1968) have been reported. Other criteria used to define MAP enzymes have been their apparent subcellular location, their sensitivity to a variety of protease inhibitors and metal ions, as well as the absence or presence of detectable enzyme activity in various organs.

Although there were areas of similarity between the proteases studied by some researchers, there were such striking differences between the proteases studied in some cases that these differences could not be attributed only to experimental nor procedural differences. Clearly, thus, there is an indication that the alkaline proteases are not a single entity, but probably a group of similar enzymes.

It is for this reason that I have investigated, after application of the purification procedures described in the previous chapter, the properties of the purified enzyme to see whether there is in fact a

single MAP enzyme and to compare the MAP which I have studied to those investigated by other workers. Whilst a demonstrably pure enzyme preparation would be ideal for a thorough study, the properties of my particular enzyme preparation have been explored to assist in the positive identification of the enzyme concerned. Furthermore, a comparison with many of the other "MAP enzymes" studied can be made.

3.2. RESULTS

3.2.1. Evidence of a second alkaline protease

3.2.1.1. Sephadex G-75 chromatography

3 ml of the dialysed supernatant (See 2.2.2.5) was placed on a 1,6 x 90 cm Sephadex G-75 column and eluted by the standard method with 0,6 M KCl - 10 mM Tris-HCl, pH 7,0 (4.4.9). The resulting enzyme elution profile showed two distinct enzyme peaks of unequal magnitude (Fig. 3.1).

3.2.1.2. CM-Sephadex C-50 chromatography

A sample (10 ml) of dialysed supernatant (See 2.2.2.5) was placed on a 1,4 x 2,5 cm CM-Sephadex C-50 column and eluted step-wise with 15 ml 0,2 M NaCl - 10 mM Tris-HCl, pH 6,0 followed by 15 ml 0,7 M NaCl - 10 mM Tris-HCl, pH 6,0. Fractions of 3,3 ml were collected. These were assayed for protein content and enzyme activity by the standard procedures (4.4.8). The enzyme activity eluted in two separate peaks of unequal magnitude (Fig. 3.2).

3.2.1.3. Gelatin gel electrophoresis

A dialysed supernatant fraction (See 2.2.2.5) was applied to a 0,1% gelatin - 11% polyacrylamide slab gel according to the procedure

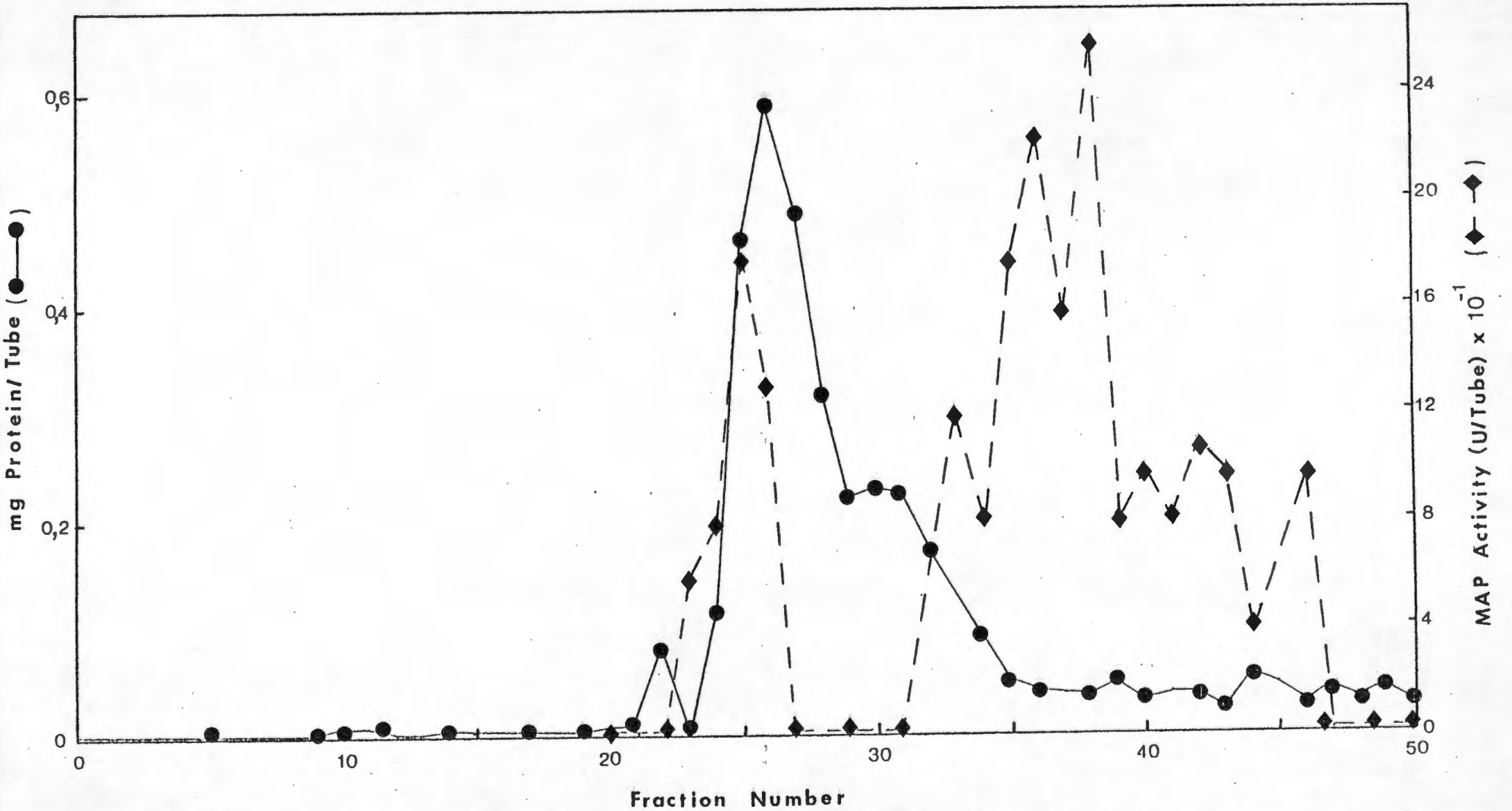


Fig. 3.1. Sephadex G-75 chromatography of enzyme preparations from rat skeletal muscle. MAP was prepared as described in 4.4.1 to 4.4.5. A sample of 3 ml (9 mg) was applied to the column and eluted with 0,6 M KCl - 10 mM Tris-HCl, pH 7.0. Elution fractions of 2,5 ml were collected. Aliquots were assayed for proteolytic activity as described in 4.2.3 and for protein concentration as described in 4.3.

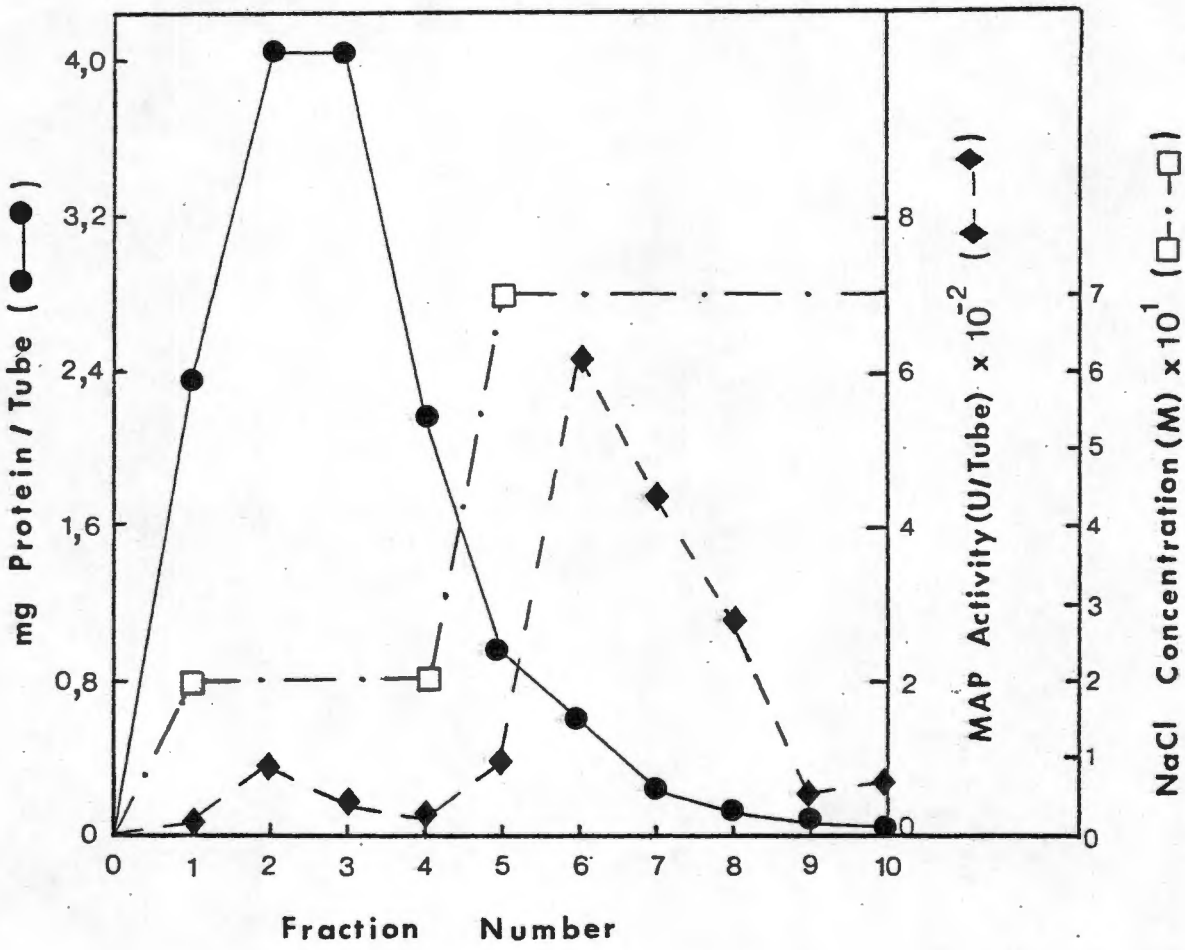


Fig. 3.2. CM-Sephadex C-50 chromatography of MAP from rat skeletal muscle. MAP was prepared as described in 4.4.1 to 4.4.5. A sample of 10 ml (19,7 mg protein) was applied to the column and elution was with a sequential step-wise gradient of NaCl from 0,2 M to 0,7 M NaCl (15 ml of each was used). The flow rate was 10 ml/hr and each fraction contained 3,125 ml. Aliquots were assayed for proteolytic activity as described in 4.2.3 and for protein concentration as described in 4.3.

described in Chapter 4 (4.5.2). In the absence of chymostatin in the incubation buffer, two clear lysis bands were observed. These corresponded to molecular weights of 46 800 and 32 900 when compared with standard myofibrillar proteins (Fig. 3.3). The presence of 10 µg/ml chymostatin in the incubation medium was associated with only one lysis band, normally that of the protease of higher molecular weight. An identical gel stained with coomassie blue showed a protein band corresponding to 32 500 at the site comparable to the chymostatin-sensitive protease, relative to standard myofibrillar protein markers. This clearly shows that the two bands do not represent artefacts due to enzyme aggregation or polymerisation, since the two enzymes are affected differently by the inhibitor, chymostatin.

3.2.2. Presence of a MAP inhibitor

Skeletal muscle was obtained by the normal procedure and a homogenate was prepared. The homogenate was centrifuged at 8000 g for 10 minutes at 4°C. The precipitate was then resuspended in pyrophosphate-enriched low-salt buffer (Fig. 3.4). The three enzyme-containing solutions were then all assayed for enzyme activity and protein concentration (Table 3.1).

The enzyme activity in the suspension showed a nearly 2-fold increase over that originally assayed in the homogenate. The addition of supernatant to the suspension caused a nett decrease in the expected combined enzyme activity (Table 3.1).

A similar sudden increase in activity was also found as shown in Fig. 3.5, following centrifugation of the "supernatant B" fraction for 60 min. at 100 000 g at 4°C. A dramatic 3-fold increase (254 → 736 units) in

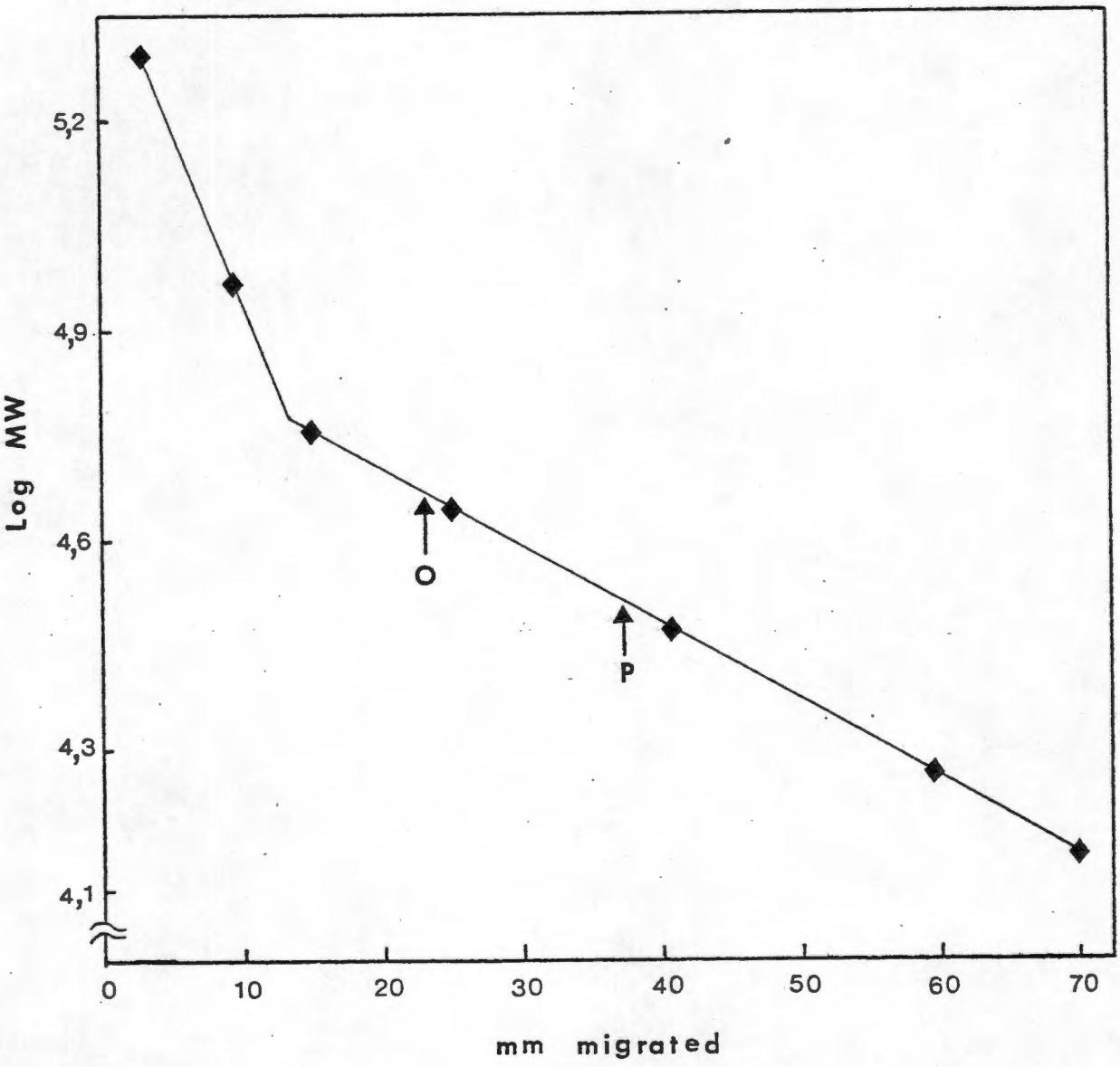


Fig. 3.3. Molecular weight calibration curve for gelatin-PAGE gel. The standards and their molecular weights were: lysozyme (14 000); soybean trypsin inhibitor (21 000); carbonic anhydrase (30 000); actin (44 000); albumin (68 000); phosphorylase (94 000) and myosin heavy chain (200 000). "O" indicates the distance migrated by the chymostatin-insensitive protease and "P" that of the chymostatin-sensitive protease.

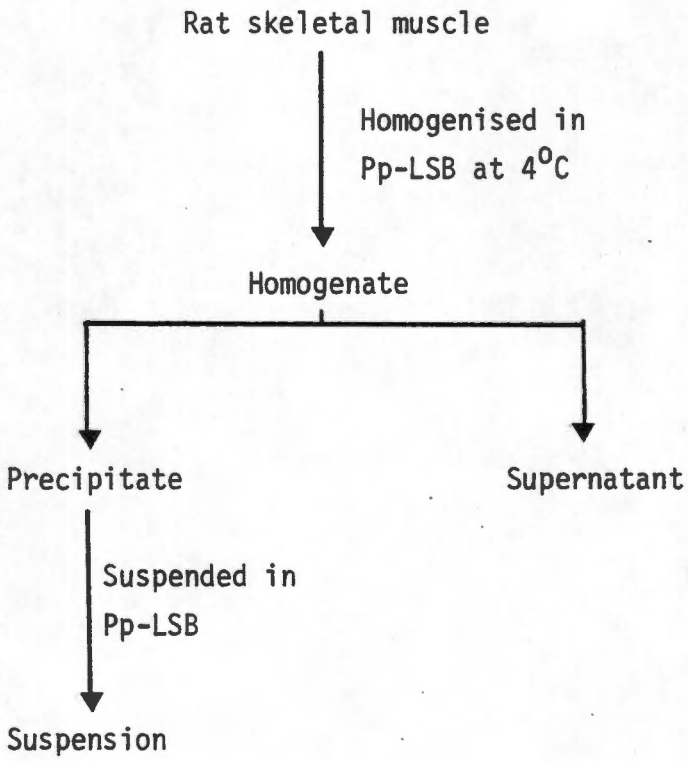


Fig. 3.4. Purification schedule to demonstrate presence of inhibitor. Pp-LSB was prepared as described in 4.4.1.

TABLE 3.1.

PROTEOLYTIC ACTIVITY SHOWING SEPARATION OF AN INHIBITOR

Fraction	Total Protein (mg)	Protein (%)	Total Activity (U)	Activity (%)
Homogenate	826	100	16 560	100
Supernatant	160	19	1 430	9
Suspension	441	53	30 580	184

enzyme activity was recorded after the ultracentrifugation.

3.2.3. Subcellular distribution of MAP activity

The rat skeletal muscle homogenate was prepared by the standard procedure as given in Methods (4.4.1. - 4.4.2.). The enzyme sample was then treated as shown in the flow chart of Fig. 3.5. All the fractions were assayed for enzyme activity and protein concentration (Table 3.2).

The results show that the MAP activity was predominantly present in the fraction which precipitates with the myofibrils; only a small proportion of the enzyme activity was associated with the mitochondrial-lysosomal pellet. The MAP activity present in the mitochondrial-lysosomal pellet may have been due to contamination as small quantities of myofibrils were detected (using PAGE) in the mitochondrial-lysosomal fraction (4.5.1.).

3.2.4. Properties of MAP

3.2.4.1. Effect of temperature on enzyme stability

Samples of the dialysed supernatant (See 2.2.2.5.) were incubated for 60 minutes at $4^{o}C$, $37^{o}C$ and $60^{o}C$ respectively. Protease activity in the samples was then determined by the standard assay method and compared with a sample which had been assayed immediately after preparation (Table 3.3.).

At $37^{o}C$ and below, no significant decrease in enzyme activity was observed for a period of 1 hour, while at $60^{o}C$ the enzyme activity was almost totally destroyed. Presumably, the enzyme activity at $37^{o}C$ was "protected" from autolytic digestion by the simultaneous presence

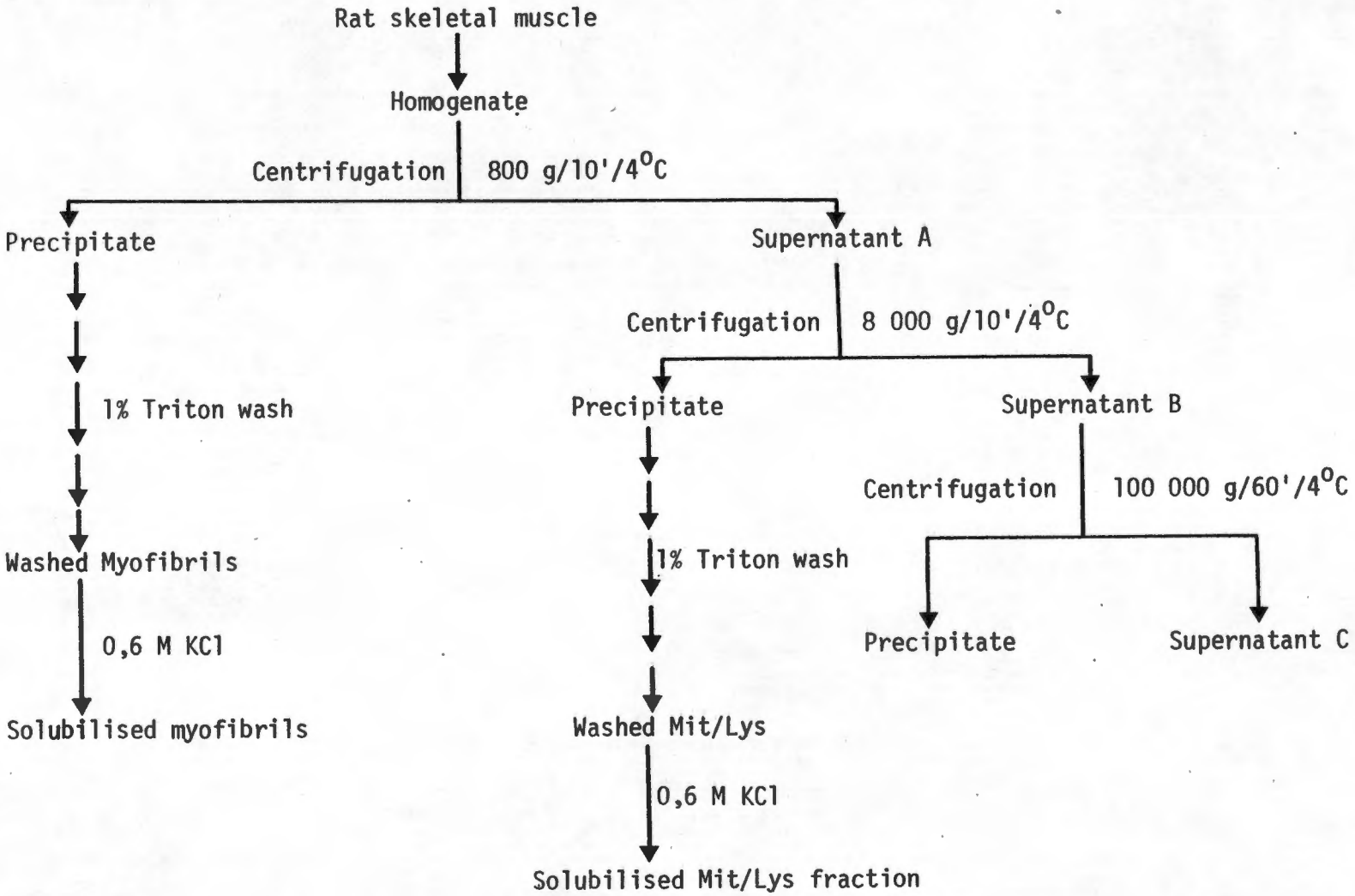


Fig. 3.5. Purification schedule for subcellular differentiation of proteolytic activity.

TABLE 3.2.

SUBCELLULAR DISTRIBUTION OF PROTEOLYTIC ACTIVITY

Fraction	Vol. (ml)	Total Protein (mg)	Total Activity (U)	Specific Activity (U/mg)
Homogenate	25	528	23 400	44
Sol. myofibrils	15	102	16 290	159
Supernatant A	17	212	4 420	21
Sol. Mit-Lys	11	12	2 838	229
Supernatant B	14,5	152	254	2
Supernatant C	11,5	102	736	7

TABLE 3.3

EFFECT OF TEMPERATURE ON ENZYME STABILITY

Temperature stored at (^{o}C)*	Proteolytic activity %
Control	100
4	100
37	98
60	1

* Enz. samples were incubated at the given temperature for 60 minutes before being assayed for protease activity.

of myofibrillar proteins and in a pure enzyme preparation a decrease in enzyme activity would be expected.

3.2.4.2. Effect of salt concentration on enzyme solubility

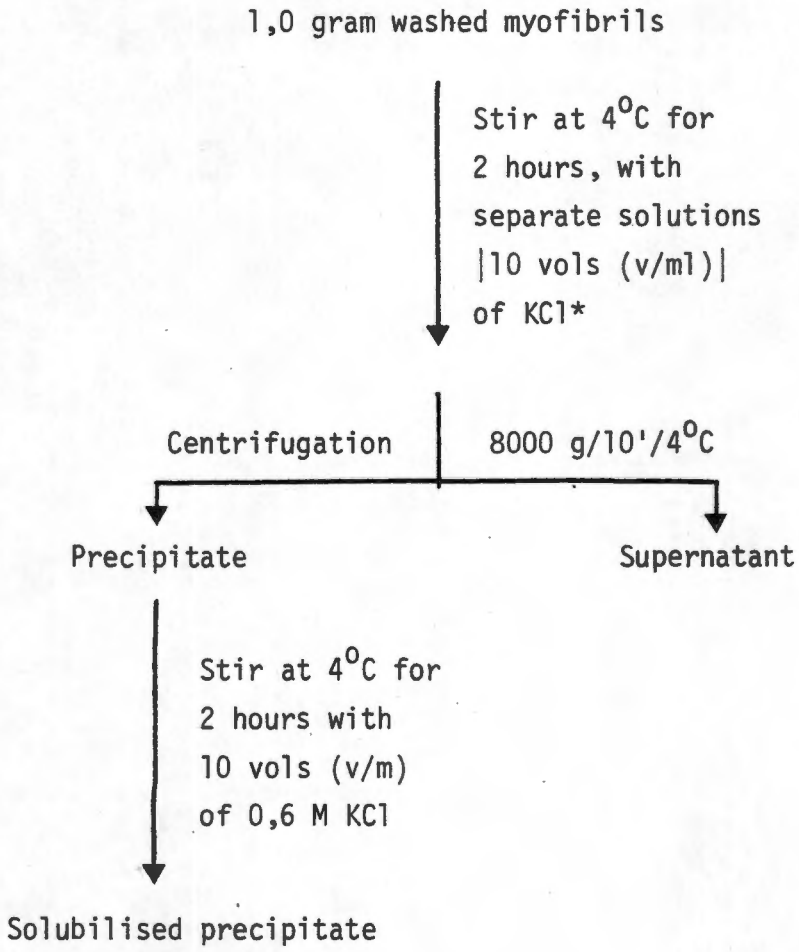
1,0 gram fractions of washed myofibril precipitates were treated at a range of KCl concentrations as shown in Fig. 3.6. The initially undissolved myofibrils were solubilised by 0,6 M KCl. The various solutions were assayed for enzyme activity and protein concentrations (Table 3.4.). The solubility of the enzyme increased as the salt concentration was raised, which paralleled the solubilization of the myofibrils.

3.2.4.3. Effect of pyrophosphate concentration on enzyme solubility

1,0 gram fractions of washed myofibril precipitate were treated at a range of fresh pyrophosphate concentrations, as shown in Fig. 3.7. The different enzyme solutions were assayed for enzyme activity and protein concentrations (Table 3.5.). These results show that at 10 mM pyrophosphate, both the enzyme and associated proteins become solubilised, while at 20 mM pyrophosphate, very little enzyme activity was solubilised despite a large amount of protein going into solution.

3.2.4.4. Effect of proteolytic enzyme inhibitors on MAP

Samples of dialysed supernatant (See 2.2.2.5.) were used to assess the effect of various proteolytic enzyme inhibitors on the activity of MAP. The inhibitors tested were chymostatin, soybean trypsin inhibitor, pepstatin, leupeptin and EGTA. The effects of these inhibitors on enzyme activity, expressed in comparison with the activity given by a control sample, are shown in Table 3.6.



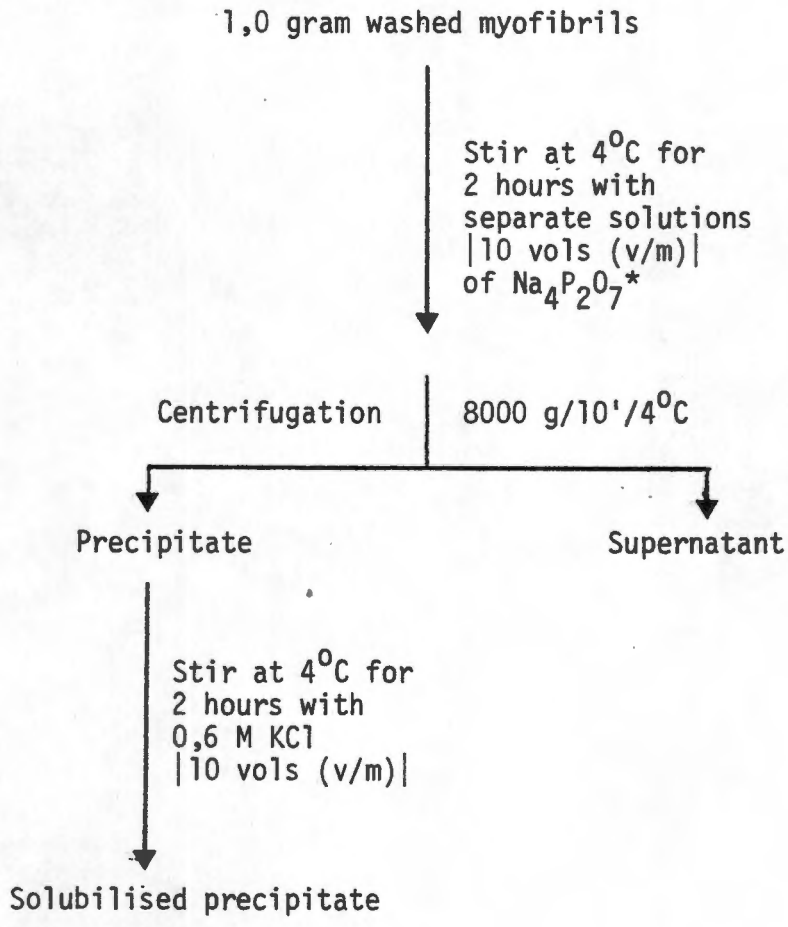
* 0 M; 0,2 M; 0,4 M and 0,6 M KCl solutions were used.

Fig. 3.6. Purification schedule for a range of KCl concentrations.

TABLE 3.4.

EFFECT OF KCl CONCENTRATION ON PROTEASE SOLUBILITY

KCl conc. (M)	Fraction	Total Prot. (mg)	Protein (%)	Total Act. (U)	Activity (%)	Spec. Activity (U/mg)
0	Supernatant	4	5	114	2	28
0	Solubilised ppt.	70	91	5 023	98	72
0,2	Supernatant	2	3	1 623	32	812
0,2	Solubilised ppt.	78	101	3 528	68	45
0,4	Supernatant	64	83	2 447	48	38
0,4	Solubilised ppt.	12	16	2 646	52	220
0,6	Supernatant	77	100	5 125	100	67



* 0 mM, 10 mM and 20 mM $Na_4P_2O_7$ solutions were used.

Fig. 3.7. Purification schedule for a range of pyrophosphate concentrations.

TABLE 3.5.

EFFECT OF PYROPHOSPHATE CONCENTRATIONS ON PROTEASE SOLUBILITY

$Na_4P_2O_7$ conc. (mM)	Fraction	Total Prot. (mg)	Protein (%)	Total Act. (U)	Activity (%)	Spec. Activity (U/mg)
0	Supernatant	—	—	—	—	—
0	Solubilised ppt.	77	100	5 125	100	67
10	Supernatant	67	87	4 392	86	66
10	Solubilised ppt.	9	12	624	12	69
20	Supernatant	63	82	532	10	8
20	Solubilised ppt.	14	18	4 312	84	308

TABLE 3.6.

EFFECTS OF INHIBITORS ON MAP ACTIVITY

Inhibitor	Inhibitor concentration (µg/ml)*	Protease activity (%)
None		100
Pepstatin	5	104
Leupeptin	10	99
Soybean Trypsin Inhibitor	10	67
Chymostatin	6,6	5
DMSO	-	100
EGTA	2	90

* All enzyme inhibitors were used in µg/ml concentrations, except EGTA which was used in millimolar concentration.

MAP activity was unaffected at the concentrations used of pepstatin (5 µg/ml), leupeptin (10 µg/ml) and to a lesser degree EGTA (2 mM). 10 µg/ml soybean trypsin inhibitor decreased enzyme activity by 33%. MAP activity was especially sensitive to chymostatin - 95% inhibition occurred at 6,6 µg/ml chymostatin. This may have been partly due to chymostatin being dissolved in DMSO, but no inhibition was observed in the presence of pure DMSO at the same concentration.

The chymostatin-sensitivity of the major MAP (MW 32 900), as compared with the chymostatin-insensitivity of the minor MAP (MW 46 800) (shown in 3.2.1.3.), was utilised in a preliminary study of the existence of the major alkaline protease in compound 48/80 treated (mast cell granule depleted) rats. The rats were treated as described in the Methods (4.4.10). Histology of skeletal muscles confirmed that compound 48/80 caused degranulation of the mast cells (Fig. 3.8). The dialysed supernatant prepared from such successfully granule-depleted rats contained significant levels of alkaline protease activity of which up to 63% was inhibited by 6,6 µg/ml chymostatin.

3.2.5. Presence of MAP in rat heart tissue

Heart muscle was removed from male fed rats and freed of excess fat and blood vessels. The heart was then cut into small pieces and homogenised as in the case of rat skeletal muscle (4.4.1.). The "heart MAP" was then further purified according to the procedure for "skeletal MAP" purification up to the formation of the dialysed supernatant (cf. 2.2.2.5). The various enzyme fractions were assayed for enzyme activity by the standard azocasein - A_{340} method (Table 3.7).

Rat heart muscle clearly contained MAP activity and the activity behaved

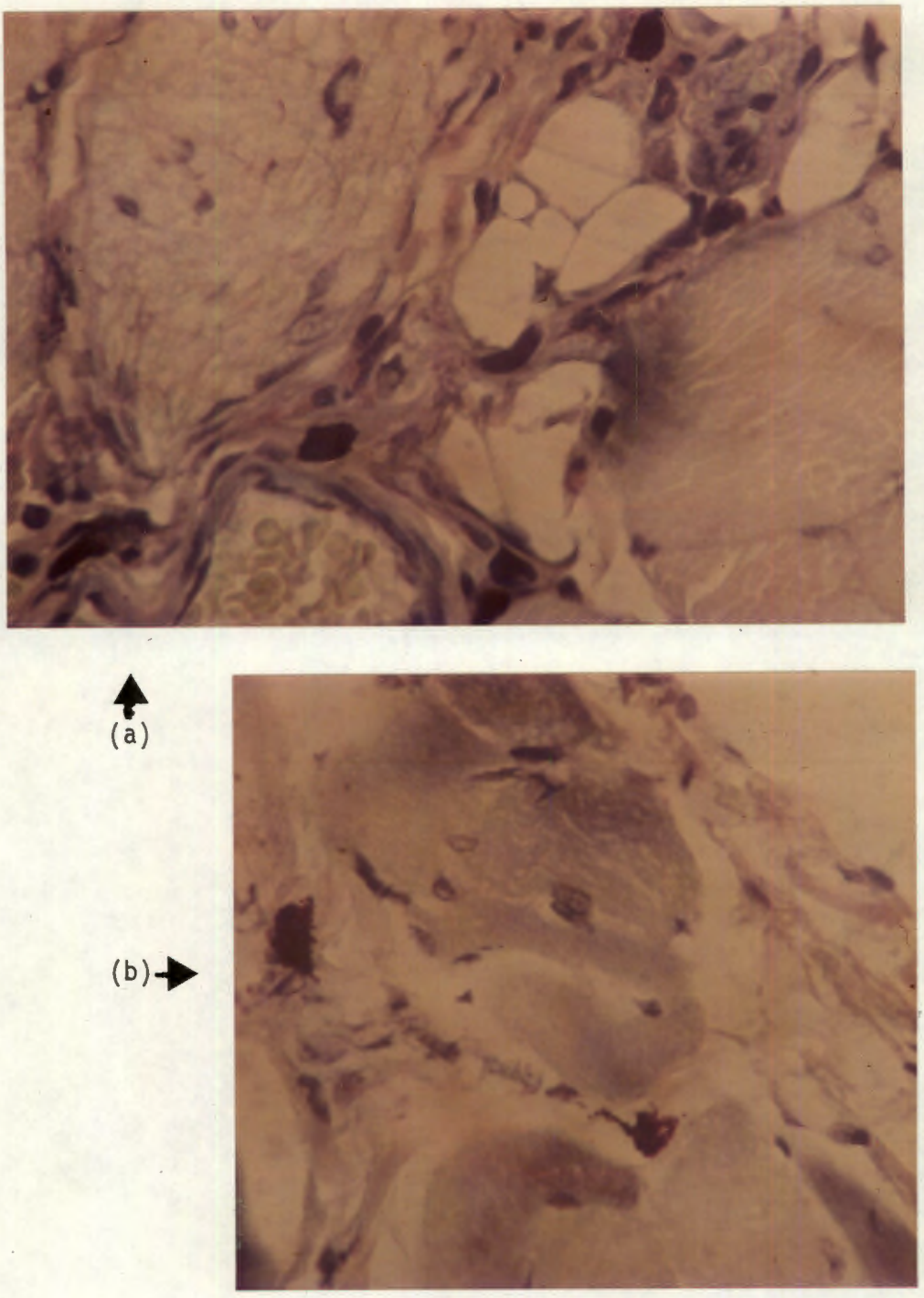


Fig. 3.8. (a) Normal rat skeletal muscle (Giemsa stain) showing the presence of mast cells with granules.

(b) Compound 48/80-treated rat skeletal muscle (Giemsa stain) showing a decrease in the number of mast cell granules.

TABLE 3.7.

PURIFICATION OF MAP FROM RAT CARDIAC MUSCLE

Sample	Vol. (ml)	Enzyme activity Units/ml	Total Activity (U)
Homogenate	21	414	8 694
Suspension	24	354	8 496
Supernatant	18	96	1 728
KCl supernatant	16	245	3 920
Dialysis supn.	15	85	1 275

very similarly to that detected in skeletal muscle. The heart form also behaves as a "myofibrillar-protease" up to the dialysis step.

3.3. DISCUSSION

The results described in this and the previous chapter point to the existence of two "myofibrillar alkaline proteases" in washed myofibrillar precipitates. Evidence of a second protease was found using three different separation methods as well as by means of inhibitor studies. The molecular masses of the two proteases were found to be 32 900 and 46 800 daltons. Inhibitor studies based on the premise that the same entity would not change its sensitivity upon polymerization showed that the two entities were indeed different: Thus only the alkaline protease of molecular mass 32 900 was sensitive to chymostatin. This molecular mass determined by gel electrophoresis corresponded favourably with the molecular mass of 31 600 found by gel filtration, to give an average estimate of 32 250 $\pm$ 650.

Evidence for the presence of a MAP inhibitor in the homogenate supernatant was shown by the 184% yield of proteolytic activity which followed on the removal of the supernatant. Upon ultracentrifugation of the supernatant at 100 000 g for 60 min, the "inhibitor" remained in the supernatant whilst a relative "yield" of 290% of the proteolytic activity was recorded in the precipitate. Earlier it was argued that the rate of protein degradation may be affected by either a change in structure of the protein being degraded and/or by an alteration in the activity of the enzyme system degrading the enzyme (cf. 1.1. and 1.3.). Thus the presence of a "physiological inhibitor" may play a significant role *in vivo* in controlling the activity of a "myofibrillar alkaline protease".

Katunuma et al (1976) have also reported the presence of a physiological inhibitor. They also showed a marked increase in total proteolytic activity during some steps in the purification of the protease. They purified a specific inhibitor, from skeletal muscle, which had a molecular mass of less than 5 000. A kinetic study of the inhibitor showed a typical non-competitive type of inhibition. The effects of the inhibitor on several other common proteases were also studied. Proteases such as pronase, trypsin and chymotrypsin were not inhibited. As the protease isolated by Katunuma et al (1976) is clearly different from the protease isolated in this study, their inhibitor is probably not the same inhibitor as that observed by me. The presence of the inhibitor does, however, clearly demonstrate a possible specific physiological control of the activity of a myofibrillar alkaline protease, which may be operative in living muscle cells.

The myofibrillar alkaline protease was present mainly in the fraction containing the myofibrils obtained by differential centrifugation. Light microscopy and PAGE confirmed that the presence of MAP in other fractions was always associated with myofibrils or myofibrillar proteins.

The protease was stable for a period of one hour at $4^{o}C$ and $37^{o}C$ but was inactivated at $60^{o}C$. The proteolytic activity decreased rapidly on standing at pH 6,0, whilst the enzyme was stable in the cold for more than a week at pH 8,5.

The protease was solubilised by 0,6 M KCl in a similar pattern to the solubilisation of the myofibrils. Upon repeated solubilisation and dialysis of the myofibrils, the protease was always found in the fraction containing the myofibrillar proteins. Incubation of the

myofibrils with 20 mM sodium pyrophosphate resulted in the release of only trace amounts of proteolytic activity. Thus, as was shown earlier, the protease is attached to the myofibrillar proteins and also is not solubilised by 20 mM sodium phyrophosphate. However, upon subsequent solubilisation with KCl and dialysis, the proteolytic activity remained in the supernatant. This implies that although the 20 mM sodium pyrophosphate did not solubilise the protease it did solubilise some factor which assisted in the attachment of the protease to the myofibrillar proteins. Thus upon subsequent dialysis of the solubilised pyrophosphate precipitate (i.e. in the absence of this factor) the protease did not sediment with the myofibrils. Therefore, the unknown "factor" played a rôle in the binding of the protease to the myofibrillar proteins, but once the protease was bound the factor could be removed without affecting the binding of the protease. This factor was apparently solubilised upon exposure to 20 mM sodium pyrophosphate, since removal of this supernatant allowed the myofibrillar proteins and the alkaline protease to be separated.

The isolated protease was found to be sensitive to chymostatin as only 5% of activity (relatively to a control) remained in the presence of 6,6 µg/ml of chymostatin. The protease isolated by Katunuma et al (1975) was also sensitive to chymostatin, 50% activity remaining at 0,023 µg/ml of the inhibitor. The protease was, however, different from that isolated by Dahlmann and Reinauer (1978) which showed 89% of its activity at a chymostatin concentration of 10 µg/ml. In the presence of 10 µg/ml soybean trypsin inhibitor, 67% of the activity was unaffected. This protease is thus less sensitive to soybean trypsin inhibitor than the protease isolated by Katunuma et al (1975), which had only 50% of its maximum activity in the presence of 6,3 µg/ml

soybean trypsin inhibitor. The protease of Griffin and Wildenthal (1978) had less than 10% activity with 25 µg/ml inhibitor, while that of Murakami and Uchida (1978) retained 26% activity in the presence of 100 µg/ml soybean trypsin inhibitor. A pepstatin concentration of 5 µg/ml had no effect on the purified proteolytic activity, whilst the protease of Katunuma et al (1975) was described as having 50% activity at a pepstatin concentration which was greater than the 200 µg/ml tested in that study. Leupeptin at a concentration of 10 µg/ml had no effect on the protease activity, while at the same inhibitor concentration the protease of Dahlmann and Reinauer (1978) only showed 73% of its activity. The protease isolated by me did not require Ca^{+2} or other divalent metal ions, since 90% of its activity was still evident in the presence of 2 mM EGTA. As neither leupeptin nor pepstatin affected the proteolytic activity, it can be concluded that the protease is not cathepsin B or cathepsin D. However, chymostatin was a potent inhibitor of proteolytic activity and thus it can be concluded that the protease was a "chymotrypsin-like protease".

The preliminary study of rats treated with compound 48/80 was found to be ambiguous in that a reproducible reaction by the rats could not be obtained. In some cases the rats died from the injections, while in others the degranulation was found to be incomplete. However, it would appear from the results that chymostatin-sensitive MAP is present in compound 48/80-treated rats. Clearly, further research is required to standardise the treatment to exclude the possibility that the alkaline protease activity detected was not due to residual mast cell granules due to incomplete degranulation by compound 48/80. Ideally the alkaline protease should be purified to homogeneity from proven

granule-free skeletal muscle and then compared to the MAP which I have purified. In this way the possible mast cell origin of the protease will be elucidated.

With the same purification procedure (up to the washed myofibrillar step) I have shown the presence of a similar protease in rat cardiac muscle. This protease is therefore clearly different from the protease purified by Banno et al (1975), since their protease was not detected in cardiac muscle.

The results presented here show that the protease isolated by me is different from the reported proteases of other researchers and thus it must be concluded that this myofibrillar alkaline protease is a different entity. It does, however, show degrees of similarity with a number of previously isolated alkaline proteases. As the protease may theoretically play an important role in protein-catabolism and as the presence of a physiological inhibitor has been demonstrated, it must be assumed that this myofibrillar alkaline protease is capable of playing an important regulatory role. Further study is thus warranted to help to establish whether the newly isolated enzyme is a true muscle-cell enzyme and whether it is involved in physiological intracellular protein degradation.

CHAPTER 4

MATERIALS AND METHODS

4.1. MATERIALS

All operations were carried out at $4^{o}C$ unless otherwise stated.

Water double-distilled in glass was used for all solutions.

Centrifugation was performed in a Beckman Model J 21-C centrifuge, except for the ultracentrifugation where a Beckman Model L 5-65 ultracentrifuge was used, and for the enzyme assays and CM-Sephadex C-50 batch steps where a Mistral 6L centrifuge was used.

Homogenisation was done with an Ultra-Turrax homogeniser.

Sephadex G-75 and CM-Sephadex C-50 were purchased from Pharmacia (Uppsala, Sweden). When not in use, columns of Sephadex G-75 were stored in the presence of sodium azide to prevent microbial growth. Fresh CM-Sephadex C-50 columns were prepared each time.

Leupeptin and chymostatin were purchased from the Peptide Institute (Osaka, Japan). Pepstatin and soybean trypsin inhibitor were obtained from Sigma (USA). Dithiothreitol, cytochrome C and bovine serum albumin (fraction V) were purchased from Miles (Cape Town). Tris base, EDTA and $Na_4P_2O_7$ were purchased from Merck (Germany).

Standards for gel electrophoresis and concentrated dye reagent for protein determination were obtained from BIO-RAD Laboratories (California).

All chemicals not further described were of "Analar" grade, or equivalent thereof, supplied by various companies.

4.2. MYOFIBRILLAR ALKALINE PROTEASE ASSAYS

4.2.1. Denatured casein substrate methods

4.2.1.1. Preparation of denatured casein

Denatured casein was prepared as follows: 2,5 gram casein was added to 10 ml of 500 mM Tris-HCl, pH 7,4 and the mixture pre-incubated at 37°C for 20 min, after which 0,75 ml of 2 M NaOH was added. After heating in a boiling waterbath for 20 min, the solution was cooled and the volume made up to 50 ml with 500 mM Tris-HCl, pH 7,4. The solution was then dialysed overnight against 10 vol. of the same buffer and the pH adjusted to 7,4.

4.2.1.2. Conditions of assay

The samples were prepared in sets of four, one for each time point. The assay incubation mixture contained the following: 1,1 ml of 1,2 M KCl - 0,1 M glycine - NaOH buffer, pH 9,0 and 100 µl denatured casein in 500 mM Tris-HCl buffer, pH 7,4. The reaction was initiated by the addition of 300 µl of the enzyme fraction. The mixture was incubated at 37°C. The reaction was terminated at 0, 15, 30 and 45 min. by the addition of TCA. Using the tyrosine-release method, 1,5 ml of 20% TCA was added, whilst for the TCA-supernatant A-280 method 0,5 ml of 20% TCA was added. All the samples were kept in the waterbath at 37°C for 60 min. The samples were then placed at 4°C for 15 min. The sample was then mixed well and then centrifuged at 800 g for 10 min.

The supernatants were then treated as described below.

4.2.1.3. Measurement of tyrosine release

The tyrosine content of supernatants was measured fluorimetrically using the method of Waalkes and Udenfriend (1957). 1,0 ml of 0,1% 1-nitroso-2-naphthol in 96% ethanol was added to 2,0 ml of TCA-supernatant containing released tyrosine. The solution was mixed and 1,0 ml of 0,05% sodium nitrite in nitric acid (diluted 5 times) was added. After mixing, the tubes were stoppered, incubated at 55°C for 30 min and then cooled. The unreacted substrate was extracted with 9 ml 1,2-dichloroethane. The tubes were shaken well and centrifuged briefly at 200 g to separate the aqueous and the organic layers. The fluorescence of the upper, aqueous layer was measured on a Perkin-Elmer fluorescence spectrophotometer (λ_{ex} = 460 nm; λ_{em} = 570 nm). Tyrosine (0 - 20 nmoles) was used as a standard. Each time point was prepared in duplicate. Enzyme activity was determined by the rate of tyrosine release.

4.2.1.4. Measurement of TCA-supernatant A-280.

The aromatic amino acid content of TCA-supernatants was determined by measuring their absorbance at 280 nm in a Unicam SP1800 UV spectrophotometer. Each time point was measured in duplicate and the 0 min time point was used as the blank. Enzyme activity was determined by the rate of increase of A280.

4.2.2. $|^{125}I|$-labelled casein as substrate

4.2.2.1. Preparation of $|^{125}I|$-labelled casein

$|^{125}I|$-labelled casein was prepared according to a modified method of

Hunter and Greenwood (1962). All solutions used were prepared in 0,05 M sodium phosphate buffer, pH 7,5. To 5 µl of $|^{125}I|$ (0,5 mCi) was added 1,0 ml of casein (2,5%). 250 µl chloramine T (4%) was then added and the mixture was allowed to stand at $4^{o}C$ for 5 min. After the addition of 250 µl 5% sodium metabisulphite, the mixture was slowly applied to a Sephadex G-75 column (0,9 x 30 cm), previously equilibrated with the same buffer and the sample washed with 3 sample volumes of 10% potassium iodide in water. The sample was eluted with the same buffer at a flow rate of 6 ml/h and 1,0 ml fractions were collected. Aliquots were counted in a Nuclear Enterprise 8312 gamma counter. $|^{125}I|$-labelled casein eluted with the void volume and the unreacted $|^{125}I|$ eluted at a later stage. The $|^{125}I|$-labelled casein peak was pooled and dialysed overnight against 10 vol of 0,05 M sodium phosphate buffer, pH 7,5.

4.2.2.2. Conditions of assay

The samples were prepared in duplicate sets of four. The enzyme activity was assayed as described in 4.2.1.2., except that 20 µl of $|^{125}I|$-labelled casein and 80 µl of denatured casein were used as substrate instead of 100 µl denatured casein. The reaction was stopped at 0, 15, 30 and 45 min by the addition of 0,5 ml 20% TCA.

4.2.2.3. Measurement of TCA-soluble radioactivity

Aliquots of 50 µl were removed from an incubation mixture containing buffer instead of enzyme and were counted directly to obtain the total radioactivity. TCA-soluble radioactivity was measured using the gamma-counter (as in 4.2.2.1.) on duplicate samples for each assay tube. Zero time radioactivity was taken as background cpm and the cpm thus

obtained were subtracted from the sample cpm. Enzyme activity was determined by the rate of increase of TCA-soluble radioactivity.

4.2.3. Azocasein as substrate

The samples were prepared in duplicate sets of four. The assay mixture containing 250 µl of 5 mg/ml azocasein dissolved in 1,2 M KCl - 0,1 M glycine - NaOH buffer, pH 9,0 to which 250 µl of enzyme solution was added to initiate the reaction. The incubation was carried out in a waterbath at 37°C. The reaction was stopped at 0, 15, 30 and 45 min by the addition of 0,5 ml 4% TCA. All samples were kept in the waterbath for a total of 60 min. The samples were then removed and kept at 4°C for 15 min. The samples were then mixed well and then centrifuged at 1 500 g for 10 min at 4°C. The absorbance of the yellow TCA-soluble fraction was then measured at 340 nm.

4.3. PROTEIN DETERMINATION

The protein concentration of samples was measured according to the method of Bradford (1976), using the colour reagent obtained from Bio-Rad Laboratories. Sephadex G-75 fractions (2.2.2.9) were assayed on the basis of their A_{220} readings relative to bovine serum albumin.

4.4. ISOLATION AND PURIFICATION OF MAP

4.4.1. Sample preparation

Fed male rats (∿ 250 gram) were killed by cervical dislocation and the hind limb muscles were immediately removed and placed in a chilled

solution of 0,1 M KCl - 2 mM $MgCl_2$ - 2 mM EGTA - 2 mM $Na_4P_2O_7$ - 10 mM Tris - Maleate buffer, pH 7,1 (pyrophosphate-enriched low salt buffer). The rat skeletal muscle was then trimmed of excess fat and connective tissue, cut into small pieces and homogenised in 10 vol (v/m) of fresh pyrophosphate-enriched low salt buffer.

4.4.2. Washed myofibril preparation

The homogenate was then centrifuged at 800 g for 10 min at 4°C. The precipitate was retained and resuspended in pyrophosphate-enriched LSB. The suspension was then centrifuged as described for the homogenate. The resulting precipitate was then treated in the same way for a further two washes with pyrophosphate-enriched LSB. Thereafter, the precipitate was suspended in 5 vol of pyrophosphate-enriched LSB containing 1% Triton X-100. This suspension was then centrifuged as before. This precipitate was subjected to a further two washes with pyrophosphate-enriched LSB. The final precipitate obtained was called the washed myofibril fraction.

4.4.3. Wash at a high concentration of pyrophosphate

The washed myofibril fraction was suspended in 10 vol of fresh 20 mM tetra-sodium pyrophosphate. The suspension was stirred at 4°C for 1 hour. The resulting mixture was then centrifuged at 8 000 g for 30 minutes at 4°C. The supernatant was discarded whilst the precipitate was retained as the pyrophosphate-wash precipitate.

4.4.4. Solubilisation at high salt concentration

The pyrophosphate-wash precipitate was then placed in 10 vol of 0,6 M potassium chloride and stirred at 4°C for 1 hour. The mixture was

then centrifuged at 48 000 g for 30 min at 4°C. The supernatant was decanted and retained.

4.4.5. Dialysis of KCl-supernatant

The high salt supernatant was then dialysed using Thomas (1,5875 cm) dialysis tubing which had been boiled in a solution containing traces of potassium bicarbonate and sodium EDTA. The supernatant was dialysed, against 10 vol of 0,1 mM DTT - 5 mM potassium phosphate buffer, pH 8,5, at 4°C overnight. The equilibrated dialysate was then centrifuged at 48 000 g at 4°C for 15 minutes. The supernatant was retained as the dialysed supernatant.

4.4.6. CM-Sephadex C-50 batch step

The dialysed supernatant was stirred at 4°C in the presence of pre-equilibrated CM-Sephadex C-50 for 30 minutes. The CM-Sephadex C-50 had been equilibrated with 3 x 10 min washes with 10 mM Tris-HCl buffer, pH 6,0. The excess buffer was decanted following each wash. The CM-Sephadex C-50 equilibration was performed at room temperature. The suspension was then centrifuged at 1 500 g for 1 min at 4°C.

The CM-Sephadex C-50 sediment, containing bound-protein, was then stirred with 10 vol of 0,7 M KCl - 10 mM Tris-HCl buffer, pH 6,0.for 30 min at 4°C. The suspension was again centrifuged at 1 500 g for 1 min at 4°C.

The resulting CM-Sephadex C-50 sediment was stirred with 5 vol of 1,5 M KCl - 10 mM Tris-HCl buffer, pH 6,0 for 30 min at 4°C. This suspension was then centrifuged at 4°C for 1 min at 1 500 g. The supernatant, in this case, was decanted and retained. The supernatant was then dialysed overnight at 4°C against 10 vol of 0,1 mM DTT - 5 mM

potassium phosphate buffer, pH 8,5.

4.4.7. Ultrafiltration

The CM-1,5 M dialysed supernatant was then filtered at 4°C, under a positive pressure of 4,5 bar (under high purity nitrogen) in an Amicon ultrafiltration Cell Model 52 fitted with a PM-10 membrane. The supernatant was concentrated by approximately 3-fold by means of ultrafiltration.

4.4.8. Sephadex C-50 column chromatography

The resulting solution from the ultrafiltration step was applied to an equilibrated CM-Sephadex C-50 column (1,4 x 5,5 cm). The resin was equilibrated as in 4.4.6. and then poured into the column at 4°C; 250 ml 10 mM Tris-HCl buffer, pH 6,0, was then passed through the column under standard conditions before the enzyme sample was applied. The column was eluted at 4°C at a flow rate of 10 ml/h and 3,125 ml fractions were collected. The sample was eluted under these standard conditions by a linear salt gradient (0 M NaCl → 0,7 M NaCl) in 10 mM Tris-HCl buffer, pH 6,0. The enzyme peak was pooled and dialysed overnight against 10 vol of 0,1 mM DTT - 5 mM potassium phosphate buffer, pH 8,5. The dialysate was then concentrated by vacuum dialysis at 4°C to a final volume of 3 ml.

4.4.9. Sephadex G-75 chromatography

The 3 ml concentrated enzyme was placed on an equilibrated G-75 column (1,6 x 90 cm) at 4°C. The column had been equilibrated with 250 ml 0,6 M KCl - 10 mM Tris-HCl buffer, pH 7,0 passed through the column under standard conditions. The sample was then eluted with the same

buffer at a flow rate of 10 ml/hr. Elution fractions of 2,5 ml were collected.

4.4.10. Treatment of rats with Compound 48/80

Rats were treated with Compound 48/80 according to the method of Riley (1959). Rats (150 g) were injected intraperitoneally, daily over five days, with compound 48/80. Each rat received 100 µg/100 g on day 1, 200 on day 2, 300 on day 3, 400 on day 4 and 500 on day 5. The animals were killed on day 6 by cervical dislocation and the muscles were quickly removed.

4.5. POLYACRYLAMIDE GEL ELECTROPHORESIS

4.5.1. SDS-Polyacrylamide gel electrophoresis

SDS-polyacrylamide gel electrophoresis was performed on 15 - 8% acrylamide slab gels according to a modified method of Laemmli (1970). The two gel solutions were poured into a gradient maker so as to give a 15% to 8% linear acrylamide gradient. The two solutions contained the following:

8% acrylamide	15% acrylamide
0,1% SDS	0,1% SDS
5% (v/v) glycerol	10% (v/v) glycerol
0,37 M Tris-HCl, pH 8,8	0,28 M Tris-HCl, pH 8,8.

The gels were polymerised chemically by the addition of ammonium persulphate and N,N,N',N'-tetramethylethylenediamine (TEMED) to a final concentration of 0,03% (m/v) and 0,025% (v/v), respectively. The stacking gel contained 5% acrylamide, 0,1% SDS and 0,1 M Tris-HCl, pH 8,8 and was polymerised by the addition of 0,05% (v/v) TEMED and

0,1% (m/v) ammonium persulphate. The electrode buffer consisted of 0,025 M Tris-HCl, 0,19 M glycine and 0,1% SDS, pH 8,8.

Protein samples were dissolved in a sample buffer consisting of 2% SDS, 10% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol and 0,05 M Tris-HCl, pH 6,8 and boiled for 2 min. Electrophoresis was carried out for 3,5 h at a constant current of 25 mA. Gels were fixed and stained overnight in 200 ml of 0,025% Coomassie blue, 25% isopropyl alcohol and 10% acetic acid. Gels were destained with shaking in 10% acetic acid.

4.5.2. Gelatin polyacrylamide gel electrophoresis

Gelatin PAGE was performed on a 0,1% gelatin - 11% polyacrylamide slab gel. The running gel solution contained the following:

11% A-bis A (30:1 = acrylamide:bis acrylamide (m/m))

0,375 M Tris-HCl buffer, pH 8,0

0,1% SDS

0,1% gelatin.

The gel was polymerised chemically by the addition of ammonium persulphate and TEMED to a final concentration of 0,02% (m/v) and 0,11% (v/v), respectively. The stacking gel contained 10% A-bis A, 0,05% SDS and 0,0625 M Tris-HCl buffer, pH 6,8 and was polymerised by the addition of 0,25% (v/v) TEMED and 0,05% (v/v) ammonium persulphate. All the gel solutions were autoclaved and kept sterile prior to use. The electrode buffer consisted of 0,19 M glycine - 0,1% SDS - 0,025 M Tris-HCl buffer, pH 8,8.

The enzyme sample was dialysed against a 0,1 mM DTT - 5 mM potassium phosphate buffer, pH 8,5. SDS and sucrose were added to the enzyme

sample to give a final concentration of 2,5% (m/v) and 8% (m/v), respectively. The sample was then incubated at 37°C for 30 minutes.

Electrophoresis was done at 4°C at a constant current of 0,8 mA, while the sample was in the stacking gel and then adjusted to 0,6 mA. The voltage was not allowed to exceed 100 V. Electrophoresis was continued for 3,5 h.

After electrophoresis, the gel was washed in 2,5% Triton X-100 for 1 h with shaking. The gel was then rinsed quickly under distilled water and then placed into warm 1,2 M potassium chloride - 0,1 M glycine - NaOH buffer, pH 9,0. Incubation was done at 37°C with shaking for 3 h.

After incubation, the gels were stained overnight in 0,1% Amido black. Gels were then destained with shaking in 10% acetic acid until clear lysis bands could be observed.

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