THE DIGESTIVE ENZYMES OF THE WEST AFRICAN GIANT LAND SNAIL, ARCHACHATINA MARGINATA SWAINSON

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CERTIFICATION

THIS IS TO CERTIFY THAT THE THESIS –

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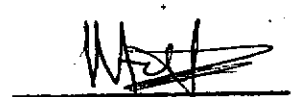
Prof. W. O. Odieta INTERNAL EXAMINER'S NAME	 SIGNATURE	30th Sept. 1992 DATE
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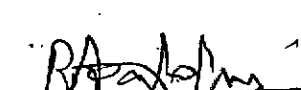
Prof. R. A. Balogun EXTERNAL EXAMINER'S NAME	 SIGNATURE	30th Sept. 1992. DATE

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D E D I C A T I O N

Dedicated to my Glory and the

lifter up of my head -

The El-Shaddai.

D E C L A R A T I O N

I declare that no portion of the work referred to in this thesis has been submitted in support of an application for another degree or qualification of this or any university or other institution of learning.

A C K N O W L E D G E M E N T S

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ABSTRACT

Digestive enzymes were studied in the West African giant land snail, Archachatina marginata with respect to their distribution in the gut, some kinetic parameters and their purification.

A survey of the activities of four disaccharidases, proteases and a lipase in the salivary gland, crop juice, crop, stomach, intestine and digestive gland revealed that by far, the greatest activity in terms of total and specific activity was found in the crop juice and very little was associated with the tissue in the different parts of the digestive tract. The specific activity and total activity of the crude crop juice were in descending order of cellulase > amylase > cellobiase > lactase > maltase > sucrase.

In contrast to the distribution pattern of the carbohydrate digesting enzymes proteolytic activity in A. marginata was mainly associated with the digestive gland, indicating that the digestion of proteins is probably more of an intracellular process.

Lipolytic activity was highest in the crop juice followed by the digestive gland extract and lowest in the salivary gland.

Kinetic studies on the crude crop juice preparation, which had a measured pH of 5.8, indicated a pH optimum of 5.7 ± 0.2 for all the enzymes tested.

The curves for the hydrolysis of B linked substrates had a broader optimal region than for the a-linked substrates. Cellulase and sucrase were inhibited by phosphate. Cellobiase and sucrase gave classic michaelis menten kinetics with Km values of 0.86 ± 0.1mM and 17 ± 0.1mM respectively. The plot of enzyme activity against substrate concentration for lactase gave a curve characteristic of two enzymes, with one enzyme having a Km value of 124 ± 6.3mM and the second one having a lower Km value of 35 ± 6.5mM. Maltase gave activity versus concentration curves which were characteristic of substrate inhibition curves, giving a calculated Km of 0.41 ± 0.1mM and an inhibition constant (Ki) of 41 ± 11.8mM.

Carbohydrate digesting enzymes were partially separated on DEAE cellulose, gel permeation and hydrophobic columns. A clear separation of maltase and sucrase (a bond hydrolysers) from lactase and cellobiase (B bond hydrolysers) was obtained. The evidence suggests that there are present at least three enzymes or groups of enzymes hydrolysing different carbohydrates.

CHAPTER 1

INTRODUCTION

Figure 1.1

1.1 The Biology and Ecology of A. Marginata

The West African giant land snail Archachatina marginata, Swainson (Figure 1.1) is an important source of animal protein much in demand for food in West Africa. The genus Archachatina belongs to the family Achatinidae (Pulmonata), sub-family Achatininae (Pilsbry, 1919; Bequaert, 1950; Crowley & Pain, 1970) which contains the largrest terrestrial snails (Bequaert, 1950). The main diagnostic feature of Archachatina spp. is the possession of a wide bulbous or dome shaped protoconch shell summit which consists of nepionic or embryonic whorls produced by the snail before the egg hatches (Bequaert, 1950).

Archachatina marginata is commonly found in the high forests and fringing forest of the derived savanah regions of West Africa. It is restricted to areas from Benin Republic to Zaire (Bequaert, 1950).

A. marginata is active during the rains, but during the dry season, remains inactive under rocks, decomposing tree trunks, and plant debris. During this state of inactivity, which is known as aestivation, the snail withdraws into the shell, the aperture of the shell is then closed up with the epiphram, a thin whitish membrane formed of calcified slime. Aestivation offers the snail increased protection from dehydration, and with the onset of the rainy season the epiphram is broken and the snail

resumes normal activities. The land snails are nocturnal and are very slow in their speed of movement. These two characteristics have hindered further behavioural studies until the introduction of time-lapse cinematographic (Newell, 1966) or video techniques, using synchronized flash or infra-red lighting.

Although the snails are hermaphrodites and each is a producer of eggs, A. marginata ova require to be cross-fertilised (Plummer, 1975). They begin to lay viable eggs at 13-15 months producing clutches of 8-9 eggs at approximately monthly intervals (Plummer, 1975; Larambergul, and Alaphilippe, 1959). Until recently, the question of total reproductive productivity of any achatinid has been purely speculative, being based on data from snails kept in captivity for only a portion of their life. Plummer (1982), who studied the reproductive potential and longevity in A. marginata ova, for a period of 8 years, found that its average reproductive potential, using the figures for numbers of clutches per year within a period of 4 years, was such that a snail would have produced 27 clutches, totalling 197 eggs. She assumed that with a hatching rate of 70% (according to Plummer,1975), 138 viable offspring would have been produced.

Although Archachatina is an important source of animal

protein, it has not been successfully cultivated, except for the only limited sporadic use of the snail for food supplements with inappreciable, or only modest effects on increasing the local snail population.

The need for the cultivation of this source of animal protein for human consumption arises from the continuing and protracted need for protein supplements as the world population grows, especially in the developing countries such as in Nigeria. Man needs nutritionally high quality proteins which contain a high proportion of essential amino acids, such that cannot be synthesised from other materials but must be taken in in the diet.

This need for accelerated and increased food production, especially in Nigeria, has led to the launching of various agricultural schemes within the past fifteen years by four successive regimes. Under these schemes, the cultivation of various types of food has been given much attention. Examples include the rearing of fish, goats, sheep, pigs and fowl, but not the West African giant land snail, although the essential amino acid content of snail meal is substantially greater than in dehydrated chicken eggs. Mead (1982), refering to earlier findings (Mead & Kemmerer, 1953), stated that a snail meal prepared from dehydrated giant land snails had about 1.3 times the lysine and 2.7 times the arginine found in

dehydrated whole chicken eggs. To be able to cultivate any animal species, successfully, a knowledge of the food and feeding habits is essential, having a direct bearing on proper management, and thus the success of cultivation on a large commercial scale for human consumption and other purposes. Although other aspects of the biology of such an animal to be cultivated are important, it has been found that, with favourable conditions of food and moisture, a population of Achatina fulica Bodwich is able to produce in a few months a comparatively remarkable biomass, because individuals can attain all but a fraction of full growth in the first of their five to six year life span (Mead, 1961; 1979).

Kekamoha (1966) reported a heavy population of this species in Hawai to have an estimated 587,600 snails in a 6.72 hectare area, or an average of 7.75 snails per m^2.

With the soft parts of the average medium size snail weighing conservatively 32g, the biomass would approximate 2.4 metric tons per hectare. Abbas & Gantam (1975) found natural aggregations of aestivating snails in Makkapha and Andaman Islands that reached a density of 10,219 snails in a 99.80m^2 area or an average of 102 snails per m^2.

On the same basis, the biomass of that population would equal 327 kilograms or 33.7 metric tonnes per hectare. When one considers the fact that feeding on vegetation generally makes up over three quarters of

the food intake, the conversion rates of this terrestrial gastropod in time and space are truly remarkable. And it is all the more remarkable that little practical use has been made of this natural machine for converting dead and decaying plant and animal substances into highly nutritious flesh.

Other species of the giant land snail have entered the commercial field. In the mid-1970's Taiwanese entrepreneurs began to export canned and frozen snail specimens of Archachatina fulica into Europe and North America (Mead, 1982). The success story is spreading to other countries. Achatina fulica has been used on a commercial scale in Thailand, and early efforts are being made in Ghana and Cote d'Ivoire, and elsewhere in West Africa, to raise the largest species of Achatina and Archachatina on a commercial scale, however, mainly for the local consumption of the full grown individuals (Mead, 1982). Until recently, Archachatina were collected from the surrounding areas by local people, which gives them additional income, but there is now an awareness to cultivate this snail in Nigeria (Ajayi et al., 1978), although only on a family consumption scale.

Apart from being widely used as human food, snails also provide an important link in many food chains (Vines & Rees, 1972). They are known to be commuters of dead, dying or fresh plant tissues, so increasing

the turnover of this material by making it more readily available for bacterial and fungal decay. In Nigeria, the shells are used as decorations in many homes, and also serve as good material for fossilization.

1.2 **Food Ingested By Gastropods And Their Chemical Nature**

Results obtained from different workers indicate that terrestrial gastropods are mainly herbivorous, feeding mainly on decomposing plant matter, fruits and a great variety of plant leaves, bulbs and roots (Stone and Morton, 1958; Ajayi et al. 1978; Bailey, 1989). These food materials are largely carbohydrates. The most widely distributed carbohydrates are built up from hexoses. Hexoses may occur freely, such as glucose in nectar, or as disaccharides, especially sucrose in fruit and lactose in milk, or in polysaccharides, such as cellulose and starch, which dominate among plant products and therefore constitute by far the most important sources of the diet of herbivores. Other examples of polysaccharides in the plant world are inulin and lignin, chitin, pectin and dextran. The key to this complex situation is the structure of the carbohydrate molecule. Many of these carbohydrate molecules are built up from glucose units. For example, maltose, which is a disaccharide,

is made up of two glucose units (Figure 1.2a).

The disaccharide cellobiose is identical with maltose, except that the cellobiose has a B(1-4) glycosidic linkage as shown in Figure 1.2b. In the cellobiose structure, one glucose residue is linked through its hydroxyl group in the 1-position to the 4th hydroxyl group of the second glucose, that is, the two glucose units are 1-4 linked. The difference between maltose with α(1-4) linkage and cellobiose with a ß(1-4) linkage lies in the configuration at the number one carbon atom.

Starch is another type of diet carbohydrate. It is a storage polysaccharide of higher plants consisting of two components, amylose and amylopectin, which are present in varying amounts. The amylose component consists of D-glucose units linked by α(1-4) linkages as shown in Figure 1.3a. Amylopectin is a branched polysaccharide. In this molecule, shorter chains of glucose units are also joined to the chain by α(1-6) linkings (Figure 1.3). Thus carbohydrates are characterised by their glycosidic bonds and are largely classified as such.

A certain percentage of the food ingested by gastropods is protein in nature. Proteins are organic substances formed by condensation of large numbers of amino acids. Heterotrophic organisms cannot synthesize all the amino acids they need, thus, some must be supplied in the diet. Ten amino acids are

Fig. 1.2a: Structure of a Maltose Unit

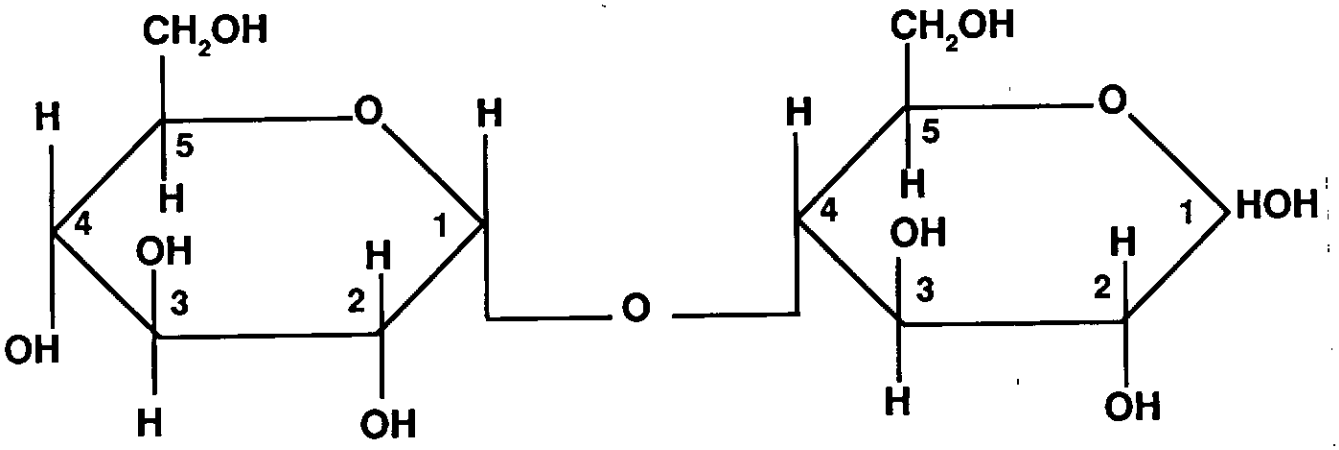


Fig. 1.2b: Structure of Cellobiose

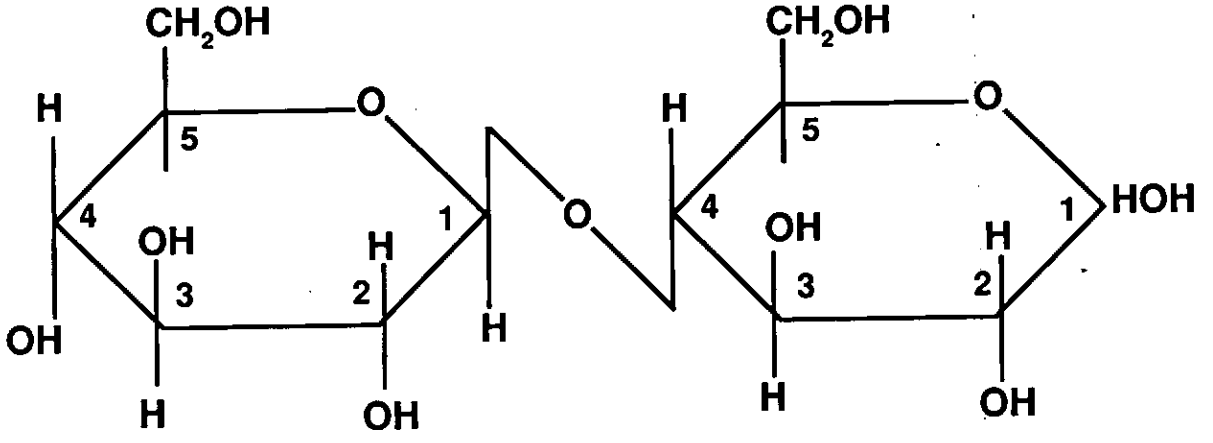


Fig. 1.3a: Structure of a Amylose

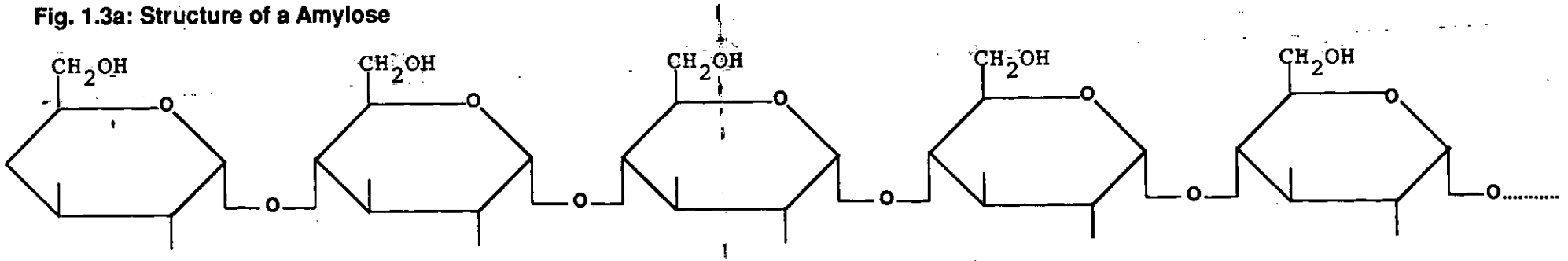
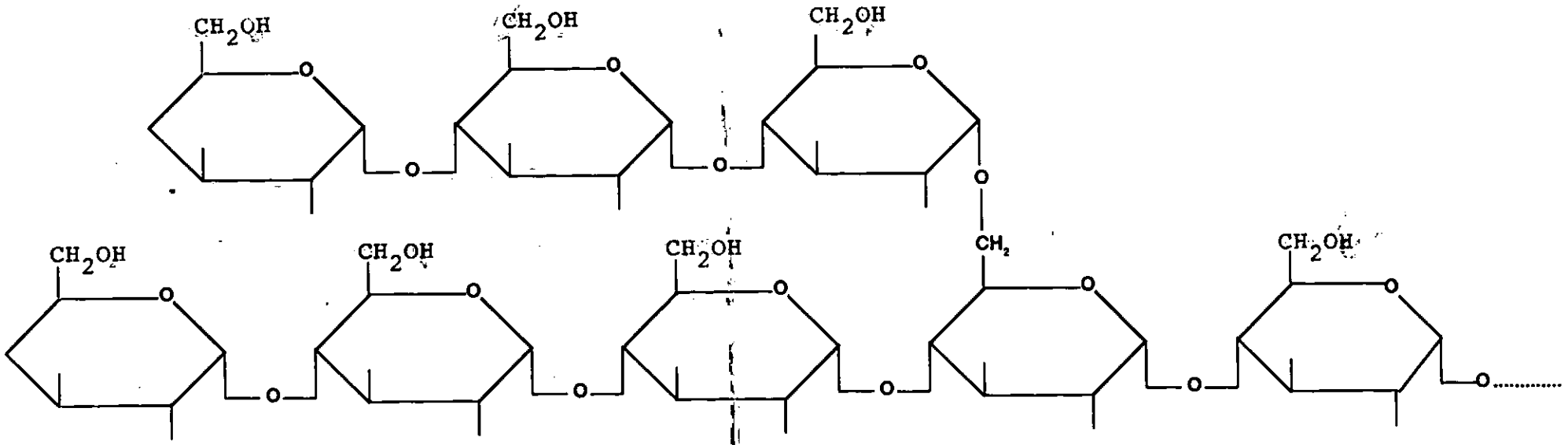


Fig. 1.3b: Amylopectin (a branched polysacharide with α-1-4 linkage and α-1-6 linkages)



generally essential in the diet of the higher animals. They are histidine, lysine, tyrosine, phenylalanine, tryptophan, methionine, leucine, isoleucine, valine and threonine. Arginine is a constituent of all proteins, and in some organisms at least, is essential for growth, and for the production of urea in the ornithine cycle. From phenylalanine, the hormones adrenaline and from tyrosine, thyroxine and the pigment melanin are produced. Tryptophan provides the pyrrole groups of haemoglobin. Methionine provides sulphur and methyl groups necessary for metabolism. Deficiency of methyl groups leads to the condition known as amethylosis, which is characterised by degeneration of the kidneys, spleen and thymus glands. In mammals a protein molecule consists of one or more chains, each made of a large number of amino acid residues which are joined together by the peptide linkage, -CO.NH-. In life, hydrolysis of the protein occurs by the action of proteolytic enzymes.

Lipids are ingested by all the groups of gastropods, be they herbivores, carnivores or deposit feeders. Lipids are organic compounds utilized by living organisms and characterised by their insolubility in water, but are soluble in certain organic solvents. An important dietary lipid is the triacylglycerol (fat) which is an ester of fatty acids and glycerol.

1.3 Digestion in Gastropods

Animals clearly choose what they eat, and can even choose among chemically different sugars. The physiological and biochemical mechanisms that constrain feeding choices are largely unknown (Martinez-del-Rio and Steven, 1989) but it is pertinent to note that, in some cases, animals take food materials which their body is unable to utilize. According to Vadziz et al. (1968), such incidental food supply denotes availability rather than need. The nutritional value of the diet of an animal is ultimately determined by the ability of the animal to digest and absorb it (Ananichev, 1959). That is, food is not useful until it is digested, absorbed and made available for metabolism and thus available for maintainance, growth and development.

The study of the enzymes hydrolysing these food materials is therefore necessary as digestion depends on both the physical state of food and the types and quantity of enzymes present in the digestive tract of the animal. Digestion in pulmonate gastropods is fairly well documented (Owen, 1958; Ghose, 1963; Myers and Northcote, 1958; Prosser and Van Weel 1958 and Odiete and Akpata, 1983). For a long time, it was

considered that primitive digestion in the Mollusca was wholly intracellular, but it has been shown by workers such as Yonge (1932) and Graham (1932) that in the members of Gastropoda, digestion takes place extracellularly in the stomach and intracellular in the digestive gland. Thus, there is the possibility that while primitively, digestion may have been partly intracellular, considerable extracellular digestion also occurred, involving secretion of enzymes by the digestive diverticula. Owen (1958) and Ghose (1963) supported the idea that the relative importance of extracellular and intracellular methods of digestion is closely related to the animal's food and feeding habits. In those herbivorous mesogastropods which feed more or less continuously, generally microfeeders, the phagocytic activity of the cells of the digestive diverticula is of prime importance, oesophageal glands are reduced or lost (Graham, 1939) and the gut possesses a true crystalline style which provides for the continuous release of amylolytic and lipolytic activity.

Although in the majority of the gastropods, the cells of the digestive diverticula are still known to retain their phagocytic activity, the ability of the cells of the digestive diverticula of Helix to phagocytose particulate materials has been questioned by Horstaduis and Horstaduis (1940). There is, however, some evidence to suggest that phagocytosis may be

induced by the presence of protein (Rosen, 1952) and it may now be taken as an established fact that phagocytosis even at the level revealed by the light microscope, does occur in pulmonates such as Helix and Archachatina (Van Weel, 1950). The importance of this phagocytic activity in an animal such as Helix which possesses an astonishing variety of extracellular enzymes, however, remains uncertain (Myers & Northcote, 1958).

1.4 The Alimentary Tract of A. marginata

Despite variations in feeding habits, the alimentary tract of gastropods show basic structural similarities, although there are differences related to their mode of feeding. Primitively, the alimentary canal in the Mollusca consists of an anteriorly situated mouth, a buccal cavity containing radula, a tubular oesophagus, a stomach, and a coiled, largely undifferentiated intestine opening at a posteriorly situated anus. The glandular areas associated with the gut are mainly the salivary gland, opening into the buccal cavity, the oesophageal pouches and the digestive glands opening into the stomach and in some cases into the crop (Odiete & Akpata, 1983).

The alimentary system of gastropods has been studied exhaustively by a number of workers. In those gastropods (prosobranchs) in which cystalline style

has been developed, mainly the microherbivores, the evolution of stomach has paralled that of the lamellibranch bivalves. The ciliary sorting areas are well developed (Yonge, 1932 a,b). Among the remaining gastropods, there has been a general trend toward simplification of the gastric region, largely related with increased extracellular digestion. Thus, among the carnivorous neogastropods, a progressive simplification of the stomach can be traced, involving the reduction or loss of dorsal caecum, so that in many genera, it is little more than a bag within which the food is mixed with digestive juices. Similarly, simplification has occurred in the pulmonates. The stomach is reduced to a passage to and from the digestive gland (Carriker, 1947; Morton 1955c). For example in the Arion ater, the complex ciliary sorting regions which are so characteristic of the prosobranchs and opisthobranch gastropods are absent (Evans and Jones 1961).

The alimentary system of A. marginata is simple and has close resemblence with those of the other stylomatophoran pulmonates already discussed above.

1.5 **Digestive Enzymes**

The digestive tract of Archachatina is known to contain many digestive enzymes (Odiete & Akpata, 1983). Generally there are three major categories of digestive enzymes based on the type of food taken and

these are the carbohydrases, proteases and lipases.

In pulmonate gastropods, and other animals, carbohydrates are absorbed as monosaccharides and enzymes which can liberate the monosaccharide units from the complex carbohydrates taken by the herbivores have been investigated in their digestive tracts (Stone and Morton, 1958). The glycosidases responsible for this function of digestion are classified according to the type of the glycosidic linkage they attack. Carbohydrases have been found to occur in the digestive juice, salivary and the digestive glands of most gastropods and also in the crystalline style where present (Stone and Morton, 1958; Cockburn and Reid, 1979). Most investigators refer only to the common carbohydrases such as amylase and maltase, but many gastropods, particularly herbivores, posses a wide array of carbohydrases.

Of the thirty or more enzymes reported in the digestive tract of Helix (Holden and Tracey, 1950; Myers and Northcote, 1958), more than twenty are carbohydrases which include α and ß amylase (pH optima 6.2 - 6.8 and 4.5 respectively), cellulases and chitinase (Myers and Northcote, 1958) and others such as xylanase, yeast, mannase, laminarinase, galactase, and pectinase.

Apart from Helix, cellulases have also been reported from many genera including Patella, Litorina, Aplysia (Stone and Morton, 1958), Oncomelania (Wrinkler and

Wagner, 1959) and Archachatina (Odiete and Akpata, 1983). The origin of these cellulases, however, is uncertain, since cellulolytic bacteria are present in the gut of most gastropods. Myers and Northcote (1958) have suggested that more than one enzyme may be involved in the digestion of cellulose in the gut, with one or more involving the gut flora, while others are produced by the animal. Parnas (1961) investigated the source of cellulases in the snail Laventia hierosolyma by comparing the cellulolytic activity of normal snails with that of snails whose digestive tract had been sterilised with antibotics. In the normal snails, all the three regions (salivary glands, crop and digestive diverticula) of the gut possessed cellulolytic activity which was greatest in the digestive diverticula and least in the salivary glands. But in the snails treated with antibiotics, the acivity persisted only in the digestive diverticula. From this and other evidence, Parnas concluded that the cellulase of the digestive diverticula of Levantia is produced by the animal, whereas the cellulolytic activity of the crop and salivary gland may have resulted either from bacteria or from the passage of enzymes from the digestive diverticula. More recently, Odiete and Akpata (1983) from their results on the origin of the digestive juice of Archachatina marginata concluded that the primary cellulase in the gut originated from the

snail, because the micro-organisms isolated from the various regions of the gut of the snail showed no exoglucanase action. Purchon (1968) commented that the development of an extracellular cellulase in browsers on land vegetation was particularly important and must have provided a major impetus in the evolution of the Pulmonata.

A somewhat similar situation exists with respect to the chitinase activity of the alimentary canal of Helix. Jeuniaux (1950 and 1954) demonstrated the presence of chitinolytic flora in the digestive juice, and further noted that an increase in the concentration of bacteria, which occurred during periods of rest and starvation, was parallelled by an increase in the chitinolytic activity. From this and other observations, it was first tentatively concluded (Jeuniaux, 1954) that the chitinase of Helix pomatia and other species of terrestrial pulmonates, was solely of bacteria origin.

However, a re-examination of this problem led Jeuniaux (1963) to an entirely different opinion. A comparison of the chitinolytic activity of gastric juice, intestinal juice and digestive diverticula extracts, showed no significant difference between the normal and sterilised animals he studied. He (Jeuniaux 1963) thus suggested that the contribution of chitinolytic bacterial flora to the production of intestinal chitinase is negligible, the digestive diverticula

being the actual site of chitinase secretion.

Although disaccharidase activity has been detected by many workers in the gastropods (Myers and Northcote, 1958; Evans and Jones, 1962a; Wojtowicz, 1972 and Teo, 1980), less emphasis has been laid on the extent of their activities and their relative importance. Myers and Northcote (1958), reported that a number of polysaccharide substrates were broken down much more rapidly than any of the disaccharides they tested. In a series of experiments by Evans and Jones (1962a), only the crop juice and digestive gland preparations hydrolysed the dissacharides they used as substrates to a recognisable extent. The hydrolytic power of the salivary gland brei they used as enzyme source on maltose and sucrose for example were quite weak. Myers and Northcote (1958) were of the opinion that, since carbohydrases are usually specific with reference both to the linkage and to the monosaccharide consistituents of the respective polymer they split, it was probable that most of the carbohydrates chosen were degraded by discrete enzyme systems. But Evans and Jones (1962a) have pointed out that although it is believed that most carbohydrases are substrate specific, it is probable that the extracts they prepared from Arion in their experiments contained relatively few enzymes, each of which was capable of hydrolysing a range of substrates similar

in chemical composition.

There have been comparatively few modern investigations on proteolytic and lipolytic enzymes of the gastropoda. As might be expected, extracellular proteases are well represented in omnivorous and carnivorous genera. In the neogastropod, Murex, proteinase, aminopeptidase and dipeptidase occur in the digestive gland (Owen, 1958). In the herbivorous pulmonates, most workers (Myers and Northcote, 1958; Odiete and Akpata, 1983) agree that the digestive juice of Helix and Archachatina showed only slight proteolytic activity, but there has been considerable discussion as to the enzymes involved. Horstaduis and Horstaduis (1940) claimed that the crop juice of the gastropods they examined contained an extracellular cathepsin which is activated by HCN, H_2S and glutathione. On the other hand, Rosen (1952) believes that in Helix cathepsin is bound intracellularly in the digestive diverticula and that the hydrolysis of peptone by the crop juice results largely from the activity of an aminopolypeptidase. Intracellular catheptic digestion was also reported in Lymnaea (Carriker, 1946) and Achatina (Van Weel, 1950). Evans and Jones (1962b) on the other hand, found strong proteinase activity in the crop juice of the slug Arion ater. The pH optimum of the proteinase, pH 5.5 - 6.0, led Evans and Jones to

suggest that, in the course of gastropod evolution, the cells of the wall of the alimentary tract have not evolved the ability to secrete a typical extracellular alkaline proteinase or strongly acidic proteinase, but in some highly specialised forms like Arion, a catheptic type of proteinase is actually secreted by the digestive cells and functions as an extracellular protease.

Lipolytic enzymes have been demonstrated in a number of gastropods (Ferreri and Ducato, 1959; Myers and Northcote, 1958; Cockburn and Reid, 1979; Teo, 1980; Odiete and Akpata, 1983). Some of the recent work on lipases and esterases by Teo (1980) and Odiete and Akpata (1983), using titrimetric methods, demonstrated high lipase activities in the digestive gland and crop juice, but very low lipase activities in the salivary glands.

Ferreri and Ducato (1959) reported lipases in the epithelium of the digestive tract of Murex trumclus, Planorbis corneus and Helix pomatia. In all the three species, lipase activity was activated by calcium chloride, and was inhibited by sodium fluoride and citrates. It's relative activity on four substrates were as follows:-

tributyrin > methyl butyrate > Tweed 60 > olive oil.

The digestive tract of molluscs have been reported to

contain a variety of hydrolases (e.g. dissacharidases, endopeptidases, estarases) (Cockburn & Reid, 1982, Odiete & Akpata, 1983). There is the problem of identifying the enzymes involved - since carbohydrases for example are not substrate specific. According to Myers & Northcote (1958), it is possible that the crude enzyme extract of the snail contained relatively few enzymes each of which can usually hydrolyse a range of substrates of similar chemical constitution. There has been much corresponding advance in the purification and isolation of hydrolases that have been found in the digestive tracts of mammals. For example, Flanagan & Fostner (1978) isolated and purified maltase/glucoamylase from the rat intestine. Pig intestinal microvillus maltase/glucoamylase was purified and characterized by Sorenses et al. (1982). Although there has been a considerable increase in the amount of work dealing with the variety of digestive enzymes present in the digestive tract of molluscs, there has been little or no corresponding effort to purify the specific enzymes that hydrolyse particular substrates and characterize them. This work was carried out in order to obtain information on:

(i) the mechanism of action of specific enzymes

(ii) the role of the enzymes under the conditions which exist in the cell and

(iii) How the activity of the enzymes can be controlled thus providing valuable pointer to mechanisms of regulation under physiological conditions.

To our knowledge the only work on the purification and characterization of digestive enzymes in molluscs is that of Myers & Northcote (1958) who partially purified and characterized a cellulase from Helix pomatia. Our interest in a successful large scale commercial culture of Archachatina marginata has led us to carry out:

(1) A general survey of the quality and quantity of the digestive enzymes present in its digestive tract.

(2) Detailed study of the carbohydrases, since carbohydrates make up a large proportion of their food intake (Ajayi et al., 1978).

(3) Separation and characterization of carbohydrases of the crop juice (since sufficient quantities of the enzymes are readily available).

(4) Kinetic studies on crop juice and purified enzymes of the crop juice.

CHAPTER 2

MATERIALS AND METHODS

2.1 SOURCE OF MATERIALS

2.1.1. Chemicals and Reagents

General reagents and chemical compounds used throughout the investigation were of analytical grade. Maltose, cellobiose and chitin were obtained from Sigma Chemicals (London, England). Glucose oxidase reagent was from Boehringer Mannheim (West Germany), inulin, casein, ammonium molybdate, soluble starch, lactose, potassium dihydrogen orthophosphate were all from BDH Chemicals (U.K.) Ltd. The Cellofas B used was kindly supplied by Courtaulds Chemicals (Derby, U.K.).

2.1.2. Sources and Maintenance of Snails

The snails used were purchased from local farmers/traders from villages near Ile-Ife, and from local markets in Lagos, Nigeria. Snails used in Manchester University, England were kept in cages which were warmed with the aid of electric bulbs located just outside the cages to obtain tropical temperatures (29-32^{0}c). The snails were fed with succulent fruits such as pawpaw, apples and bananas and vegetables such as lettuce and onions. The snails and the soil were kept moist by sprinkling water on them. Decaying food substances and faeces were removed about twice a week.

2.2 Preparation of Crude Enzyme Extract

The snails were deshelled, dissected on ice and the alimentary tracts divided into five different parts as

follows: the salivary gland, the oesophagus and crop, the stomach, the intestine, and the digestive gland. The crop juice was also collected.

These different parts of the alimentary tract were weighed separately and homogenised with five times their volume of distilled water. The homogenates were centrifuged at 20,000g at 4°C for 30 minutes and the clear supernatants were poured off and kept for use as the enzyme extract. The crop juice was also diluted to five times its volume and centrifuged at 20,000g at 4°C for 30 minutes. In order to minimize deteriorative changes that might result from repeated freeze-thaw cycles, the homogenates from the snail's alimentary tracts were divided into aliquots and stored at -70°C.

2.3 Qualitative Survey of Enzymes in Crude Extracts

2.3.1. Carbohydrases

A 2% (w/v) solution of each of the following carbohydrates was used as substrates: sucrose, cellulose, raffinose, cellobiose, maltose, lactose, starch, inulin and chitin. Each reaction mixture consisted of:

2% buffered substrate (appropriate pH)	1.0 ml
Enzyme extract	1.0 ml

The pH of the crop juice was 5.8, while that of the extracts of salivary gland, oesophagus/crop, stomach and the intestine had pH 6.8 and the digestive gland extract had a pH around 7.3. Corresponding buffered substracts

were used for each of the crude enzyme reaction mixtures. The control experiments consisted essentially of the same materials except that the enzyme extract was heat-inactivated for 20 minutes in a boiling water-bath. The whole set-up was incubated for two and a half hours at 37°C. The products of the reaction were analysed using the appropriate methods described below.

1. **Reducing Sugar Test using Benedict's solution**

The production of reducing sugars from hydroysis of the non-reducing substrates in the reaction mixture was tested with Benedict's solution as described by Teo (1980). Essentially it involved the addition of 5 ml of alkaline copper reagent of Benedict's solution to 2 ml of reaction mixture. This was then heated in a boiling water-bath for one hour. The appearance of a brick-red precipitate was used as an index of a positive test.

2. **Barfoed's Test for Reducing Sugars**

The semimicro Barfoed's test was used to test the results of the hydrolysis of reducing substrates such as maltose, lactose, and cellobiose as described by Teo (1969). The Barfoed's reagent contained 13.3g of neutral copper acetate dissolved in 200 ml 1% (v/v) acetic acid. A few drops of the undiluted sugar solution was added to 2 ml of the reagent and heated to boiling with a boiling chip present.

3. **Iodine Test**

The iodine test was used to test the result of starch digestion. All extracts were allowed to act on starch for 15 minutes and then the blue-black colour formed on addition of an iodine-iodine solution was compared with the colour of an amylase free control.

2.3.2 Proteases

Proteases were assayed qualitatively according to the methods described by Balogun and Fisher (1970).

1. **Alkaline Proteinase Activity**

Each reaction mixture for both test and control consisted of 0.5 ml of alkaline casein solution and 0.5ml of the appropriate enzyme extract. The enzyme extract for the control experiment was boiled before the assay. Both test and control samples were incubated at 37^{o}C for 48 hours. The presence of protease activity was tested by adding 1% (v/v) acetic acid drop by drop to the test and the control mixtures. Increase in the turbidity of the test solution was used as an index for the presence of tryptase.

2. **Acid Proteinase Activity**

The method is essentially the same as the one for alkaline tryptase assay except that the substrate for the assay was acidic casein at pH6.0 and 10% sodium acetate solution was used as the test solution instead of the 1% acetic acid

used for alkaline tryptase assay. An increase in turbidity of the test solution indicates the presence of acid proteinase.

3. **Peptidase**

The methodology for peptidase assay was similar to that of protease, except that the substrate consisted of 1% peptone solution and Biuret reagent was used as the test solution. A bluish-purple colour is an indication of a positive result.

2.3.3. **Lipase**

Lipase activity was determined by a titrimetric method as described by Sigma Chemicals Ltd (U.K.) 1989. The substrate for lipase was an emulsion of olive oil (lipase substrate supplied by Sigma Chemicals ready prepared as an emulsion). The reaction mixture consisted of 2ml of oil emulsion and 1ml enzyme extract, and was incubated for three hours at 37^{o}C. Sodium hydroxide (0.05M) was used in titration with phenolphthalein as an indicator after the incubation period. Both the test and control mixtures were titrated to a similar pink colour. The quantity of alkali used in the titration is equivalent to the quantity of fatty acids released from the triglyceride by lipase activity, this can be calculated using the equation below.

Olive oil (triglyceride) ---------> fatty acid +
Diglyceride

NaOH + R-COOH ----------> R-COOna + N20

2.4 Quantitative Determination of Digestive Enzymes in the different Parts of the Gut of A. marginata

The various digestive enzymes in the gut extracts of A. marginata were determined quantitatively using the assay procedures described below. All assays were carried out in duplicate.

2.4.1 Dissacharidase Activity

The release of glucose from some disaccharides was measured according to a modification of the method of Dahlqvist (1968).

The substrates used were maltose, sucrose, lactose and cellobiose. Each sugar (1.37g) was dissolved in 20ml of 0.1M phosphate buffer pH 5.7 and dispensed in 100ul amounts into test tubes containing 100ul of enzyme extract from different parts of the gut (already preincubated to 37^{o}C for a few minutes). All tubes were shaken and incubated at 37^{o}C for 30 minutes.

Release of glucose was measured by the addition of 2.5ml of glucose oxidase reagent pH 7.0 containing 0.5M Tris which interrupts enzyme activity and develops the chromogen (green colour), as a result of glucose oxidation, to a maximum within 45 minutes at room temperature. The control was treated similarly to the test except in the control the substrates were added after the addition of Tris-glucose oxidase reagent.

A glucose standard tube and a blank tube were routinely made up by mixing 200μl of 0.555mM glucose and 200μl distilled water respectively with 2.5ml of Tris-glucose oxidase reagent. After 45 minutes of standing at room temperature, the absorbance was read at 670nm in an LKB spectrophotometer.

In order to check the linearity of the test, a series of standard glucose concentrations were also prepared and treated with Tris-glucose oxidase as in the enzyme assays, and the absorbance read at 670nm. A glucose standard curve was plotted, using glucose concentrations ranging from 0-0.555mM.

A unit of enzyme catalyses the transformation of 1μmole of substrate per minute. The enzyme activity was estimated according to the following formula:

Total enzyme activity in the tissue (μmole glucose released min^{-1})

$$= \frac{A1 \times Std.concn \times vol.incub.mix. \times df \times Tol.Vol.hom.}{A2 \times Vol.Enz. \times T.Incub.}$$

A1 = Absorbance of the Test minus Absorbance of the control at 670 nm.

A2 = Absorbance of the standard minus Absorbance of the blank.

std.concn = concentration of glucose standard (μmole/ml).

Vol.enz. = volume of enzyme used in reaction mixture (ml).

vol.Incub.mix = volume of the incubation mixture (ml).

T.Incub. = time of incubation at 37°C (minutes).

df = dilution factor.

tol.Vol.hom. = total volume of homogenate from each part of the gut.

The enzyme activity was also expressed in units of μmole min^{-1} g^{-1} tissue calculated by dividing the total activity by the weight of the tissue.

$$\frac{\text{Total Enzyme activity (umole.min}^{-1})}{\text{Total weight of tissue used (g)}}$$

The specific activity of each part of the gut was also calculated.

$$\text{Specific Activity} = \frac{\text{Total Enzyme activity (μmole min}^{-1})}{\text{total mg Protein(mg)}}$$

2.4.2 **Polysaccharidases**

The amount of reducing sugar produced from each of the enzyme reactions was estimated using the colorimetric procedures of Nelson (1944) and Somogyi (1945). The pH used for the assays was determined from preliminary investigations of the effect of pH on enzyme activity. Each reaction mixture consisted of 1% (w/v) substrate in 250 ul of the appropriate buffer solution and 100 ul of enzyme. The control experiment was treated in a similar manner, except that the enzyme was boiled for about 20 mins. before the experiment. The reaction mixture was mixed thoroughly and incubated at 37^{o}C for 30 mins. The substrates used were Cellofas B, soluble starch, chitin, inulin and raffinose. A standard tube containing 600 ul of 0.555 umole/ml of glucose and a blank tube containing 600 ul of distilled water were prepared. The reaction was terminated by adding 600 ul of alkaline copper reagent of Somogyi (1945) to each test tube and transferring immediately into a boiling water-bath for 20 minutes. The tubes were brought out, cooled rapidly under a running tap and to all the test tubes, 600 ul of arsenomolybdate reagent (Nelson, 1944) was added and shaken thoroughly until effervescence stopped. The contents of each test-tube was made up to 6ml with distilled water and mixed well. The absorbance was then read against the blank at 540nm. Glucose was used as the standard. The enzyme

activities were estimated using the fomulae described previously in section 2.4.1.

2.4.3 **Protein Determination**

The amount of protein in each part of the gut of A. marginata was determined using the method of Lowry et al. (1951).

Reagents and Solutions

The reagents and solutions were made as follows:

Reagent A 2% (w/v) NaC03 in distilled water

Reagent B 1% (w/v) CuSO4 5H2O in distilled water

Reagent C 2% sodium tartarate in distilled water

2M NaOH

Alkaline copper reagent was made by mixing 0.5ml reagent B with 0.5ml reagent C and 50ml reagent A. Folin reagent was diluted with an equal volume of distilled water. A bovine serum albumin (BSA) standard solution containing 1mg/ml was routinely used. The assay was performed by pipetting 0.5ml of suitably diluted enzyme extract into a test tube, 0.5ml of 2M NaOH was added and allowed to stand at room temperature for 30 minutes. To 0.3ml aliquots of this mixture, 3ml of alkaline copper reagent was added and left at room temperature for 15 minutes. Finally 0.3ml of freshly diluted Folin reagent was added with immediate mixing with a vortex mixer which is essential for proper colour development. After 30 minutes of standing the absorbance was read at 750nm. A protein standard curve was

plotted using a series of concentrations of bovine serum albumin (BSA) values ranging from 0 to 0.5mg/ml. And the absorbances plotted against the concentration of BSA.

2.4.4 **Estimation of Proteolytic Activity**

The proteolytic activities of the enzyme extracts were determined using the method of Lowry et al. (1951). The substrate used was casein.

The assay was carried out by putting 100 ul of enzyme extract in a screw cap plastic bottle (e.g. 7ml bijon) and 0.4ml of standard casein solution was added. This was left for 24 hr at 37^{o}C. The reaction was terminated by adding 0.5ml of TCA (25% w/v) rapidly to each tube which was then mixed carefully and stored on ice for 30 minutes for the insoluble material to precipitate. These samples were then spun on a bench centrifuge at top speed for approximately 5 minutes.

Liberated peptides were then assayed for by the method of Lowry et al. (1951) by taking 0.3ml of each supernatant and adding 3ml of Folin reagent. This was left for 10 mins.

mins. after which the absorbance was read at 750nm. Where the absorbances were very low, when treated with the Lowry reagent, direct absorbances at 230nm were also taken.

2.5 Kinetic Studies on Crude Crop Juice

2.5.1 Effect of pH on the enzyme activity in the Crude Crop Juice

The effect of hydrogen ion concentration of the reaction media on the activity of enzymes in the crude crop juice was investigated using different buffer systems with different pH ranges. Acetate buffer was used for pH ranging from 3.5-6.0 and phosphate buffer within the pH range 6.0-8.0.

2.5.2 Effect of Substrate Concentration on the Enzyme Activity of Crude Crop Juice

The effect of the concentration of different substrates on the activity of the crude crop juice of A. marginata was investigated. In this set of experiments various substrate concentrations ranging from 1-100mM, were used for the disaccharides while concentrations ranging from 0.005% (w/v) - 1% (w/v) were used for cellulose and starch.

The Michaelis constant (Km) and maximum velocity (Vmax) are useful kinetic constants in the study of effect of substrate concentration on enzyme activity.

(1) Michaelis-Menten equation which states that:

$$V = \frac{Vmax\ [S]}{Km + [S]}$$

where v = Velocity of reaction

Vmax = maximum velocity

Km = Michaelis constant

[S] = substrate concentration

These kinetic constants were obtained from the double reciprocal plot of Lineweaver-Burk equation which gives a straight line slope of Km/Vmax, while 1/Vmax was obtained by extraprolation to the 1/v axis. The Lineweaver-Burk equation states that:

$$1/V = \frac{1}{[S]} \cdot \frac{Km}{Vmax} + \frac{1}{Vmax}$$

Other equations used in this study, where necessary were:

(2) the substrate inhibition equation.

Where there was evidence of substrate inhibition, this equation was used in preference to the simple Michaelis-Menten equation. The equation states that:-

$$v = Vmax/(1 + \frac{Km}{[S]} + \frac{[S]}{Ki})$$

(3) the equation for two enzymes.

This was used depending on the shape of the graph obtained from the double reciprocal plot.

The equation states that:

$$v = \frac{Vmax_1}{(1 + \frac{Km_2}{[S]})} + \frac{Vmax_2}{(1 + \frac{Km_2}{[S]})}$$

2.6 Purification of Crop Juice

2.6.1 Extraction of Crop Juice

The shells were carefully removed to expose the visceral mass which was then uncoiled and dissected to expose the crop, stomach and the part of the mid-gut immediately following the stomach. To remove the crop juice, the anteriorend of the oesophagus was cut and the tip was pulled away from the buccal mass and the crop decanted so that the juice will pour into a petridish. The crop juice was then diluted with about five times its volume of water and centrifuged at 20,000g at 4°C for 30 minutes. The supernatant was then dialysed against a large volume of 10mM potassium phosphate buffer at pH 6.8 at 4°C overnight.

2.6.2 Ion Exchange Chromatography on DE-52

Ion exchange chromatography was performed at room temperature on a column of DEAE cellulose (DE-52) (5.5cm long by 2.5cm diameter). The DEAE was washed

with several changes of 500mM phosphate buffer pH6.8, in order to decant the fines. The column was then packed with the washed slurry and allowed to settle under gravity.

After equilibration of the column with several bed volumes of 10mM potassium phosphate buffer pH6.8, the dialysed supernatant was applied to the column and washed through with 100 ml of the equilibration buffer to remove the unbound protein (Travel et al., 1983). The protein was eluted with 500 ml of linear gradient of 10mM - 80mM or 10mM - 200mM potassium phosphate buffer pH 6.8 at a flow rate of 1ml/min or 0.5ml/min and fractions were collected every 10 mins. The number of fractions collected varied from 80-90.

The protein content of each fraction was estimated from the absorbance at 280nm and the disaccharidase activities of the fractions were assayed as earlier described. In the initial experiments, the fractions were also tested for amylase and cellulase activities. From the results of the enzyme assays on the fractions, the fractions with substantial enzyme activities were pooled and concentrated using polyethlene glycol (15,000-20,000) (PEG) for about 3 to 4 hours depending on the total volume. The fractions were pooled separately in respect of the substrate they hydrolysed.

The concentrated fractions were then dialysed against a large volume of 10mM potassium phosphate buffer

pH6.8, and applied to a Bio-Gel P200 column or equilibrated with 2M ammonium sulphate in 10mM phosphate buffer and applied to a hydrophobic column.

2.6.3 Gel Filtration on Bio-Gel P200

Dry Bio-Gel P200 powder was gradually added to potassium phosphate buffer pH6.8. The amount of powder added was 1g to 25ml of appropriate buffer to pack a column of the required volume. The gel was then decanted and the rest of the solution was degassed without using a stirring bar.

Before packing the column, one centimeter of Bio-Gel P-4 was used as the bottom layer to secure the bottom of the column from being clogged. Enough buffer was then used to fill 20% of the column and the stirred slurry was smoothly poured into the column. Excess gel was removed, and the column was washed through with about two bed volumes of 10mM sodium phosphate buffer PH6.8 at a flow rate of 10ml/hr. The sample was then added and fractions of 6ml were collected and measured for protein content on a spectrophotometer at 280nm. The fractions were then analysed for the presence of sucrase, maltase, lactase, cellobiase, amylase and cellulase.

The Bio-Gel P200 column was calibrated with molecular weight markers, and the approximate molecular weights of each "enzyme" were estimated by comparison with a standard graph. This column was standardized using

standard blue dextran (Mr 2,000,000), Bovine Serum Albumin (Mr 68,000) and potassium chromate.
A second set of standards was also applied. This consisted of alcohol dehydrogenase (Mr 150,000), carbonic anhydrase (Mr 29,000) and myoglobin (Mr 17,000). The column was then washed thoroughly before it was re-used.

2.6.4 Hydrophobic Chromatography on Octyl Sepharose CL 4B Column

About 10ml of Octyl Sepharose CL 4B was dissolved in 10mM potassium phosphate buffer containing 2M ammonium sulphate to make a total volume of 20ml. This slurry was then poured into the column and equilibrated with 10mM potassium phosphate buffer containing 2M ammonium sulphate (pH6.8) overnight. After the sample was applied, the column was washed through with more 10mM potassium phosphate buffer containing 2M ammonium sulphate (50ml) and eluted with a reverse gradient (200ml) of ammonium sulphate from 2M to 0M at a flow rate of 5ml/hr and 2ml fractions were collected.

2.6.5 Tris Inhibition of Enzyme Activity

Tris inhibits the activity of mammalian disaccharidases and has also been used in their purification by affinity chromatography. Before attempting affinity chromatography of the snail enzymes an experiment was carried out to determine whether they too are inhibited by Tris.

A range of concentrations of Tris was prepared from 0mM to 250mM. 0.2ml of suitably diluted enzyme was mixed with 0.2ml of tris solution and 0.2ml of each mixture was added to 0.2ml of respective substrates, and tested for the enzyme activity and read at 670nm. A graph of absorbance against concentration of Tris was plotted.

2.6.6 Affinity Chromatography on a Sepharose 6B Tris Column

The column was prepared by washing 5g of epoxy-activated Sepharose 6B on a sintered-glass funnel with 150ml of water, followed by 200ml of 250mM Tris (pH9.5). The beads were re-suspended in 50 ml of the Tris solution and incubated at 35°C for 20 hours in a water bath, with gentle shaking, or left at room temperature overnight. Unbound Tris was removed with 150ml water and 250ml of 10mM potassium phosphate buffer (pH6.8). The beads were then packed in a 1x5cm column. The sample was applied to the Tris affinity column and eluted with 50ml of 10mM potassium phosphate buffer (pH 6.8) followed by 50ml of 150mM Tris (pH 7.4).

In some experiments the 10mM potassium phosphate wash through was followed with 100mM phosphate buffer before elution with 150mM Tris (pH7.4) or washed through with 100mM phosphate buffer (pH7.4) right away, instead of the initial wash through with 10mM sodium phosphate buffer. Fractions of 2ml were

collected at a flow rate of 6ml/hr.

The protein content of each fraction was measured at 280nm and fractions analysed for the presence of the various enzymes being investigated. The Tris affinity column was regenerated after use by washing with 50ml of 100mM sodium acetate buffer (pH4.0) containing 100mM sodium chloride, followed by 50ml of 100mM sodium borate buffer (pH8.0) containing 500mM sodium chloride.

2.6.7 Mixed Substrate Experiments

Are lactose and cellobiose/maltose and sucrose hydrolysed by the same enzyme ?. To be able to answer this question a simple experiment was performed to investigate further (in conjunction with the kinetic studies, and the chromatography of the crop juice) whether there was one or more enzymes hydrolysing lactose/cellobiose and maltose/sucrose.

2.6.7.1 Lactose/Cellobiose Hydrolysis Experiments

If it was the same enzyme hydrolysing each of these pairs, we would have lactose and cellobiose competing for the binding site as illustrated below:-

Cellobiose $\xrightarrow{E_1}$ Glucose

Lactose $\xrightarrow{E_1}$ Glucose + Galactose

But if there are two different enzymes, then the substrates will be hydrolysed independently as shown below:-

Cellobiose $\xrightarrow{E_1}$ Glucose

Lactose $\xrightarrow{E_2}$ Glucose + Galactose

In these experiments, two different substrate concentrations (10mM and 1mM) were used. This is because if there are two enzymes, a high substrate concentration could give a misleading result which indicates the presence of a single enzyme. In that at high substrate concentrations, it tends towards saturation, such that there is competition for binding site because there is not enough room for both substrates. But at low concentrations of these substrates, if there are two enzymes, the result should be additive (as explained later in this section) because then, there is room for both the substrates to bind to their respective enzymes independently. The reaction mixtures consisted of:

1(a) 0.05ml of 40mM cellobiose + 0.05ml phosphate buffer + 0.1ml of enzyme preparation = X_1

(b) 0.05ml of 4mM cellobiose + 0.5ml phosphate buffer + 0.1ml of enzyme preparation = X_2

2(a) 0.05ml of 4mM lactose + 0.05ml phosphate buffer + 0.1ml enzyme preparation = Y_1

(b) 0.05ml of 4mM lactose + 0.05ml phosphate buffer + 0.1ml enzyme preparation = Y_2

3(a) 0.05ml of 40mM cellobiose + 0.05ml of 40nM lactose + 0.1ml enzyme prearation = Z_1

(b) 0.05ml of 4mM cellobiose + 0.05ml of 4mM lactose + 0.01ml enzyme preparation = Z_2

But if $Z < (X + Y)$, then there are two possible explanations:

(a) Lactose could be inhibiting the hydrolysis of cellobiose (while not itself being hydrolysed/ being hydrolysed alongside) or vice versa,

or

(b) purely because there is just one enzyme reacting differently with two substrates.

2.6.7.2 **Maltose / Sucrose Hydrolysis Experiments**

The same experiments were performed using maltose and sucrose as substrates (instead of lactose and cellobiose). In all cases crude crop juice and partially purified enzyme solutions were used as enzyme sources.

2.6.8 Polyacrylamide Gel Electrophoresis in SDS (SDS-PAGE)

SDS-PAGE was performed using Laemmli's (1970) method using the following solutions and reagents.

Electrode Solution (5 times final Strength):

Tris (60.6g), SDS (20g) and glycine (288g) were dissolved and made up to 2 litres with water. The final pH was 8.3.

Solution A

Acrylamide (30g) and NN-methylene-bis-acrylamide (0.8g) were dissolved and made up to 100ml in water.

Solution B

Tris (22.72g) and SDS (0.5g) were dissolved in water and adjusted to pH8.8 with HCl and made up to 100ml.

Solution C

Tris (6.04g) and SDS (0.4g) were dissolved in water. The pH was adjusted to pH6.8 with HCL and made up to 100ml.

Sample Buffer

Gel electrophoresis was performed in 7.5% acrylamide slab gels 1.5mM in thickness and 13cm in length.

Stock Solution for 7.5% Polyacrylamide Separating Gel

A stock solution for 7.5% polyacrylamide separating gels were made by mixing the following:

Water	66ml
Solution A	30ml
Solution B	24ml

For each separating gel (lower) preparation, 50ml of the stock solution mixture was taken out and 250μl of 10% Ammonium persulphate (APS) was added and mixed thoroughly. This was followed by the addition of 30μl of TEMED.

Stock Solution of 3% Stacking (upper) Gel.

A stock solution consisting of the following formulation was prepared.

Water	32.5ml
Solution A	5.5ml
Solution C	12.5ml

Then a 3% stacking gel was prepared from this stock solution when needed by taking 25ml of the stock solution and adding 250μl of ammonium persulphate and 30μl of N,N,N'N' -tetramethylene diamine (TEMED).

To form the separating gel, acrylamide solution was poured between the glass plates to about 5cm from the top of the plates. Then the gel was layered with 1ml butanol to remove the concave meniscus. After

polymerization of the gel, butanol was poured off the gel, and then the gel was rinsed with Solution B diluted 5 fold.

Stacking gel solution was added to the top of the separating gel, and 15 sample wells were made in the stacking gel with a comb during polymerization.

When the stacking gel had set (60 minutes) samples were applied (20μl-75μl). Samples were previously prepared by mixing an equal volume of both sample solution and sample buffer, and the protein was denatured by heating at 100 C for 3-5 minutes. The following molecular weight markers were used;

carbonic anhydrase Mr 29,000, ovalbumin Mr 45,000, bovine serum albumin Mr 66,000, phosphorylase B Mr 97,400, B-galactosidase Mr 116,000 and myosin Mr 205,000.

Electrophoresis was performed with an LKB vertical gel apparatus set at limiting values of 6mA, 50V, 5 Watt per slab, and it was cooled with a water system. The electrophoresis was allowed to proceed (overnight) until the dye had reached to within 1 to 2cm of the bottom of the gel.

Staining and Destaining

Gels were immersed in staining solution which contained coomassie blue dissolved in 50% (v/v) methanol, 7% (v/v) acetic acid for 2 hours, with

occasional shaking. The gels were then destained with 10% (v/v) methanol, 7% (v/v) acetic acid for several hours, and finally allowed to stand in 7% (v/v) acetic acid. The distance migrated by bands were measured in order to estimate the molecular weights of each protein.

CHAPTER 3

RESULTS

3.1 Gross Anatomy of the Alimentary Canal of A. marginata

The alimentary canal is divisable into buccal mass oesophagous, crop, stomach, intestine and rectum. The appendages of the alimentary canal are salivary and digestive glands. The dissection of the alimentary system of Archachatina marginata (Fig.3.1a) starts anteriorly with the mouth, and ends posteriorly with the anus. The mouth, dorsally convex and semicircular, is bounded by thick brownish oral lappets and opens into the buccal cavity. The latter leads into a long tubular oesophagous, at the sides of which lies a pair of salivary glands.

The salivary glands are paired, elongated, cream-white, much-lobed structures placed dorso-ventrally on the posterior end of the oesophagous and dorsally on the anterior portion of the crop. The glands are separate and narrow anteriorly, but they are held together by connective tissue at their broader posterior ends.

The oesophagous is narrow and thick-walled. The crop is a large, elongated, thin-walled sac. The posterior part of the crop is narrower and opens in the right side of the stomach.

A short and broad duct coming from the anterior lobe of the digestive gland opens on the right side of the dorsal surface of the posterior chamber, slightly anterior to its opening in the stomach.

The stomach is almost heart-shaped, and the rounded posterior end is slightly towards the right. The stomach remains embedded on its dorsal and lateral surfaces in the mass of the digestive gland. A large duct coming from the posterior lobe of the digestive gland opens on the ventral surface of the stomach, slightly left and towards its posterior end.

The intestine arises from the left anterior border of the stomach. The posterior part of the crop and the first part of the intestine with the stomach, form a 'U', the right and left arms being formed by the crop and the intestine respectively, and the base by the stomach itself (Fig 3.1). Shortly after its origin, the intestine takes an anterodorsal course, passing under the kidney, it turns slightly to the right and becomes more dorsal, to be completely embedded in the mass of the anterior lobe of digestive gland. The intestine runs posteriorly, along the right side of the roof of the mantle cavity, where it enlarges to form the rectum. The latter terminates near the mantle's edge, in the anus (Fig. 3.1).

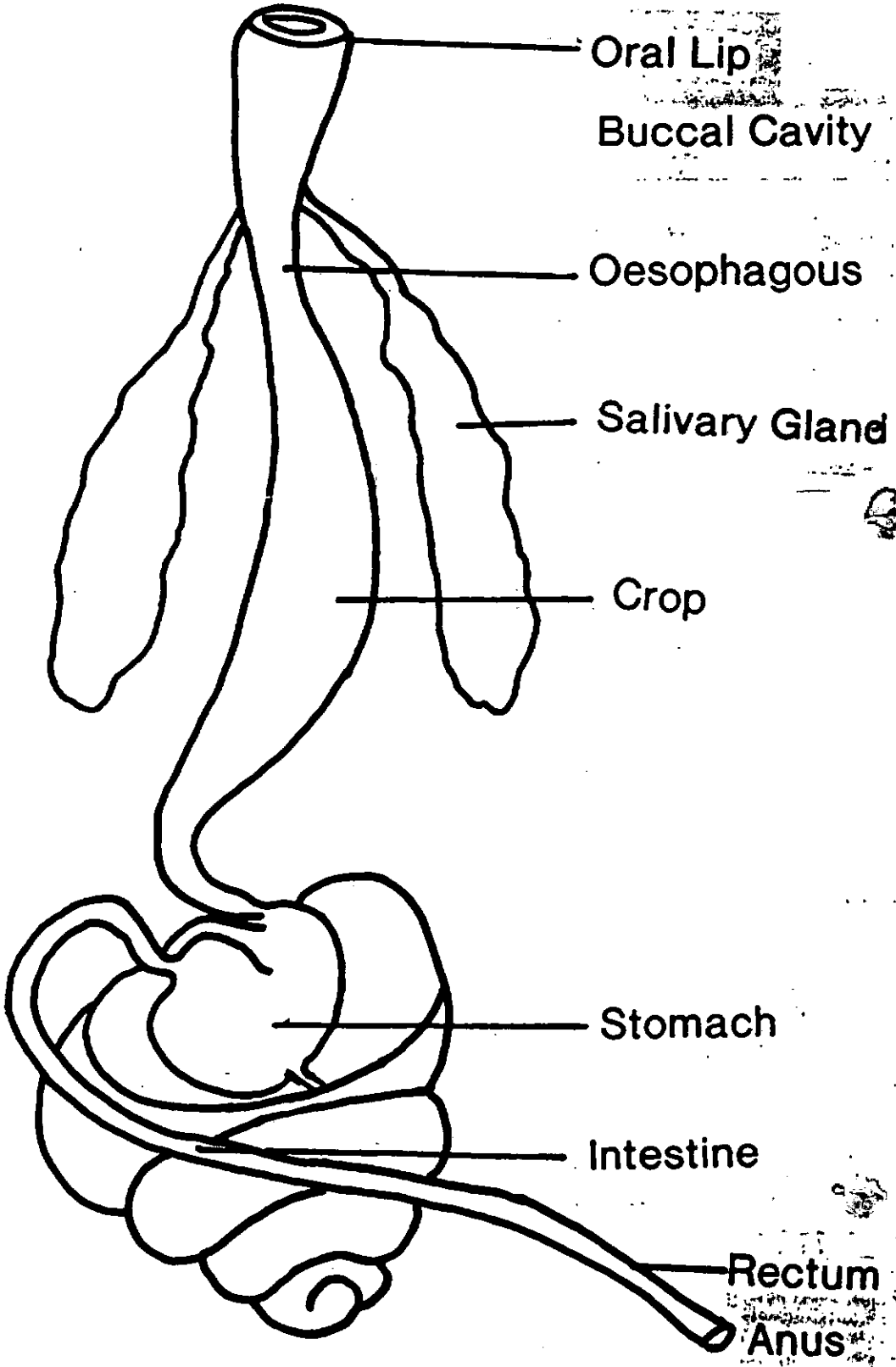

Oral Lip
Buccal Cavity
Oesophagous
Salivary Gland
Crop
Stomach
Intestine
Rectum
Anus

Figure 3.1

The alimentary canal of A. marginata

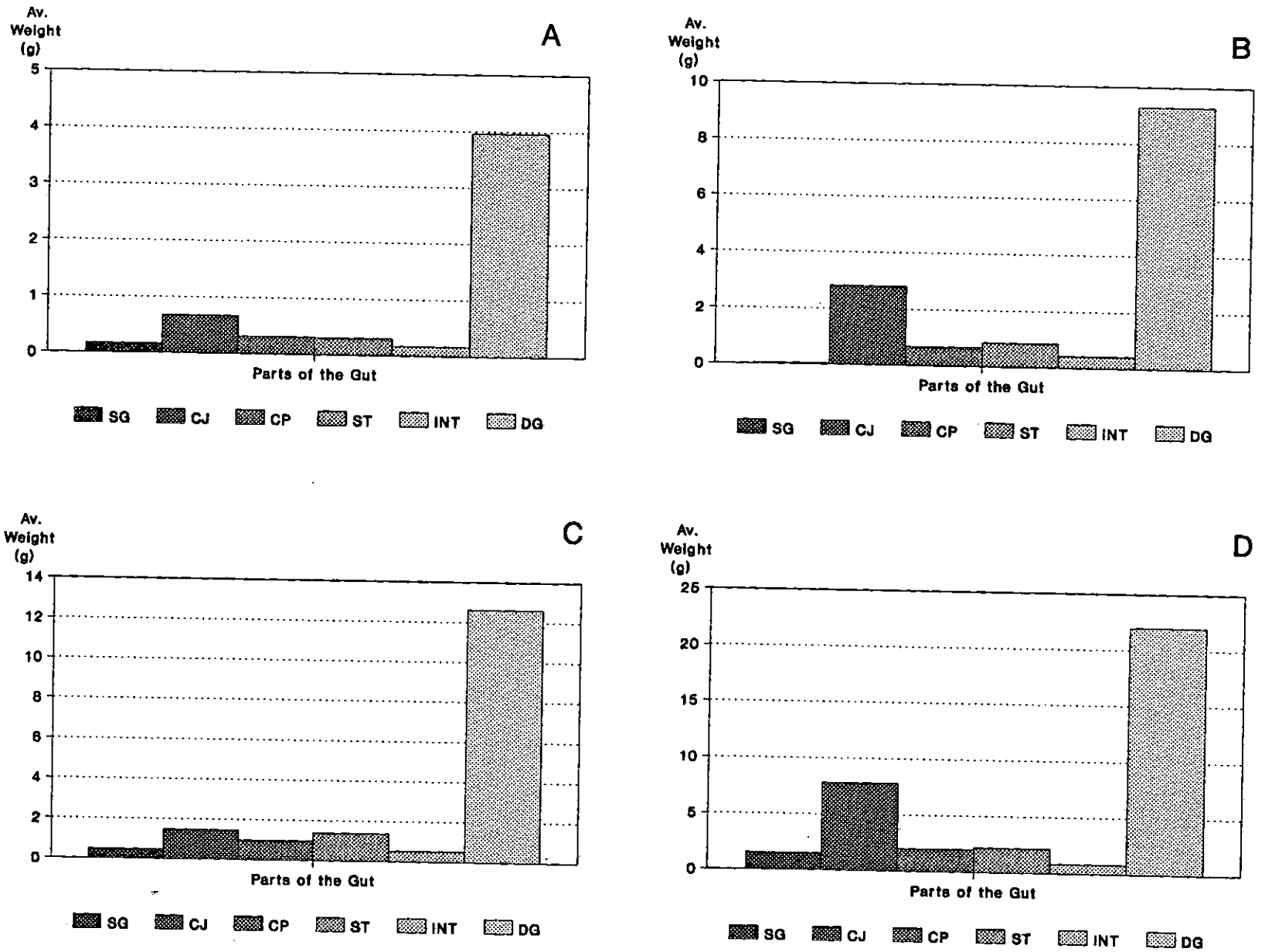
3.2 Comparison of the Average Weight of Different Parts of the Gut in Relation to the Weight of the Snail

The size of the different parts of the gut varied according to the weight of the snail (Fig. 3.2 a, b, c and d). The amount of juice in the crop showed a similar pattern, but was not as consistent with the weight of the different tissues. For example, the amount of crop juice produced by some snails with an average weight of 250 grams was less than that produced by a snail weighing about 150 grams.

3.3 Qualitative Determination of the Digestive Enzymes in A. marginata

3.3.1 Carbohydrases

Tables 3.1 and 3.2 summarize the results of the qualitative experiments on the different carbohydrases found in the alimentary tract of A. marginata. From Table 3.1, it can be seen that the crop juice hydrolysed a wide range of a-linked carbohydrates. The activities in the salivary gland and other parts of the gut were weak, but with greater hydrolytic power in relation to starch than the other substrates.



Compartaive average weights of gut parts in diff snail sizes

Figure 3.2

Comparison of the Average Weight of the Different Parts of the Gut in Relation to the Size of the Snail (A. marginata) Used.

a) Average weight of the different parts of the gut of snails with an average weight of 77g.

b) Average weight of the different parts of the gut ofsnails with an average weight of 150g.

c) Average weight of the different parts of the gut of snails with an average weight of 250g.

d) Average weight of the different parts of the gut of snails with an average weight of 353g.

SG - Salivary Gland

CJ - Crop Juice Preparation

CP - Oesophagus - Crop Tissue

ST - Stomach Tissue

INT - Intestinal Tissue

DG - Digestive Gland

The hydrolytic power of the crop juice was quite high with most of the substrates except inulin and chitin. The result of the experiment on chitin with crop juice was weak or negligible. Cellofas B, a water-soluble sodium carboxymethyl derivative of cellulose was hydrolysed by all the extracts from the different parts of the gut, except in the stomach where the difference between the control experiment and test was quite small or insignificant.

Table 3.1 Digestion of α-linked Carbohydrates by Crude Enzyme Extracts

	Maltose	Sucrose	Starch	Raffinose
Salivary gland	+	trace	±	-
Crop juice	++	++	++	trace
Crop	±	±	±	-
Stomach	±	±	±	-
Intestine	±	±	±	-
Digestive gland	±	±	±	-

KEY : Hydrolytic "Power"
++ Very strong
+ Strong
± Weak
- Absent

Table 3.2 Digestion of ß-linked Carbohydrates

	Lactose	Cellobiose	Cellofas B	Inulin	Chitin
Salivary gland	±	±	±	-	-
Crop juice	++	++	++	-	?
Crop	±	±	±	-	-
Stomach	±	±	trace	-	-
Intestine	±	±	±	-	-
Digestive gland	±	±	+	-	-

3.3.2 Proteases

None of the gut part extracts showed acid or alkaline protease activity, as shown in Table 3.3. The digestive gland extract showed a stronger peptidase action than the other parts of the gut.

Table 3.3 Digestion of Proteins

	S.G.	C.J.	Crop	ST.	INT.	D.G.
Alkaline Protease Activity	-	-	-	-	-	-
Acid Protease Activity	-	-	-	-	-	-
Peptidase	±	±	±	±	±	+

3.3.3 Lipases

The assay for lipolytic activity in the gut of A. marginata was for both the qualitative and quantitative estimation and the result is as shown in Table 3.4.

Table 3.4 Titrimetic Determination of Lipase activity in A. marginata

	S.G.	C.J.	Crop	ST	INT	D.G.
μmoles of fatty acid released min^{-1}	0.19	6.65	0.55	0.68	0.55	1.00

Table 3.4 shows the distribution of lipases which hydrolysed the emulsion of olive oil used as substrate. The oil emulsion wasn't much hydrolysed by the salivary gland extract, but there were high digestion rates with the crop juice and digestive gland, relative to the crop, stomach and the intestine.

3.4 Quantitative Determination of Various Digestive Enzymes in A. marginata

3.4.1 Glucose Standard

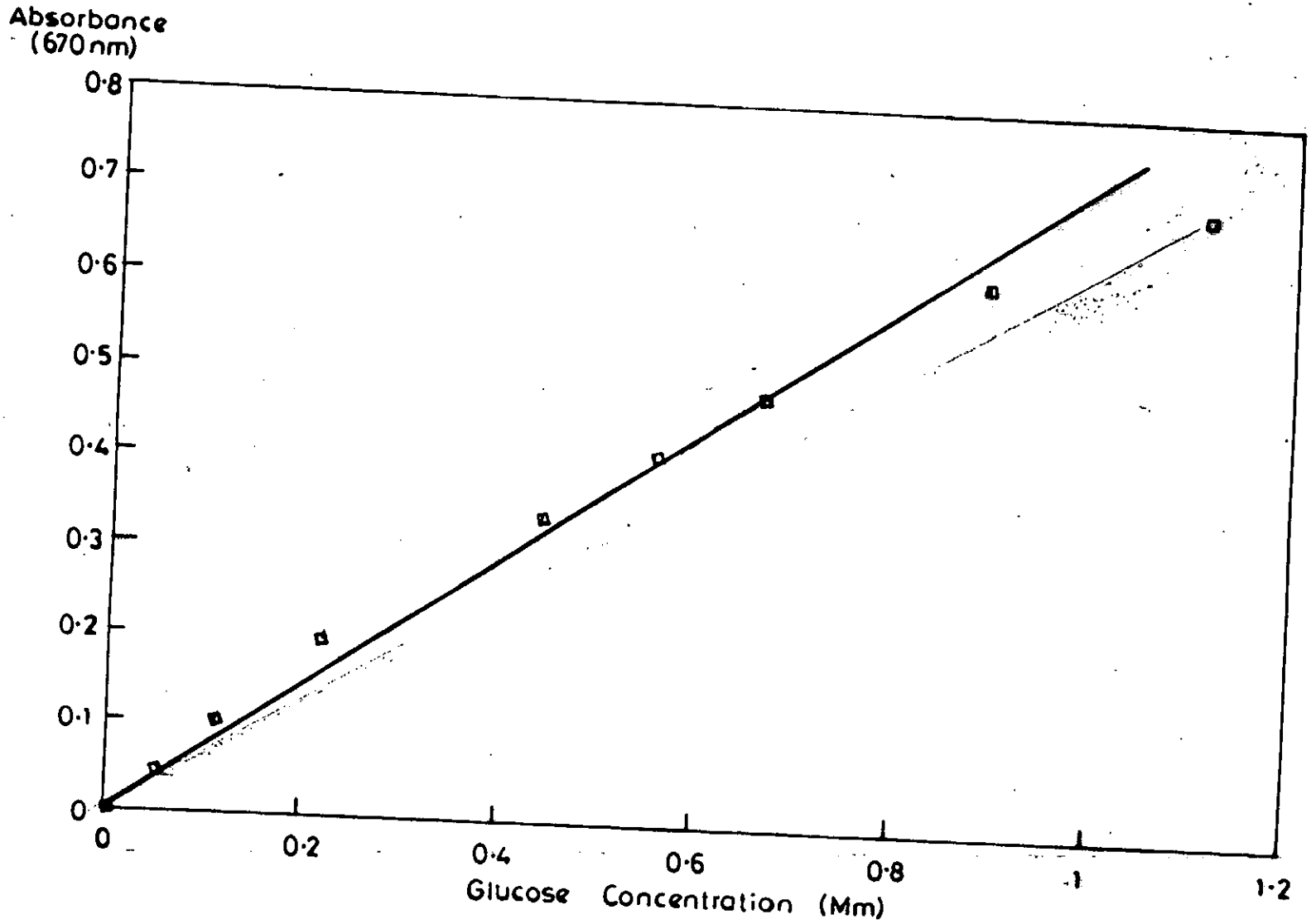
When the absorbances of a series of concentrations of glucose at values ranging from 0mM to 1.11mM were plotted against the concentration of glucose, the lines shown in Figs. 3.3 and 3.4 were obtained.

3.4.2 Protein Standard

A protein standard graph was plotted using bovine serum alumin (BSA) solutions, ranging from 0 to 1mg/ml. A linear graph was obtained when the absorbances were plotted against concentration of BSA as shown in Fig. 3.5 Fig. 3.6 shows the protein content of each part of the gut.

3.4.3 Quantitative Determination of some Carbohydrase Activities

The results of the hydrolysis of various carbohydrates are presented in terms of total activity and specific activity. Total activity enables us to know the contribution of each part of the gut to actual digestion of particular molecules, while the specific activity gives a measure of the concentration of the enzyme in that part of the gut.


Absorbance
(670nm)
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
0
0
0·2
0·4
0·6
0·8
1
1·2
Glucose Concentration (Mm)

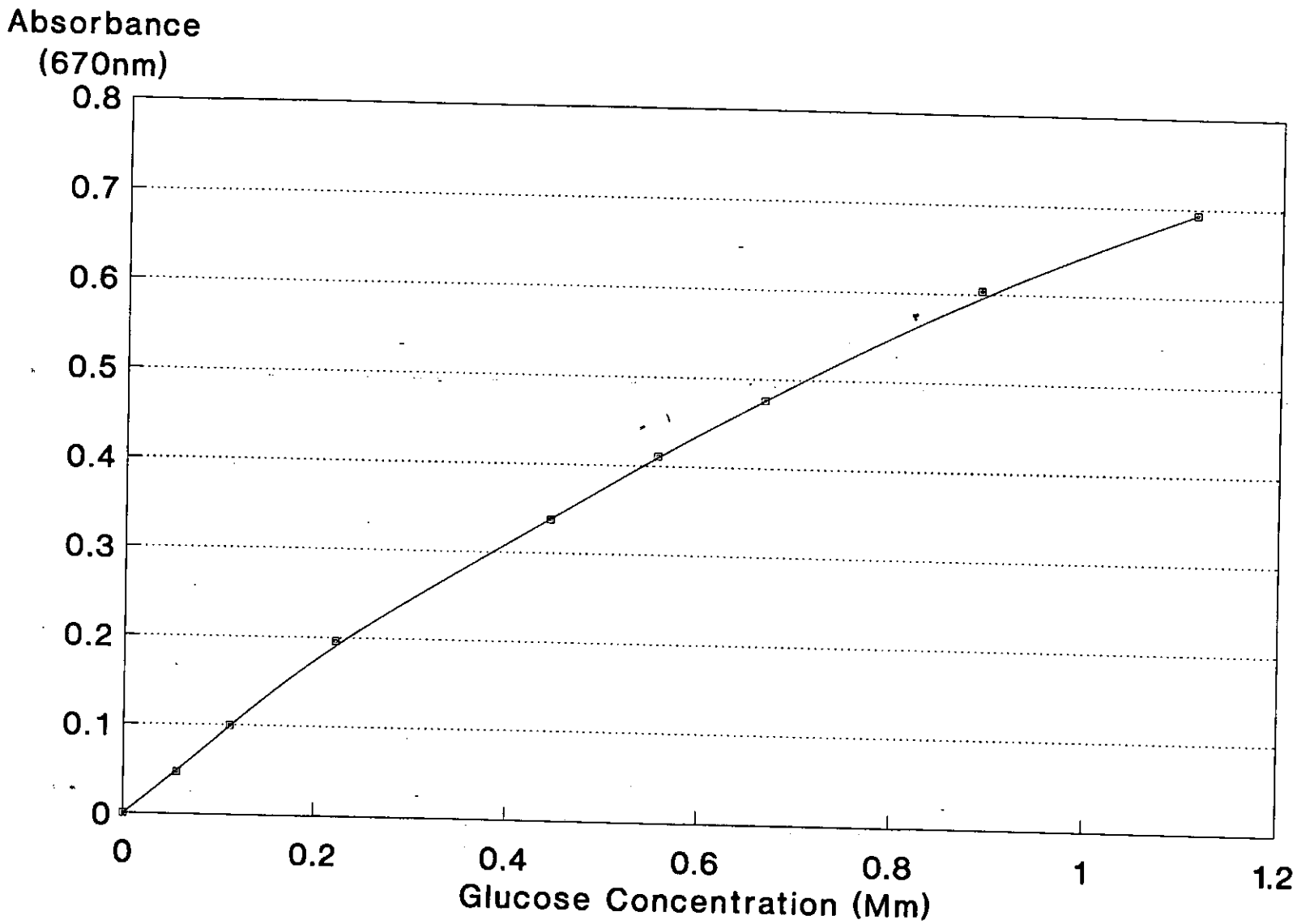
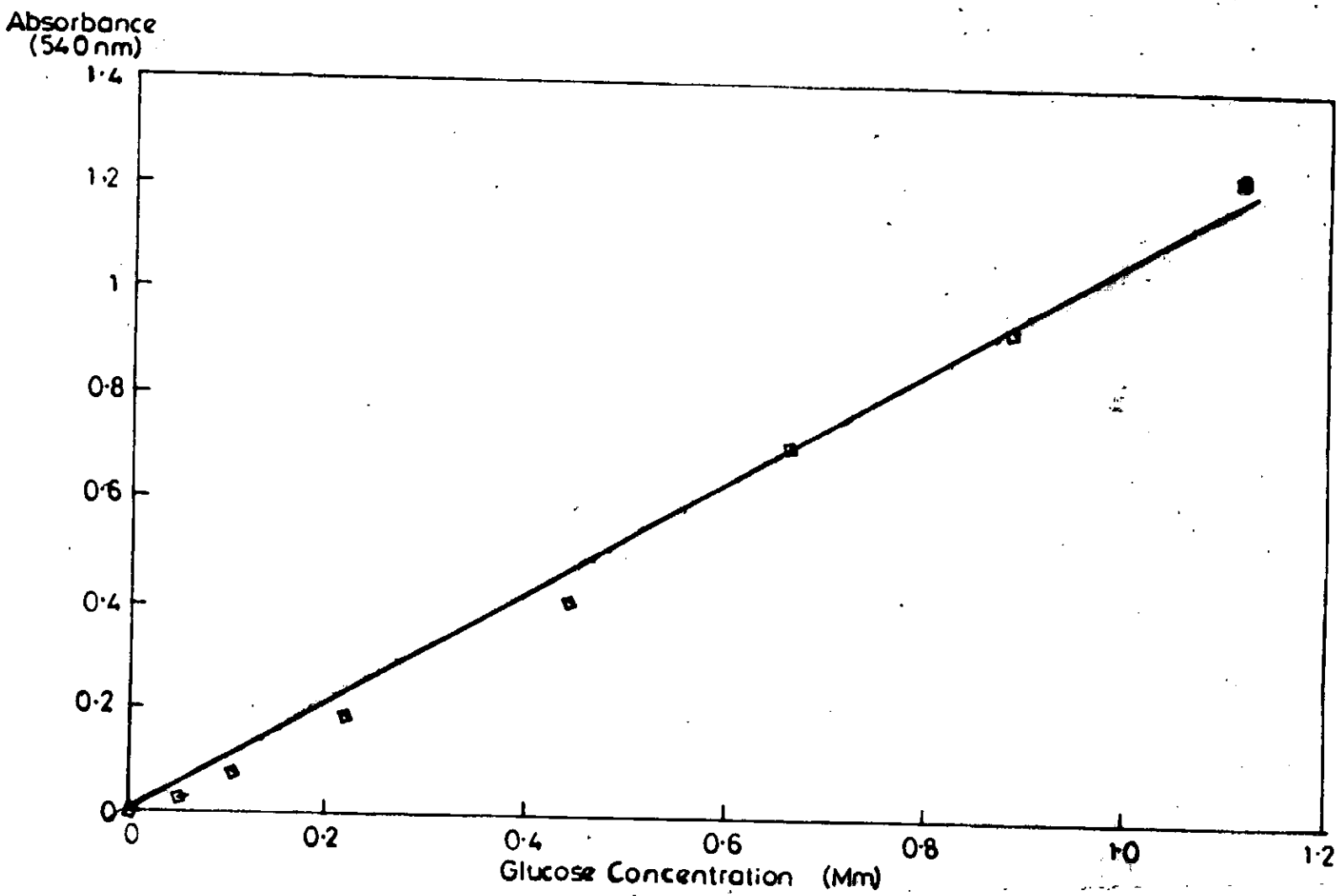

Absorbance
(670nm)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
0.2
0.4
0.6
0.8
1
1.2
Glucose Concentration (Mm)

Figure 3.3

(a) Glucose standard curve I

(b) Glucose standard graph I

The amount of glucose released was measured using glucose oxidase at 670nm


Absorbance
(540 nm)
1·4
1·2
1
0·8
0·6
0·4
0·2
0
0
0·2
0·4
0·6
0·8
1·0
1·2
Glucose Concentration (Mm)

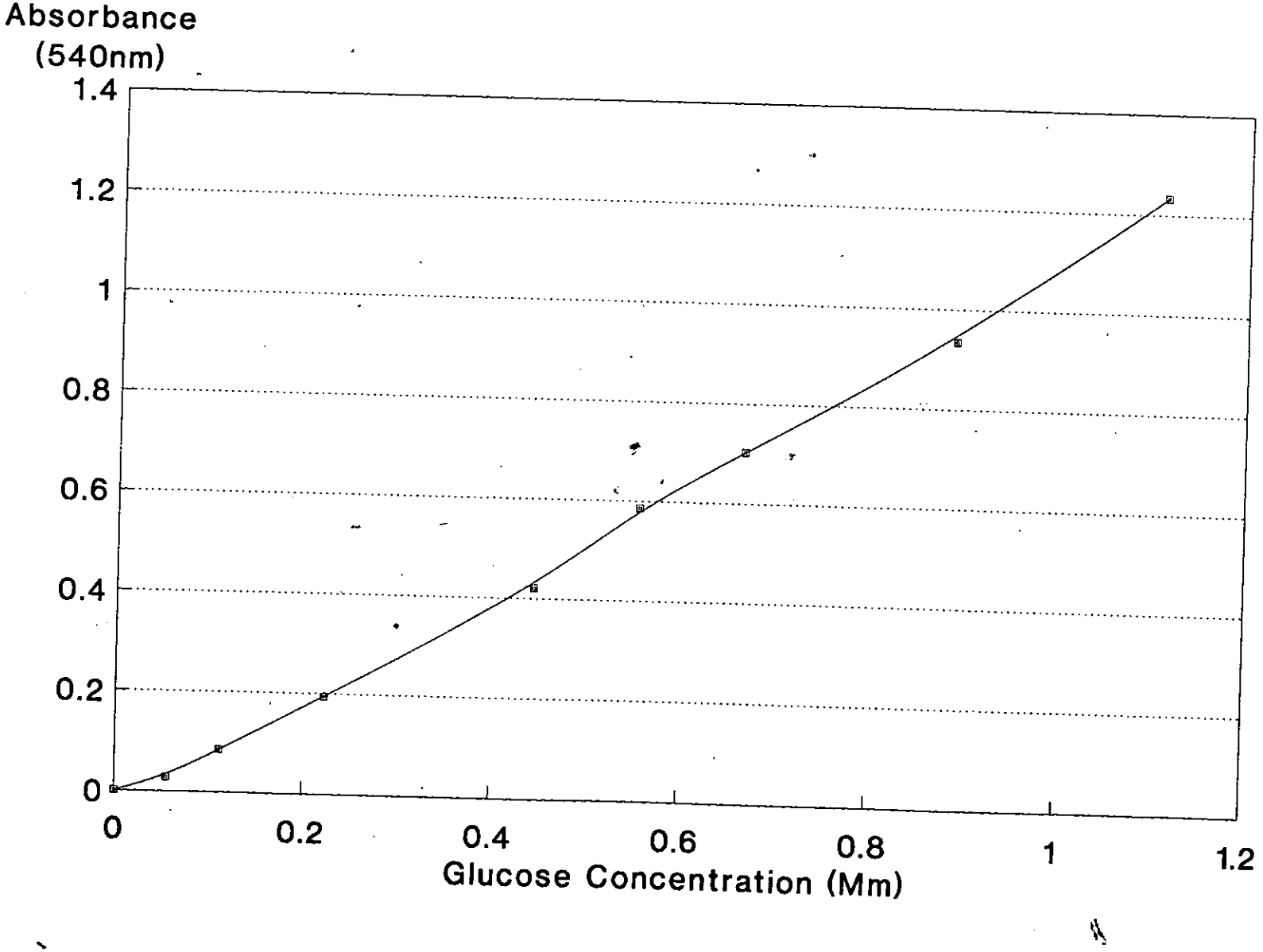
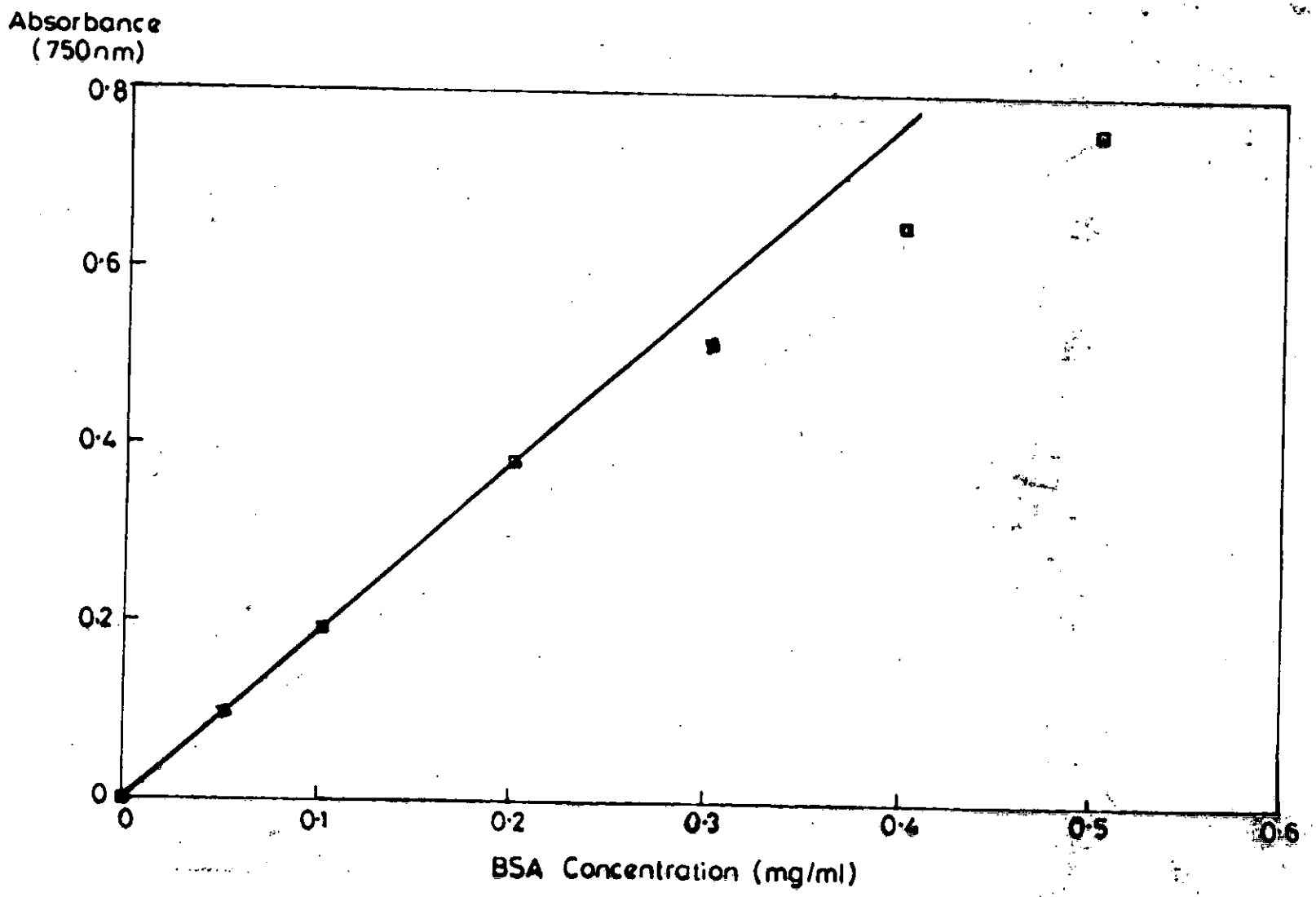

Absorbance
(540nm)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
0.2
0.4
0.6
0.8
1
1.2
Glucose Concentration (Mm)

Figure 3.4

(a) Glucose standard curve II

(b) Glucose standard graph II.

The amount of glucose released was measured by the reducing sugar method at 540nm (Nelson, 1944)


Absorbance
(750nm)
0·8
0·6
0·4
0·2
0
0
0·1
0·2
0·3
0·4
0·5
0·6
BSA Concentration (mg/ml)

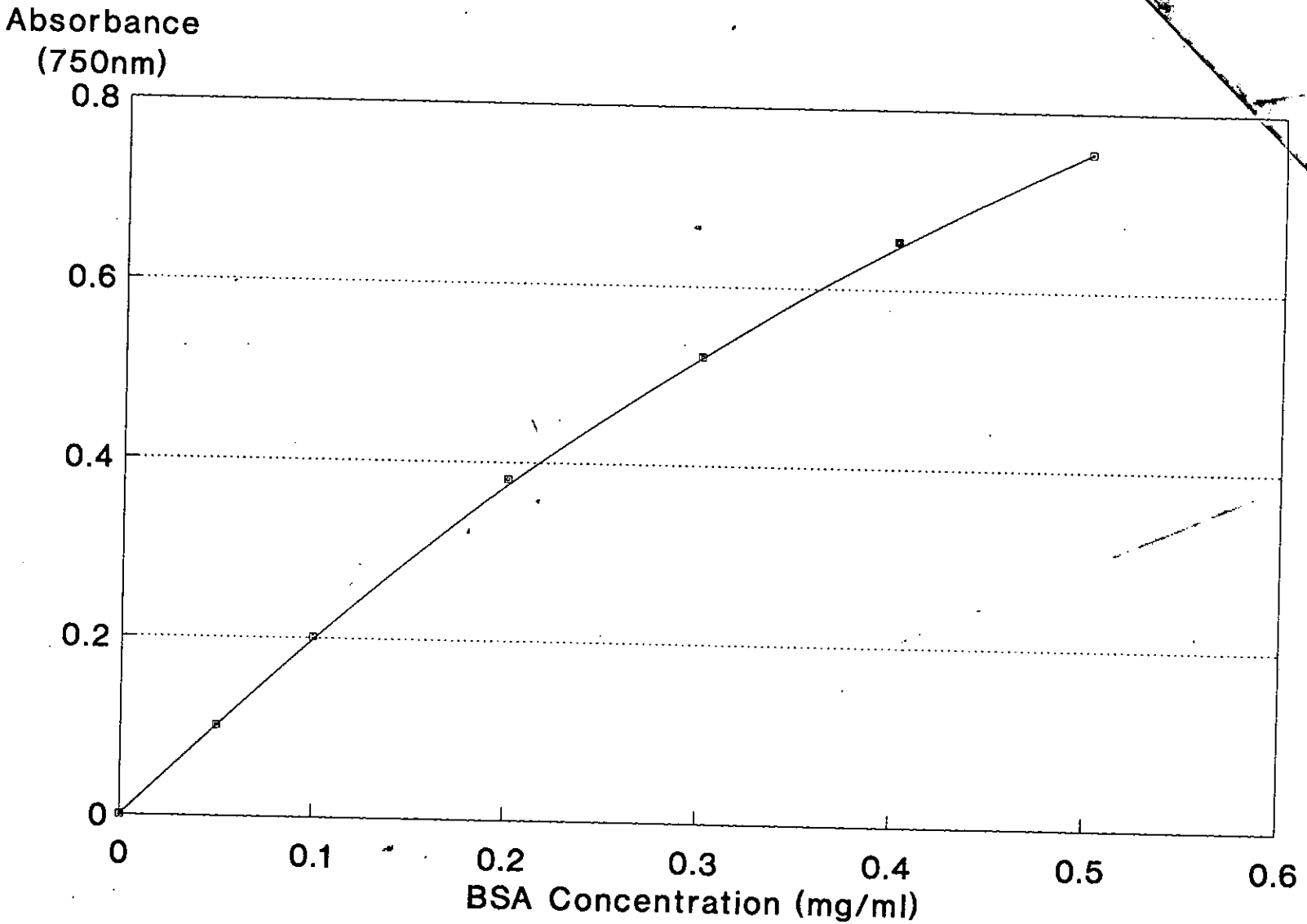

Absorbance
(750nm)
0.8
0.6
0.4
0.2
0
0
0.1
0.2
0.3
0.4
0.5
0.6
BSA Concentration (mg/ml)

Figure 3.5

(a) Protein standard curve

(b) Protein standard graph.

The amount of protein was measured by the Lowry *et al.* (1951) method.

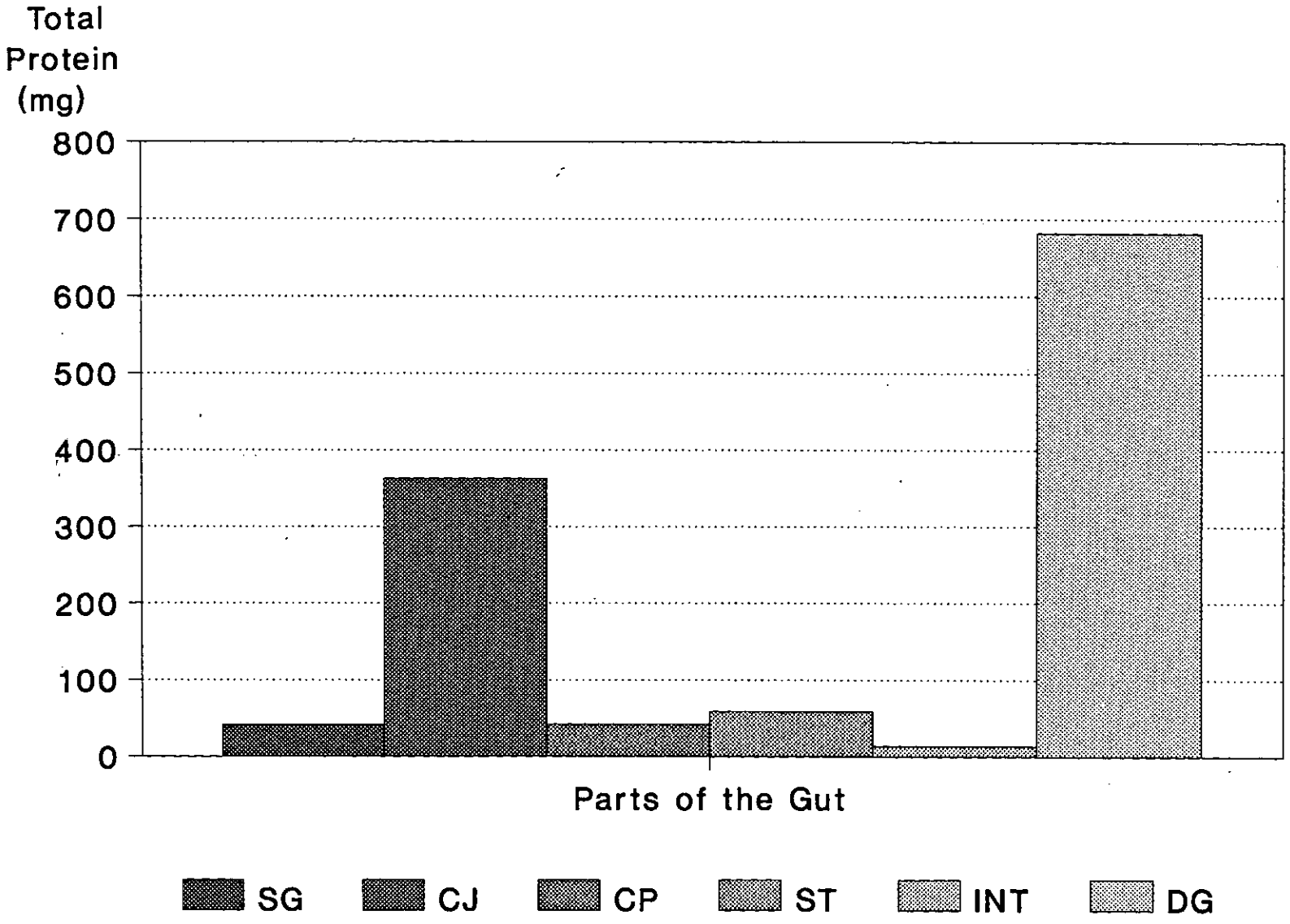

Total Protein (mg)
800
700
600
500
400
300
200
100
0
Parts of the Gut
SG
CJ
CP
ST
INT
DG

Figure 3.6

Protein content of the different gut part extracts. The protein contents of each part of the gut was estimated using the method of Lowry et al. (1951).

SG - Salivary gland extract

CJ - Crop juice

CP - Crop tissue extract

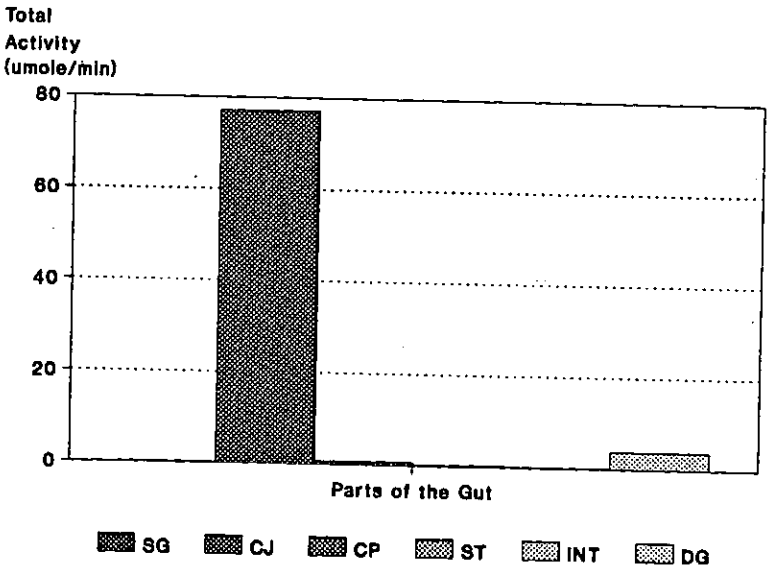
ST - Stomach tissue extract

INT - Intestinal tissue extract

DG - Digestive gland extract

Strong hydrolytic power was found in the crop juice both in terms of the total activity and the specific activity. The activity of the rest of different parts of the gut was quite low, except in the digestive gland, where the total activity was about one tenth of that of the crop juice. With the specific activity of the different parts of the gut, the crop juice had the strongest activity, followed by the crop tissue extract. The specific activity of the digestive gland was quite low, although it was the second strongest in terms of the total activity, Figures 3.7a and b summarise the quantity of sucrose hydrolysed in each part of the gut.

Fig. 3.8a and b show the distribution of the enzyme which hydrolysed maltose. The total activities in each part of the gut apart from the crop juice and the digestive gland, were quite low. The highest total activity and specific activities were detected in the crop juice. Lactose gives galactose and glucose on hydrolysis, but the two molecules are coupled via a ß-link. The quantitative study revealed the highest total activity and specific activity in the crop juice, with the digestive gland showing much lower activities both in total and specific terms.


Total
Activity
(umole/min)
80
60
40
20
0
Parts of the Gut
SG
CJ
CP
ST
INT
DG

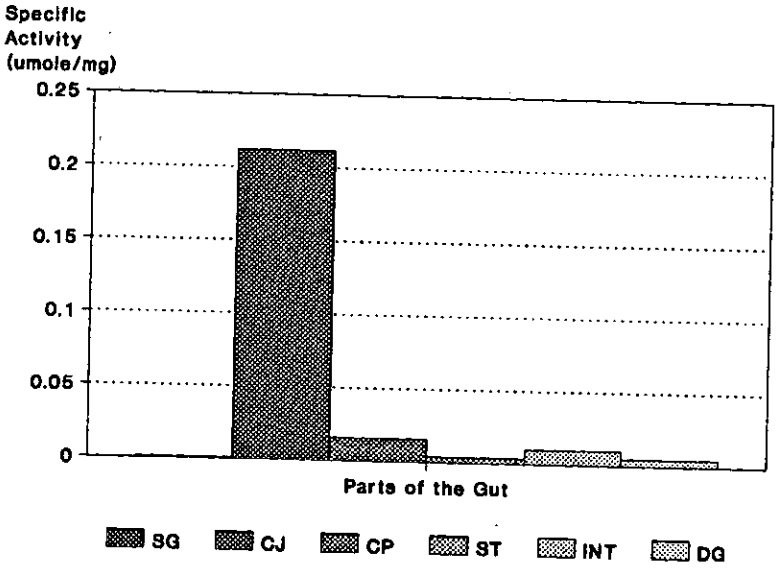

Specific
Activity
(umole/mg)
0.25
0.2
0.15
0.1
0.05
0
Parts of the Gut
SG
CJ
CP
ST
INT
DG

Figure 3.7a

Total sucrase activity in different crude tissue extracts and crop juice preparation of A. marginata gut.

Total activity was calculated in terms of μmoles of glucose released per minute from sucrose.

Figure 3.7b

Specific Sucrase Activity in Different Crude Tissue Extracts and Crop Juice Preparation

Specific Activity Values are Calculated as μmole of Glucose Released per mg Protein from Sucrose

SG - Salivary Gland Extract

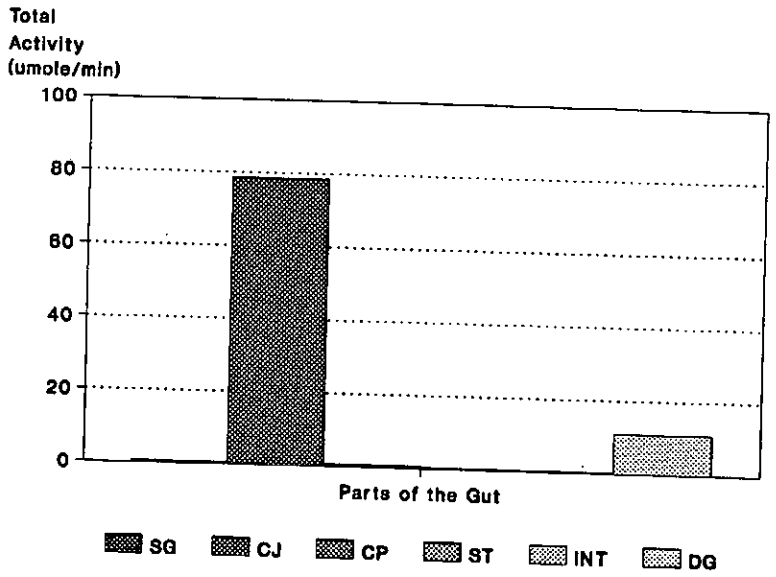
CJ - Crop Juice Preparation

ST - Stomach Tissue Extract

DG - Digestive Gland Extract

CP - Oesophagus - Crop Tissue Extract

INT - Intestinal Tissue Extract


Total
Activity
(umole/min)
100
80
60
40
20
0
Parts of the Gut
SG
CJ
CP
ST
INT
DG

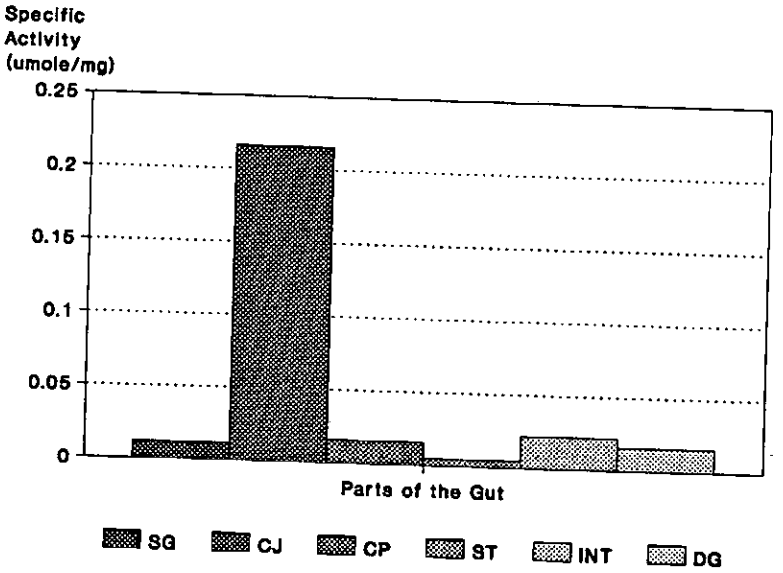

Specific
Activity
(umole/mg)
0.25
0.2
0.15
0.1
0.05
0
Parts of the Gut
SG
CJ
CP
ST
INT
DG

Figure 3.8a

Total maltase activity in different crude tissue extracts and crop juice preparation of A. marginata gut.

Total activity is calculated in terms of umoles of glucose released per minute for maltose substrate used.

Figure 3.8b

Specific Maltase activity in different crude tissue extracts and crop juice preparation of A. marginata gut

Specific activity values are calculated as umoles of glucose released per minute per mg. protein from maltose substrate used.

SG - Salivary Gland Extract

CJ - Crop Juice Preparation

CP - Oesophagus - Crop Tissue Extract

ST - Stomach Tissue Extract

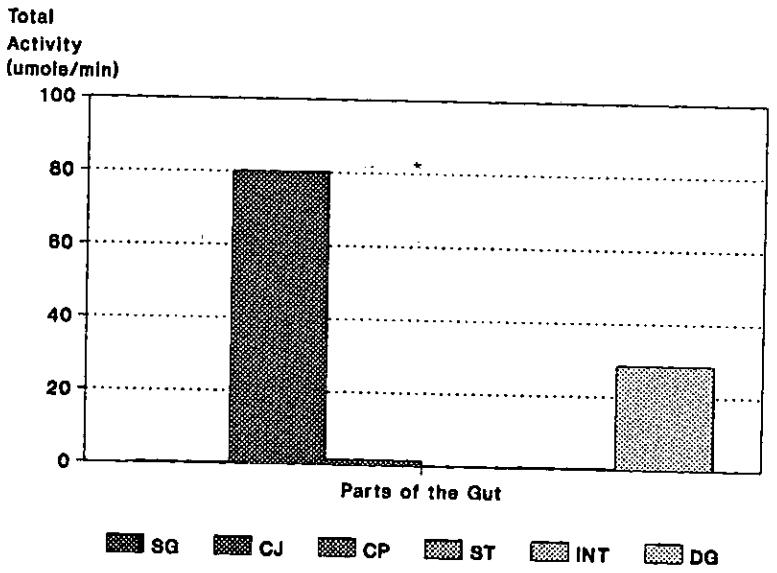
INT - Intestinal Tissue Extract

DG - Digestive Gland Extract

The specific activity in the salivary gland, stomach and intestine was low as shown in Fig. 3.9a and b for lactase. Cellobiose was significantly hydrolysed by the crop juice preparation, but poorly digested by the extracts of other parts of the gut examined. Fig. 3.10a and b summarize the results of the quantitative study on cellobiose hydrolysis.

In summary, the distribution of the activity of the different disaccharidases in the different parts of the gut was similar. In general, there were very high disaccharidase activities in the crop juice and relatively very low activities in the other parts of the gut. The digestive gland showed the second strongest total activity, but the results on specific activity showed that the concentration of the enzymes was much lower in this gland.

The digestion of the different polysaccharides by the salivary gland, stomach and intestine was negligible in comparison with that of the crop juice. Figures 3.11a and b summarize the results on the digestion of starch by the extracts from the different parts of the gut. The results are expressed in terms of total and specific activities. In both cases, the main digestion occurred in the crop juice preparation with low activities in the other parts of the gut extracts.


Total
Activity
(umole/min)
100
80
60
40
20
0
Parts of the Gut
SG
CJ
CP
ST
INT
DG

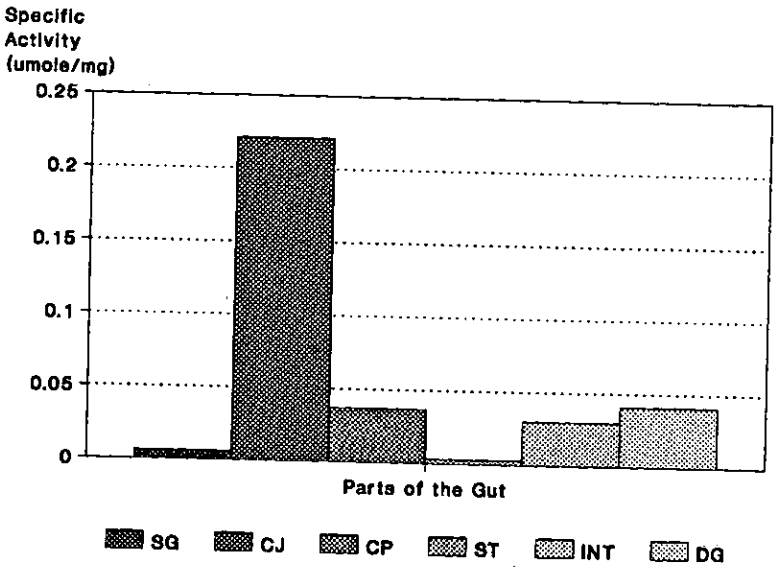

Specific
Activity
(umole/mg)
0.25
0.2
0.15
0.1
0.05
0
Parts of the Gut
SG
CJ
CP
ST
INT
DG

Figure 3.9a

Total lactase activity in different crude tissue extracts and crop juice preparation of A. marginata gut.

Figure 3.9b

Specific Lactase activity in different crude tissue extracts and crop juice preparation of A. marginata gut.

SG - Salivary Gland Extract

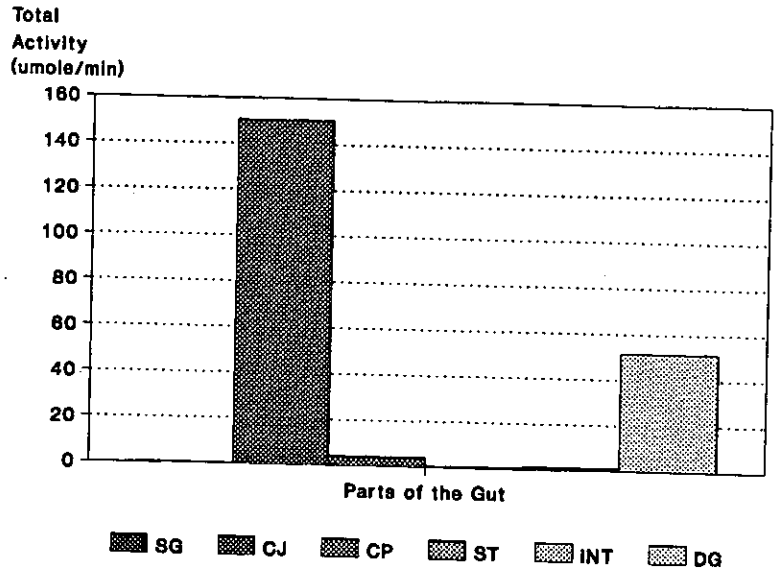
CJ - Crop Juice Preparation

CP - Oesophagus - Crop Tissue Extract

ST - Stomach Tissue Extract

INT - Intestinal Tissue Extract

DG - Digestive Gland Extract


Total
Activity
(umole/min)
160
140
120
100
80
60
40
20
0
Parts of the Gut
SG
CJ
CP
ST
INT
DG

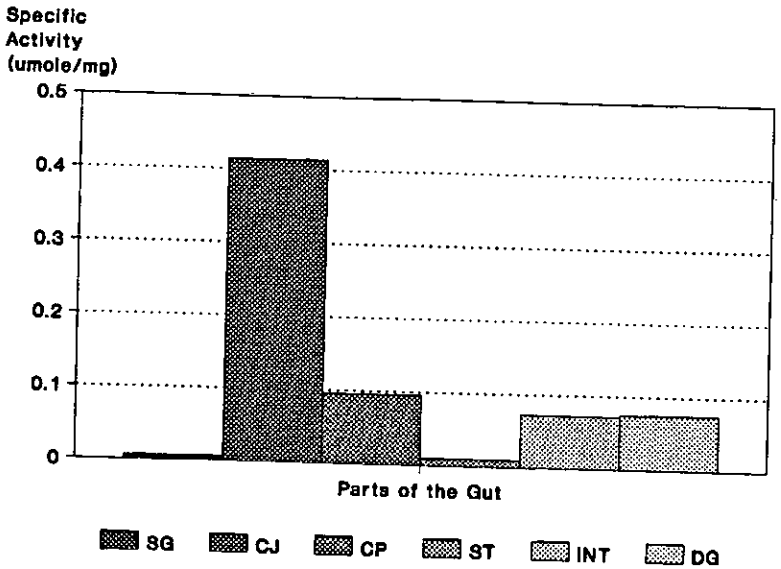

Specific
Activity
(umole/mg)
0.5
0.4
0.3
0.2
0.1
0
Parts of the Gut
SG
CJ
CP
ST
INT
DG

Figure 3.10a

Total cellobiase activity in different crude tissue extracts and crop juice preparation of A. marginata gut.

Total activity is calculated in terms of μmole of glucose released per minute from cellobiose substrate used. cellobiase in different tissue extracts (crude preparation) and crop juice preparation of A. marginata gut.

Figure 3.10b

Specific cellobiase activity in different crude tissue extracts and crop juice preparation of A. marginata gut.

Specific activity values are calculated as μmoles of glucose released per minute per mg. protein from cellobiose substrate used.

SG - Salivary Gland Extract

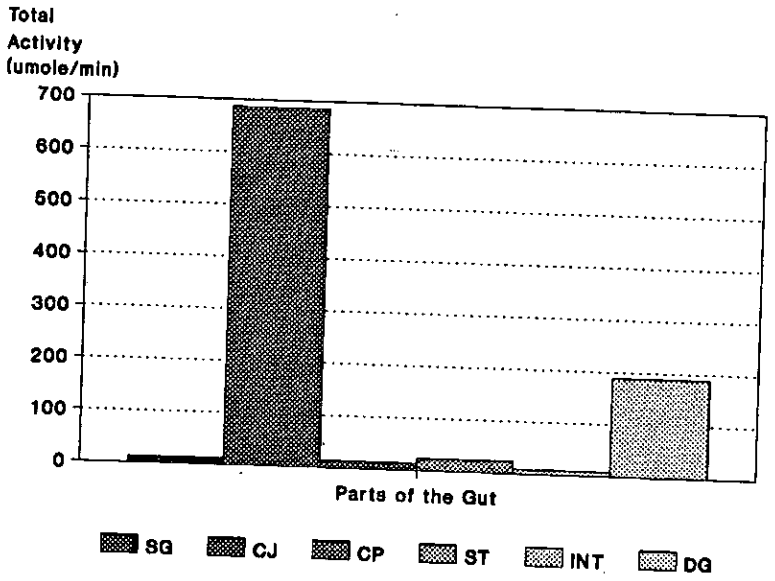
CJ - Crop Juice Preparation

CP - Oesophagus - Crop Tissue Extract

ST - Stomach Tissue Extract

INT - Intestinal Tissue Extract

DG - Digestive Gland Extract


Total
Activity
(umole/min)
700
600
500
400
300
200
100
0
Parts of the Gut
SG
CJ
CP
ST
INT
DG

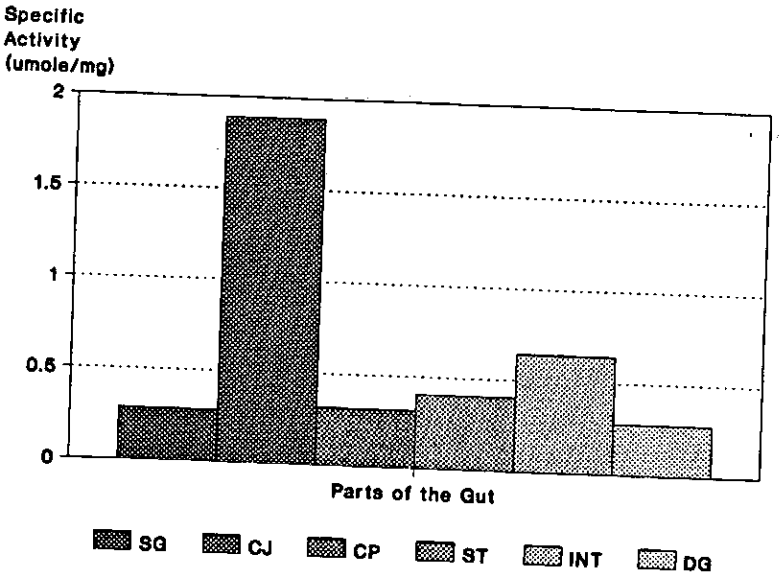

Specific
Activity
(umole/mg)
2
1.5
1
0.5
0
Parts of the Gut
SG
CJ
CP
ST
INT
DG

Figure 3.11a

Total amylase activity in different crude tissue extracts and crop juice preparation of A. marginata gut.

Total activity is calculated in terms of μmoles of glucose released per minute from soluble starch substrate used.

Figure 3.11b

Specific amylase activity in different crude tissue extracts and crop juice preparation of A. marginata gut.

Specific activity values are calculated as umoles of glucose released per minute per mg. protein from soluble starch.

SG - Salivary Gland Extract

CJ - Crop Juice Preparation

CP - Oesophagus - Crop Tissue Extract

ST - Stomach Tissue Extract

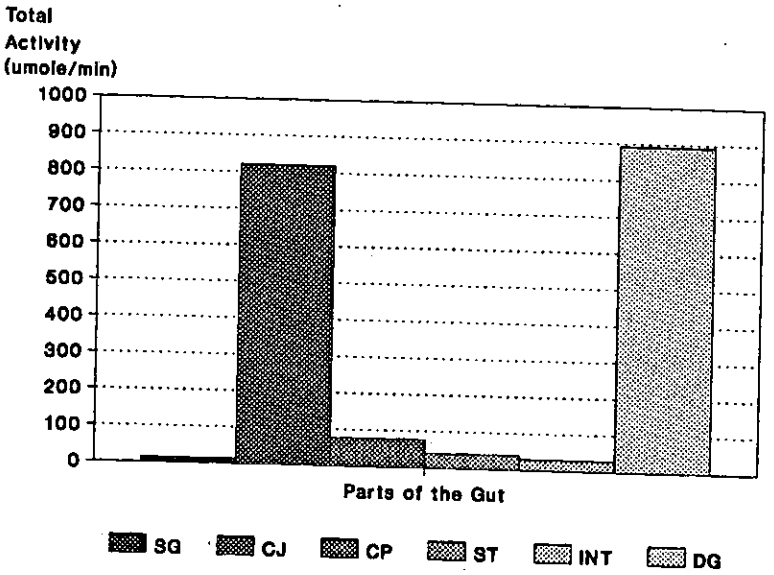
INT - Intestinal Tissue Extract

DG - Digestive Gland Extract

The enzymatic hydrolysis of cellofas B, which is a soluble carboxylmethyl derivative of cellulose was very high with the crude crop juice. The pattern of distribution of this ß(1-4) linked glucan activity is shown in Figure 3.12a and b.

In the qualitative study of chitin digestion, the result of hydrolysis was not quite clear because there was very little or no difference between the test and the control tubes. Each time the experiment was carried out with the crop juice preparation. But in the quantitative study, this polymer, composed of ß(1-4) linked N-acetylglucosamine units was weakly split by the crop juice preparation only. The result is shown in Table 3.5.

The hydrolysis of the trisaccharide, raffinose, could not be demonstrated in any part of the gut. Although there were very low readings for the intestinal extract, these were not convincing enough.


Total
Activity
(umole/min)
1000
900
800
700
600
500
400
300
200
100
0
Parts of the Gut
SG
CJ
CP
ST
INT
DG

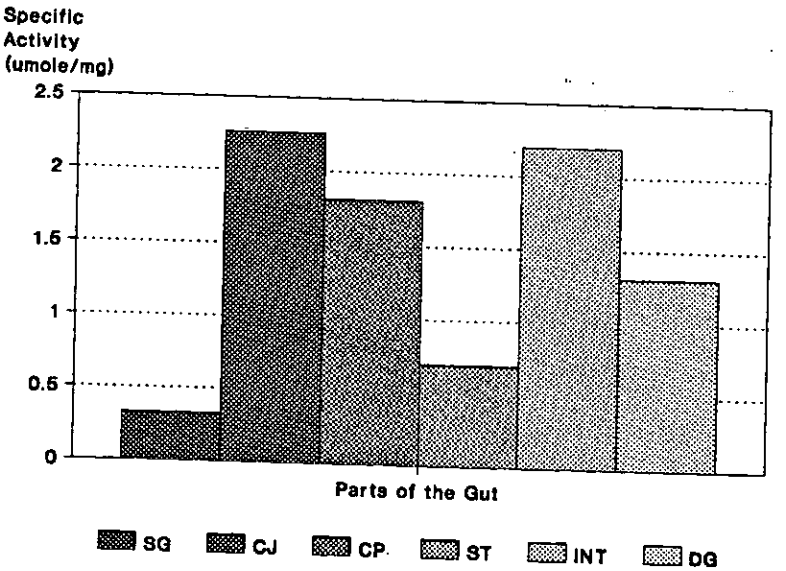

Specific
Activity
(umole/mg)
2.5
2
1.5
1
0.5
0
Parts of the Gut
SG
CJ
CP
ST
INT
DG

Figure 3.12a

Total cellulase activity in different crude tissue extracts and crop juice preparation of A. marginata gut.

Total activity is calculated in terms of umoles of glucose released per minute from cellafas B

Figure 3.12b

Specific activity in different crude tissue extracts and crop juice preparation of A. marginata.

Specific activity values are calculated as umoles of glucose released per minute per mg. protein from the substrate used (cellafas B).

SG - Salivary Gland Extract

CJ - Crop Juice Preparation

CP - Oesophagus - Crop Tissue Extract

ST - Stomach Tissue Extract

INT - Intestinal Tissue Extract

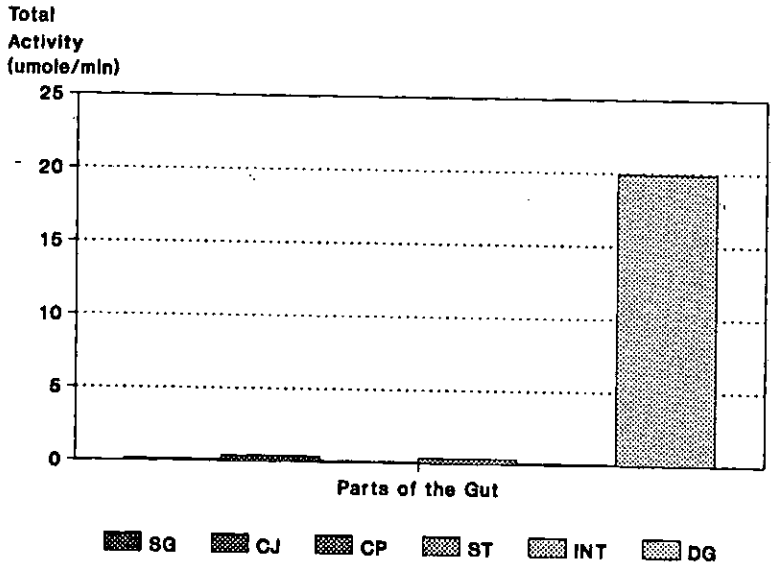
DG - Digestive Gland Extract

Table 3.5 Chitinase Assay Result

	Sal gland	Crop	Crop Juice	Stom	Intestine	Digestive
Total protein (mg)	41.60	263.2	42.49	58.60	13.55	682.05
Activity in/g tissue umole min^{-1} g^{-1}	-	0.084	-	-	-	-
total activity umole min^{-1}	-	0.295	-	-	-	-
Specific activity umole min^{-1} mg^{-1} protein	-	0.008	-	-	-	-

3.4.4 **Proteolytic Activity**

Figure 3.13a shows the proteinase activity of the enzymatically active extracts from the digestive tract of A. marginata. In terms of total proteolytic activity, the capacity of the digestive gland seemed to be the highest, while the respective capabilities of the other parts of the gut were relatively low.


Total
Activity
(umole/min)
25
20
15
10
5
0
Parts of the Gut
SG
CJ
CP
ST
INT
DG

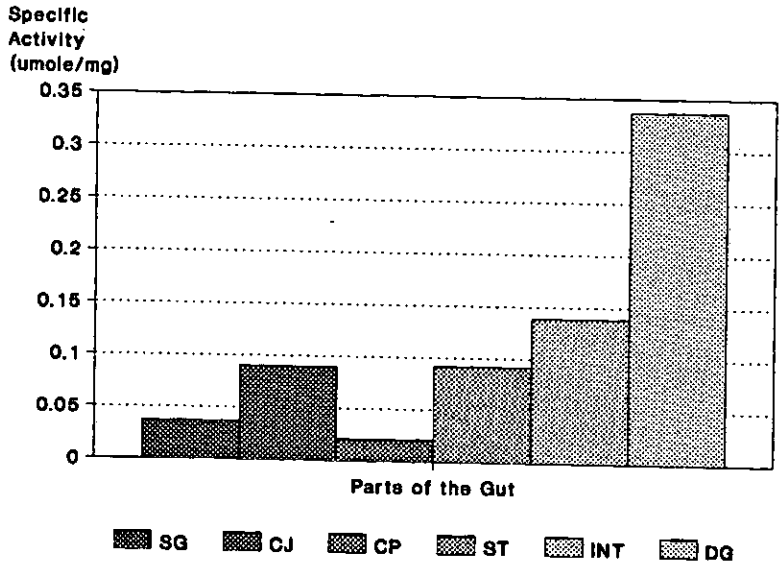

Specific
Activity
(umole/mg)
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Parts of the Gut
SG
CJ
CP
ST
INT
DG

Figure 3.13a

Total proteolytic activity in different crude tissue extracts and crop juice preparation of A. marginata gut.

Total proteolytic activity is calculated in terms of mg of BSA released per minute from casein which was used as a substrate.

Figure 3.13b

Specific activity in different crude tissue extracts and crop juice preparation of A. marginata gut for protease.

Specific activity values are calculated as mg of BSA released per minute per mg. protein from casein which was used as a substrate.

SG - Salivary Gland Extract

CJ - Crop Juice Preparation

CP - Oesophagus - Crop Tissue Extract

ST - Stomach Tissue Extract

INT - Intestinal Tissue Extract

DG - Digestive Gland Extract

From Figure 3.13b the actual hydrolysis of casein in the digestive gland is still highest followed by the intestine, the crop juice and the stomach. The salivary gland and crop tissue extracts were very low in their specific proteolytic activity.

3.4.5 Lipase Activity

The result of the titrimetric determination of lipase in the different parts of the alimentary canal of the snail is illustrated in Figure 3.14. Over 90% of the lipase activity was found in the crop juice followed by the digestive gland. Although the salivary gland had relatively very weak lipase activity, the other tissue extracts gave some appreciable activity.

3.5 The Influence of pH on Enzyme Activities

The pH activity curves for the hydrolysis of three α-linked and three ß-linked carbohydrates by the crude crop juice preparation all indicated a pH optimum at 5.7 (Figures 3.15-3.20). Compared with the ß-linked substrates, the α-linked substrates showed more definitive optimal points. Although the pH optimum for maltose was 5.7, the enzyme activity was not very much affected at lower pH values but the enzyme activity fell gradually when the pH value exceeded 6.0. Similar results were obtained for all the other enzyme

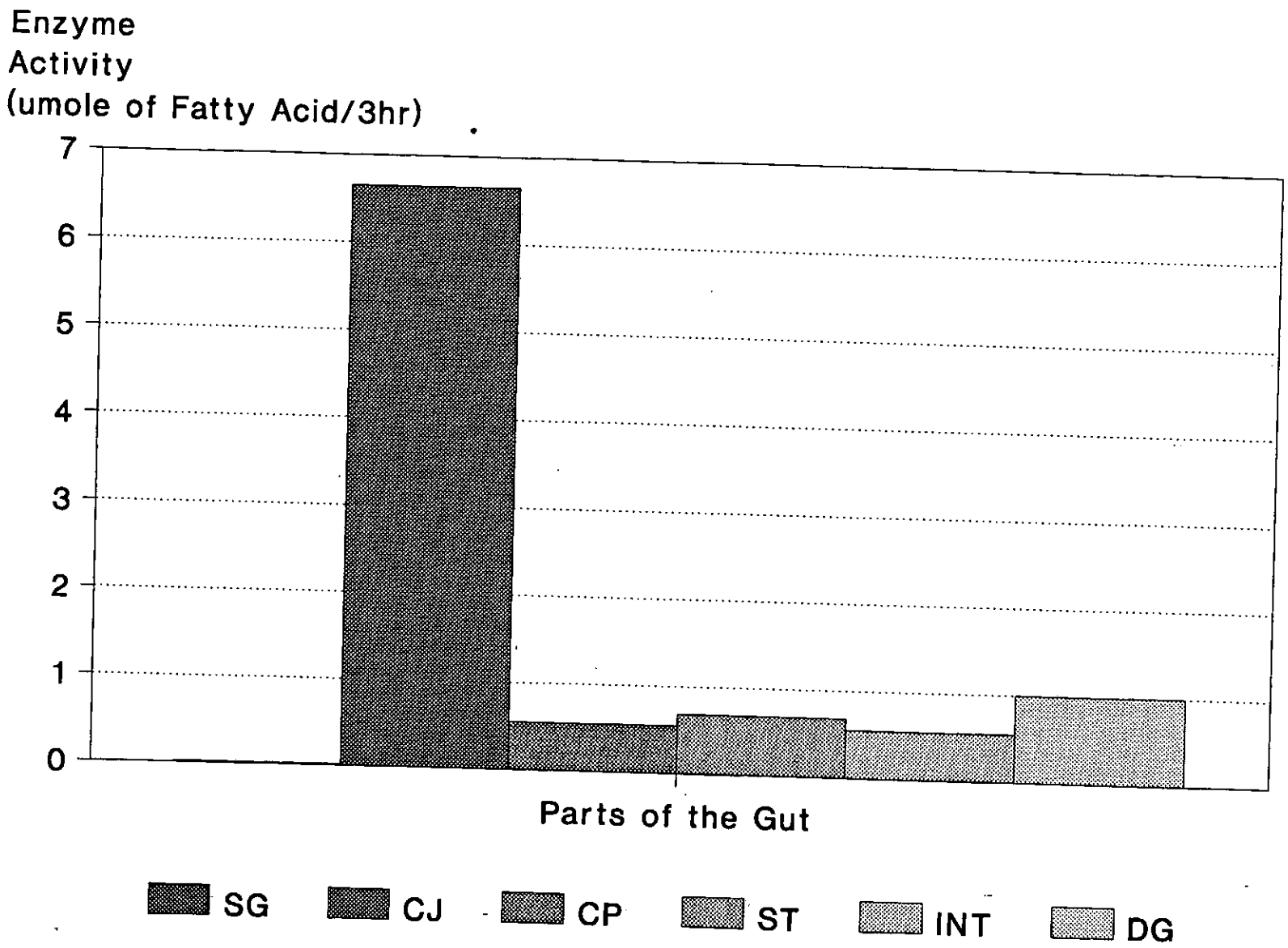

Enzyme
Activity
(umole of Fatty Acid/3hr)
7
6
5
4
3
2
1
0
Parts of the Gut
SG
CJ
CP
ST
INT
DG

Figure 3.14

Distribution of Lipase (activity) in the different parts of the gut of A. marginata. Lipase activity values are calculated as the μmoles of fatty acid released per three hours.

SG - Salivary Gland Extract

CJ - Crop Juice Preparation

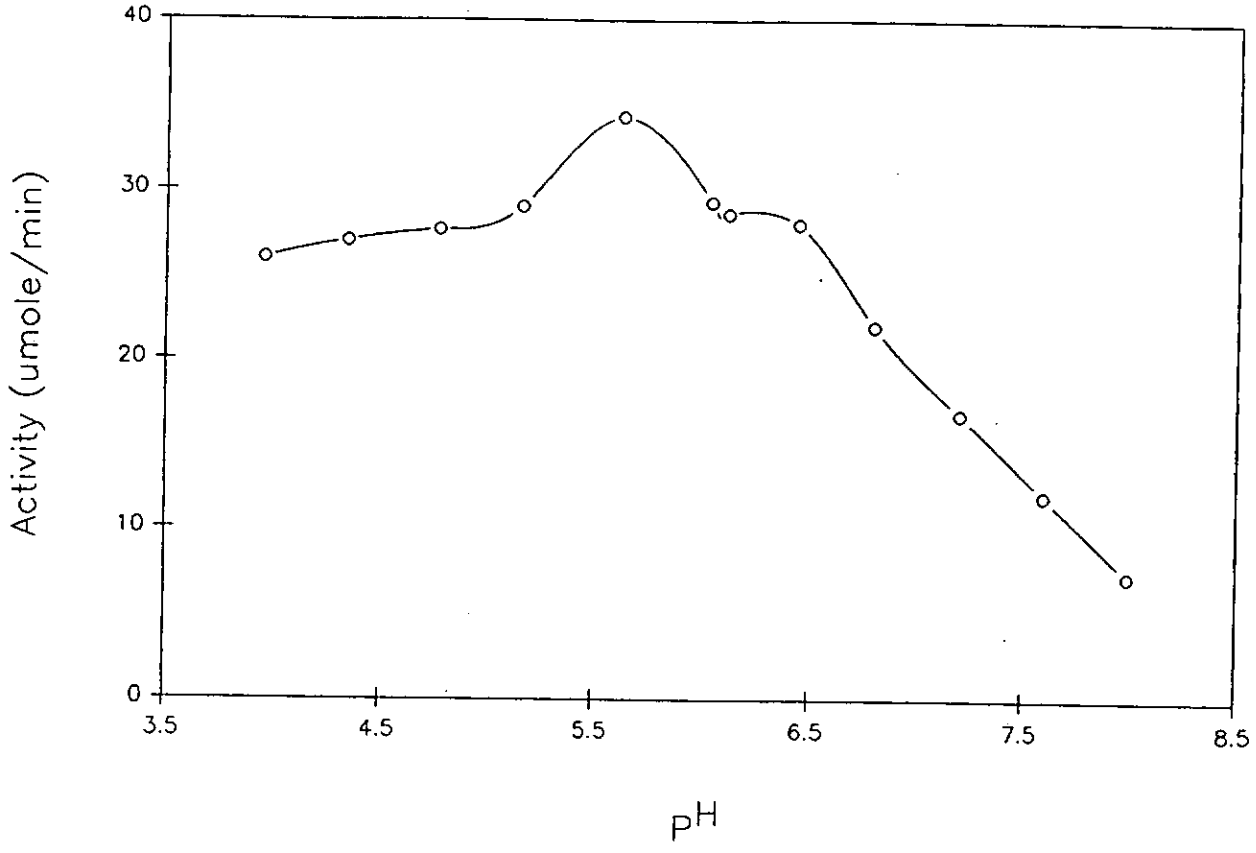
CP - Oesophagus - Crop Tissue Extract

ST - Stomach Tissue Extract

INT - Intestinal Tissue Extract

DG - Digestive Gland Extract

activities, except for amylase (Figure 3.17) and sucrase (Figure 3.16) where at low pH values equal to or less than 4.5, the hydrolytic power was considerably lower. The inhibitory effect of high pH values was more pronounced with sucrose, starch and cellofas B hydrolysing enzyme activities (Figures 3.16, 3.17, 3.20 respectively)


Activity (umole/min)
40
30
20
10
0
3.5
4.5
5.5
6.5
7.5
8.5
pH

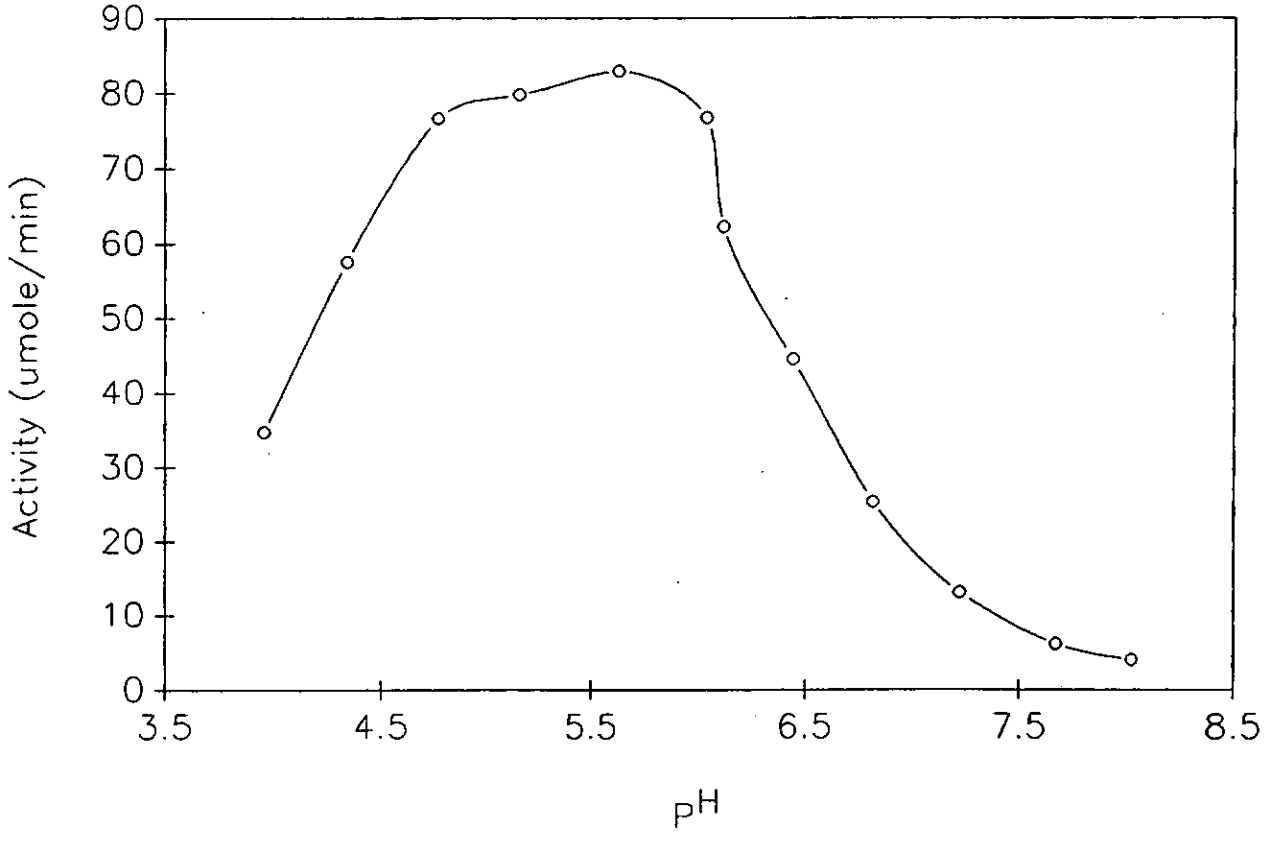
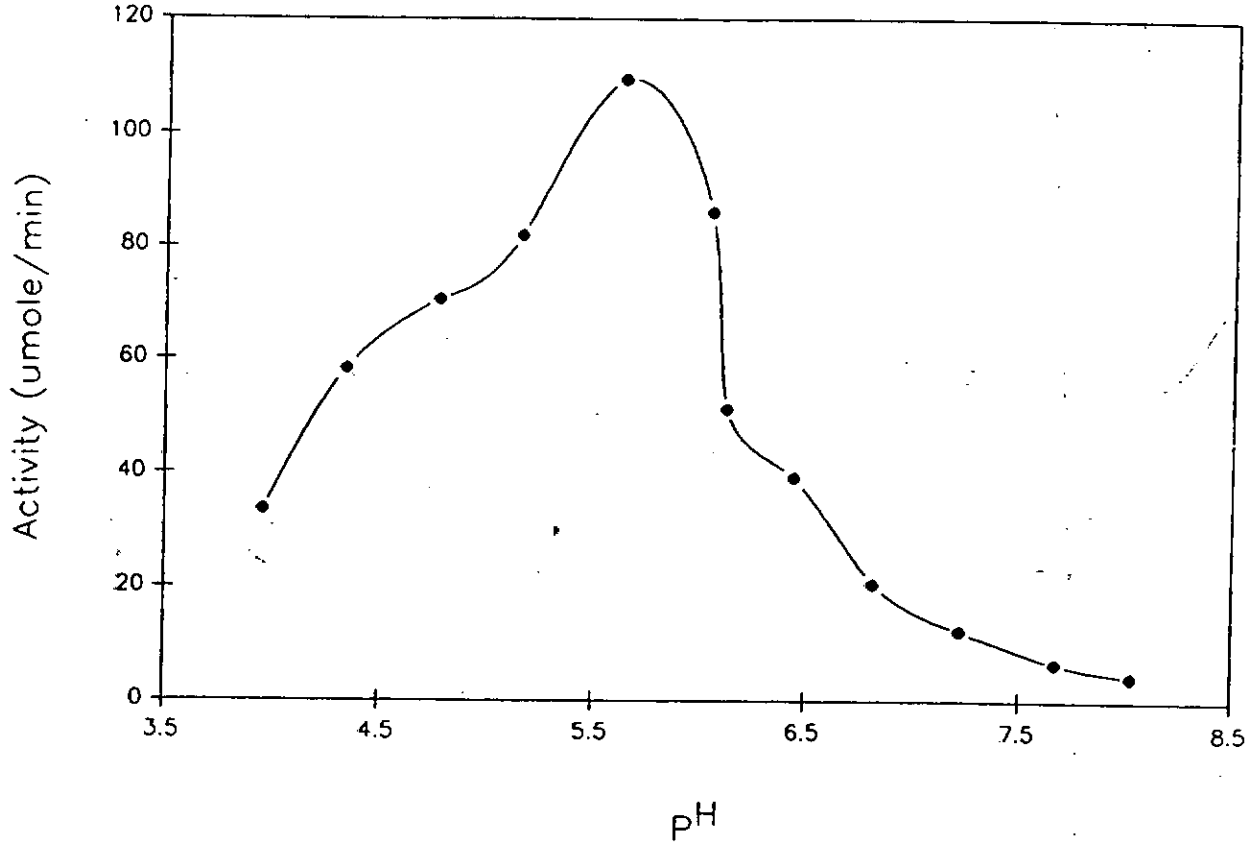

Activity (umole/min)
90
80
70
60
50
40
30
20
10
0
3.5
4.5
5.5
6.5
7.5
8.5
pH

Figure 3.15

pH - Activity curve for the hydrolysis of maltose by the crude crop juice of A. marginata

Figure 3.16

pH - Activity curve for the hydrolysis of sucrose by the curde crope juice of A. marginata


120
100
80
60
40
20
0
Activity (umole/min)
3.5
4.5
5.5
6.5
7.5
8.5
pH

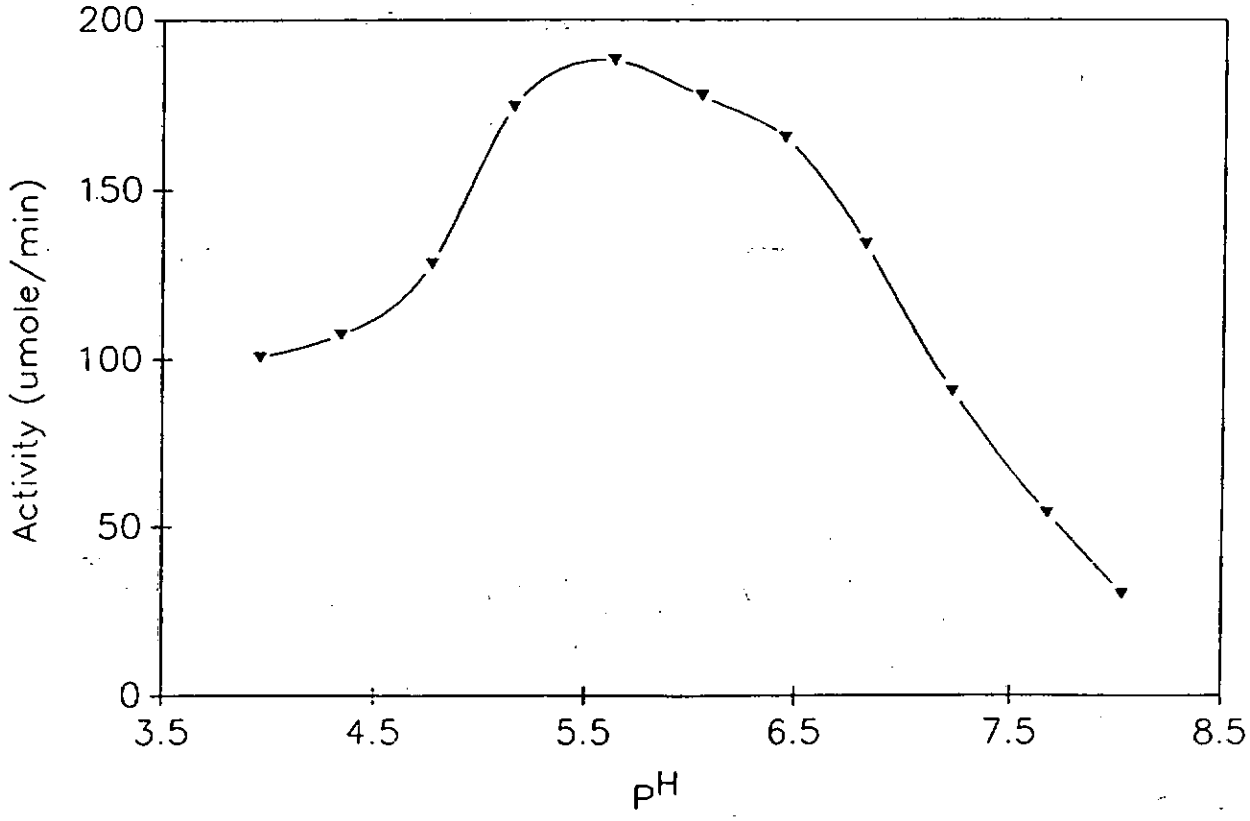
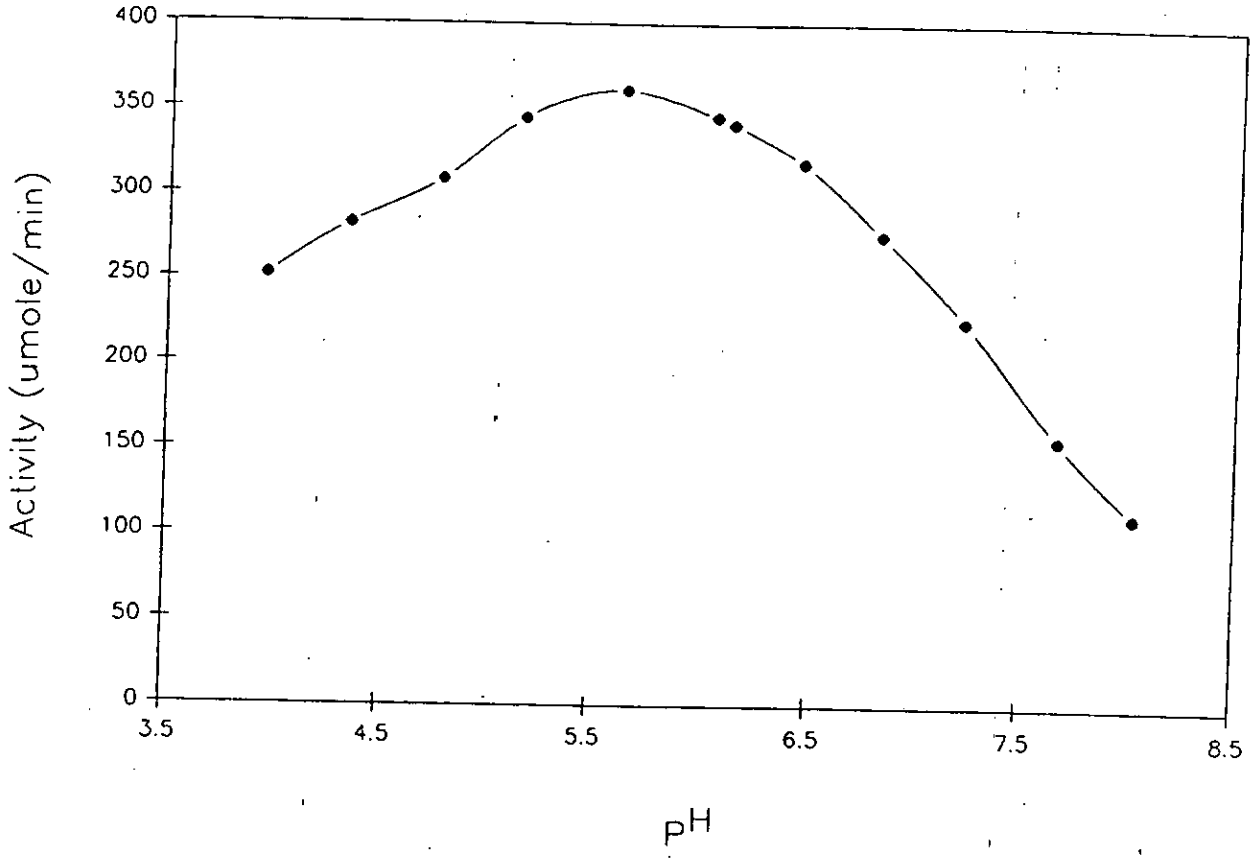

200
150
100
50
0
Activity (umole/min)
3.5
4.5
5.5
6.5
7.5
8.5
pH

Figure 3.17

pH - Activity curve for the hydrolysis of starch by the crude crop juice of A. marginata

Figure 3.18

pH - Activity curve for the hydrolysis of lactose by the crude crop juice of A. marginata


Activity (umole/min)
400
350
300
250
200
150
100
50
0
3.5
4.5
5.5
6.5
7.5
8.5
pH

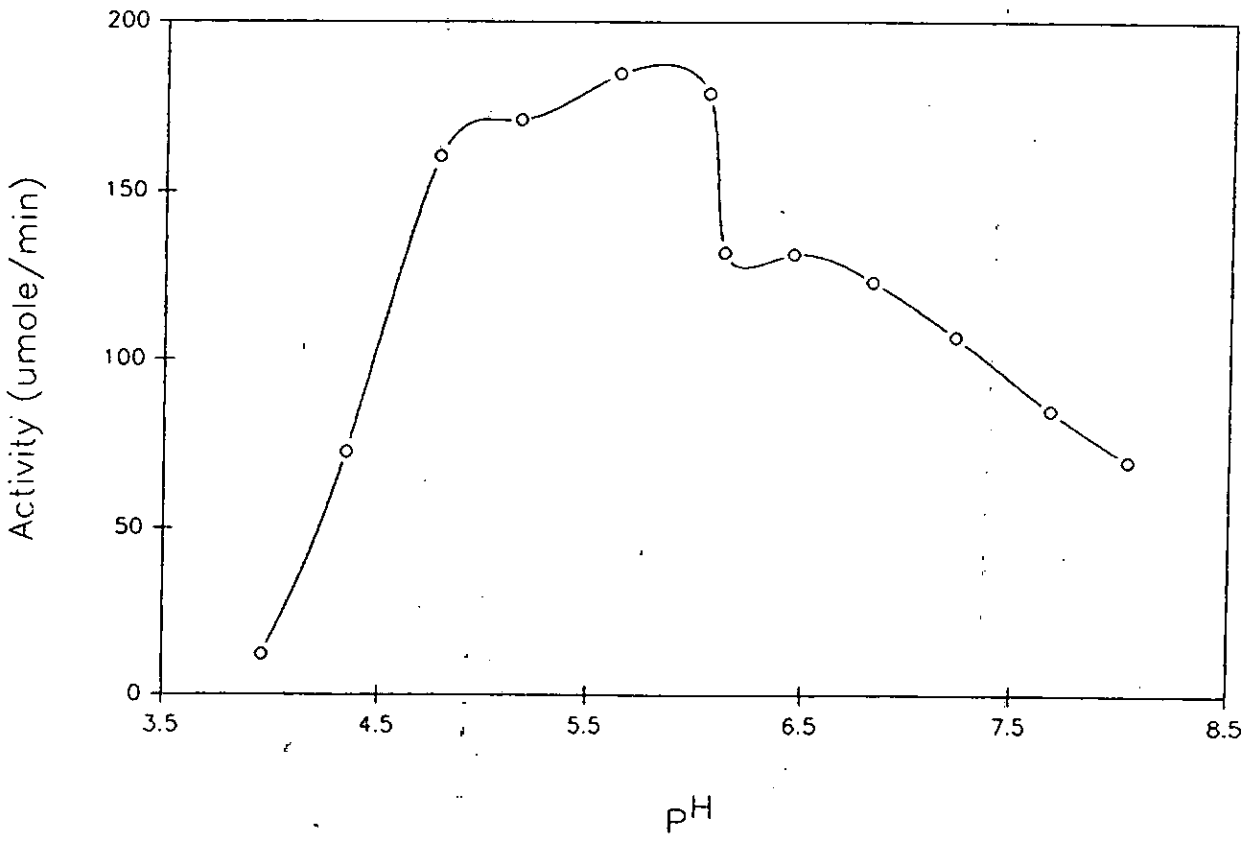

Activity (umole/min)
200
150
100
50
0
3.5
4.5
5.5
6.5
7.5
8.5
pH

Figure 3.19

pH - Activity curve for the hydrolysis of cellobiose by the crude crop juice of A. marginata

Figure 3.20

pH - Activity curve for the hydrolysis of Cellofas B by the crude crop juice of A. marginata

3.6 The Effect of Substrate Concentration on Enzyme Activities

The effect of substrate concentration on enzyme activities varied. Figure 3.21a-3.26b show the effect of substrate concentration on enzyme activities. An analysis of the kinetics of the various enzymes was attempted by examining the closeness of fit of the data to three different equations relating to enzyme behaviour. Use was made of the Michaelis-Menten equation to obtain values for the maximum velocity, (Vmax) value, which represent the velocities of reaction when the enzyme is fully saturated with substrate, and the Km value, which can be calculated from the straight-line plots of 1/v against 1/[s].
This procedure follows from the transformation of the Michaelis-Menten equation to the form:

$$V = \frac{V_{max}}{Km + [S]}$$

$$\frac{1}{v} = \frac{Km}{V_{max}} \cdot \frac{1}{[S]} + \frac{1}{V_{max}}$$

However, some of the data on the effect of substrate concentration on the rate of activity did not fit into this equation. Thus, based on the shape of the (curve formed) graph, alternative equations were used. Only the sets of data from the hydrolysis of sucrose, cellobiose and starch gave reasonable fits into the

simple Michaelis-Menten equation (using the Multifit computer programme) as show in Figures 3.21b, 3.22b and 3.23b.

Figure 3.24a shows the curve obtained when the enzyme activity values were plotted against a range of maltose concentration. This curve shows there is inhibition of the enzyme activity by the substrate. The substrate inhibition equation was used to calculate the maximum velocity and the inhibition constant.

This equation states that:-

$$v = \frac{V_{max}}{1 + \frac{Km}{S} + \frac{S}{Ki}}$$

Considering all the variables used, a reasonable fit into the equation was obtained. The values of Vmax, Km, and Ki respectively are shown in table 3.6. When the double reciprocal plot of reciprocal rate (1/v) against reciprocal substrate concentration (1/[s]) was plotted the curve was shaped in such a way that it turns upwards (Fig. 3.24b) which is characteristic of high substrate inhibition plots.

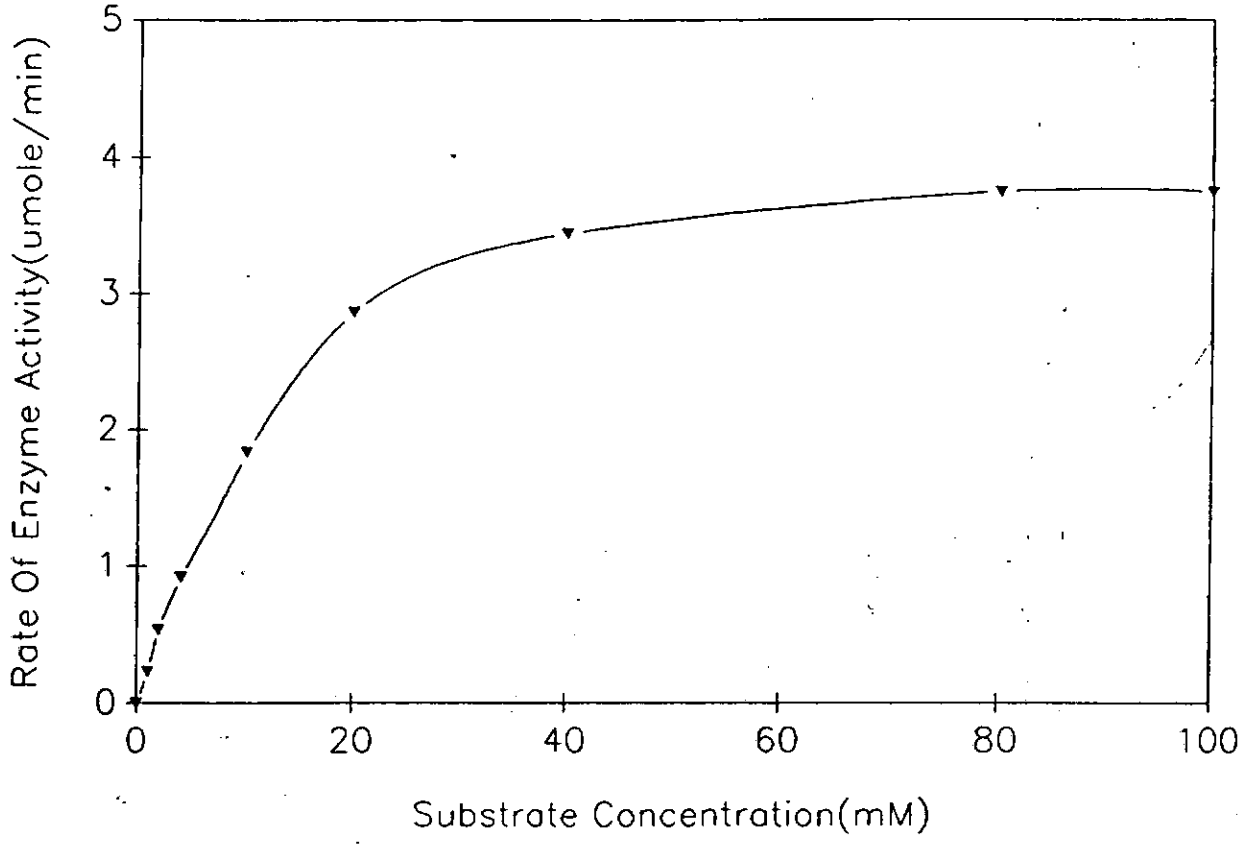
The double reciprocal plot (Figure 3.25b) of lactose hydrolysis data did not fit the simple Michaelis Menten

equation, neither did it fit the high substrate inhibition equation. But from the shape of the graph, there are 3 possible explanations:-

First, there could be two enzymes present, in which case one has a high Km and Vmax, and the other has a low Km and Vmax.

Second, there is a non enzymic background hydrolysis. However, because of the control being subtracted from the test each time the experiment was performed, the latter is most unlikely.

The third explanation could be that of a negative co-operativity, in which case the enzyme has two or more sub-units.


Rate Of Enzyme Activity(umole/min)
0
1
2
3
4
5
0
20
40
60
80
100
Substrate Concentration(mM)

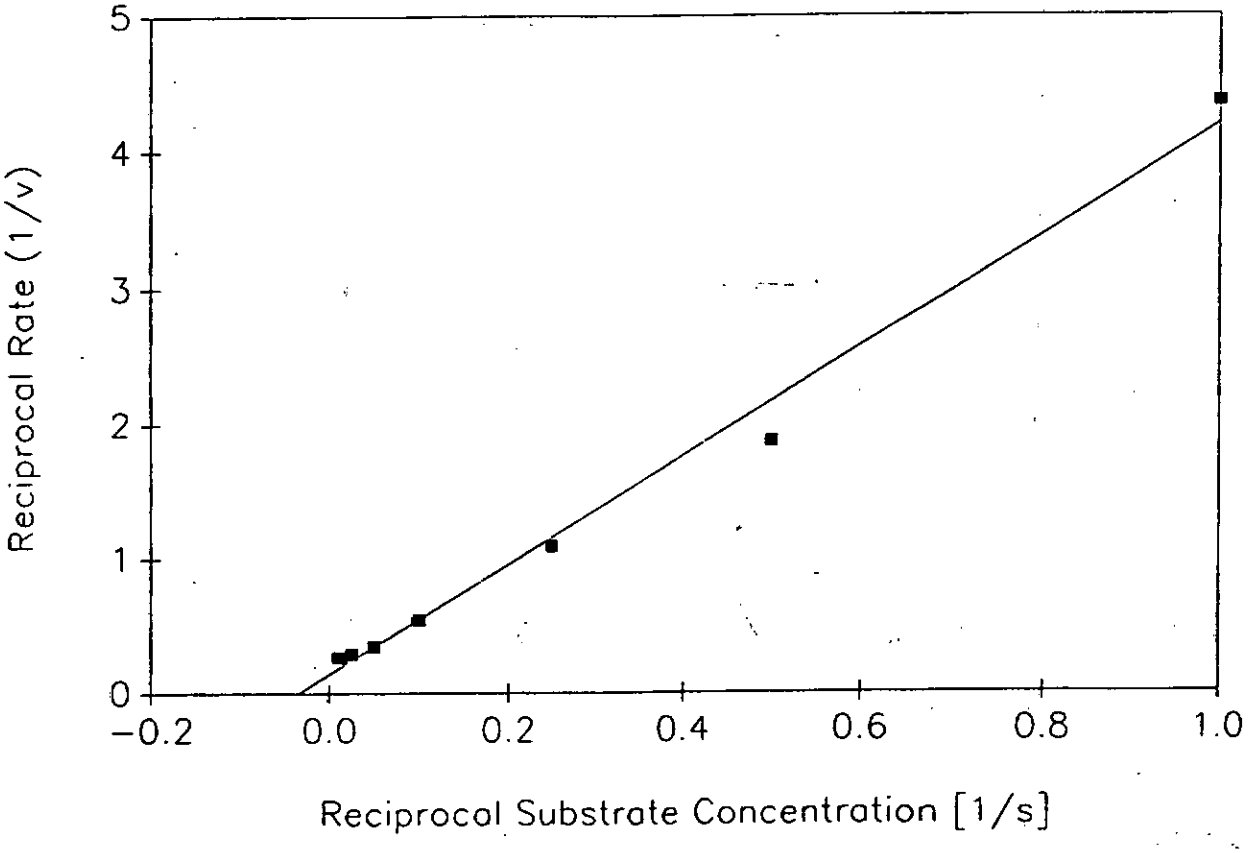
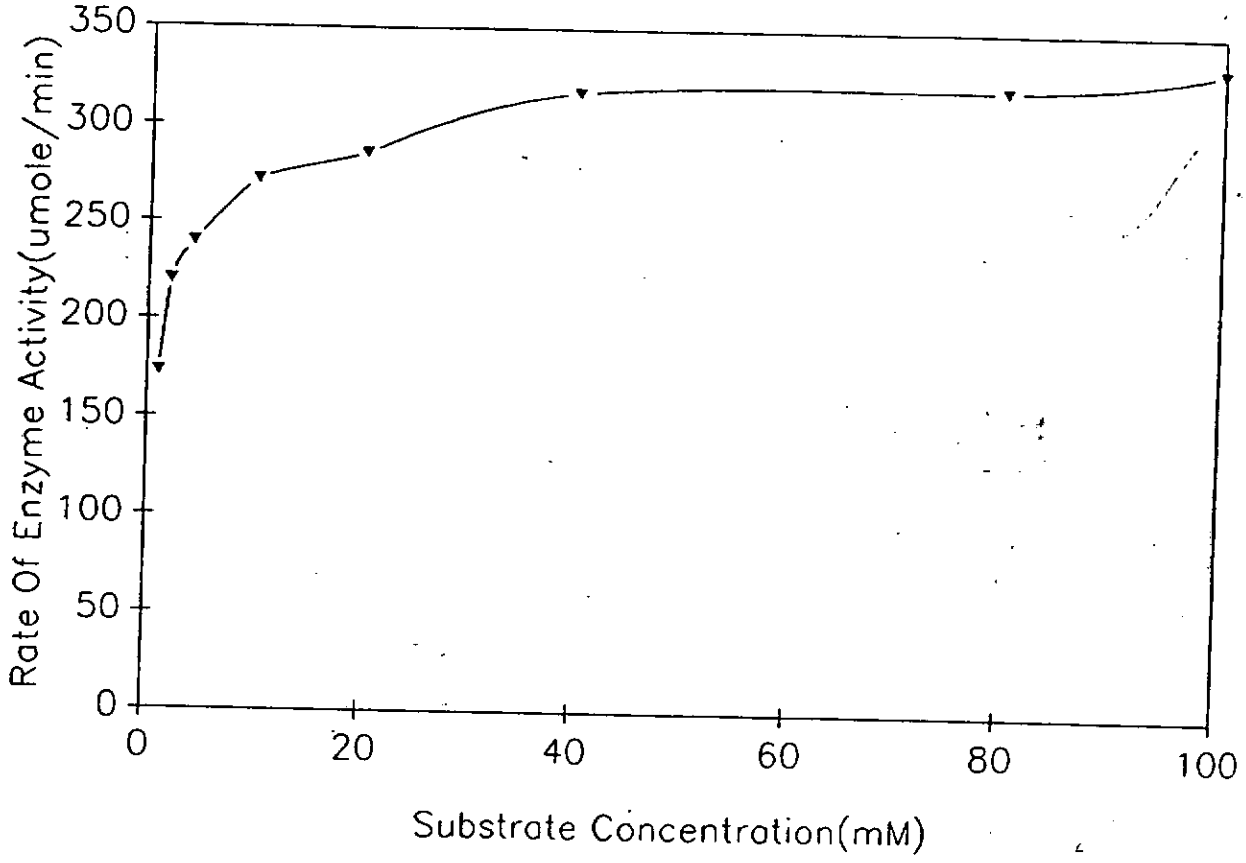

Reciprocal Rate (1/v)
0
1
2
3
4
5
−0.2
0.0
0.2
0.4
0.6
0.8
1.0
Reciprocal Substrate Concentration [1/s]

Figure 3.21a

Dependence of rate (μmole min^{-1} g^{-1}) on substrate concentration (mM) for sucrase activity

Figure 3.21b

Double reciprocal plot of reciprocal rate (μmole min^{-1} g^{-1}) against reciprocal substrate concentration (mM) for sucrase activity


Rate Of Enzyme Activity(umole/min)
350
300
250
200
150
100
50
0
0
20
40
60
80
100
Substrate Concentration(mM)

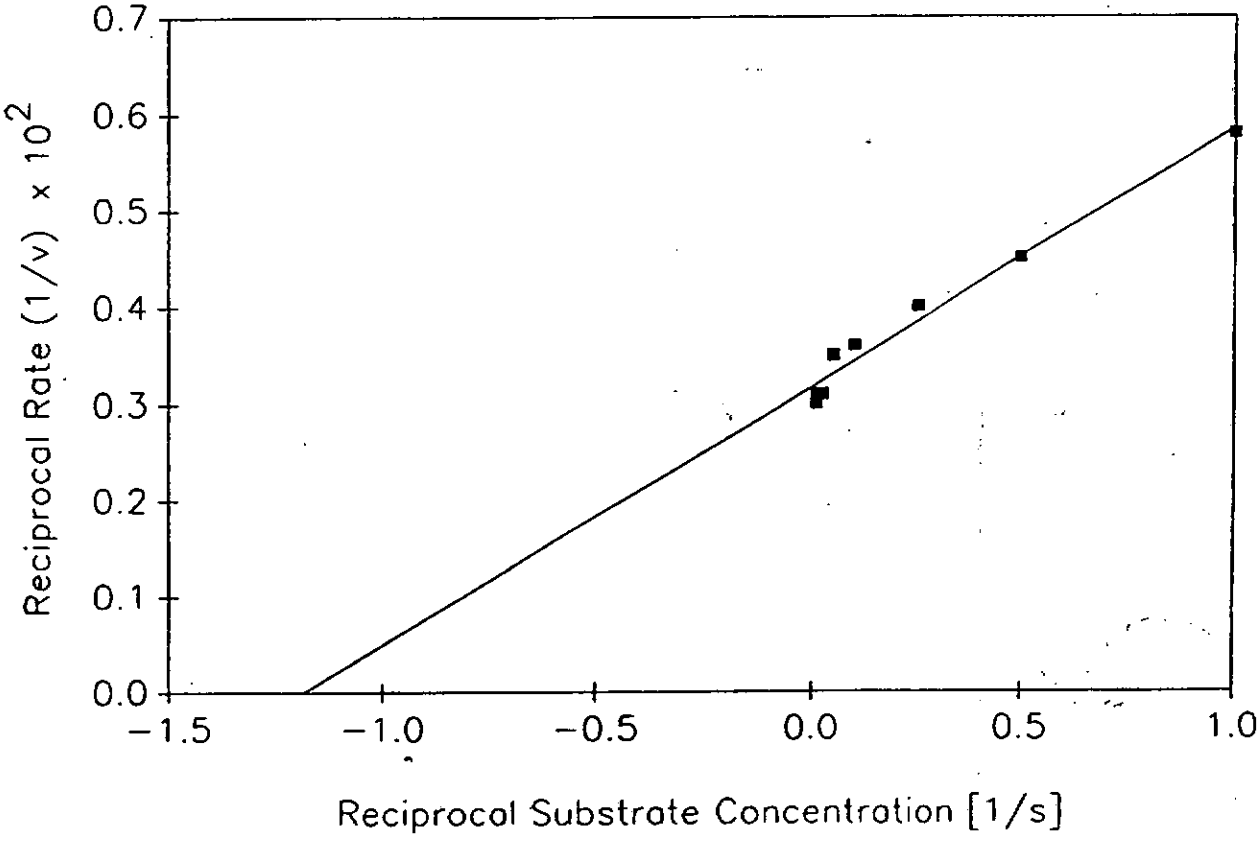
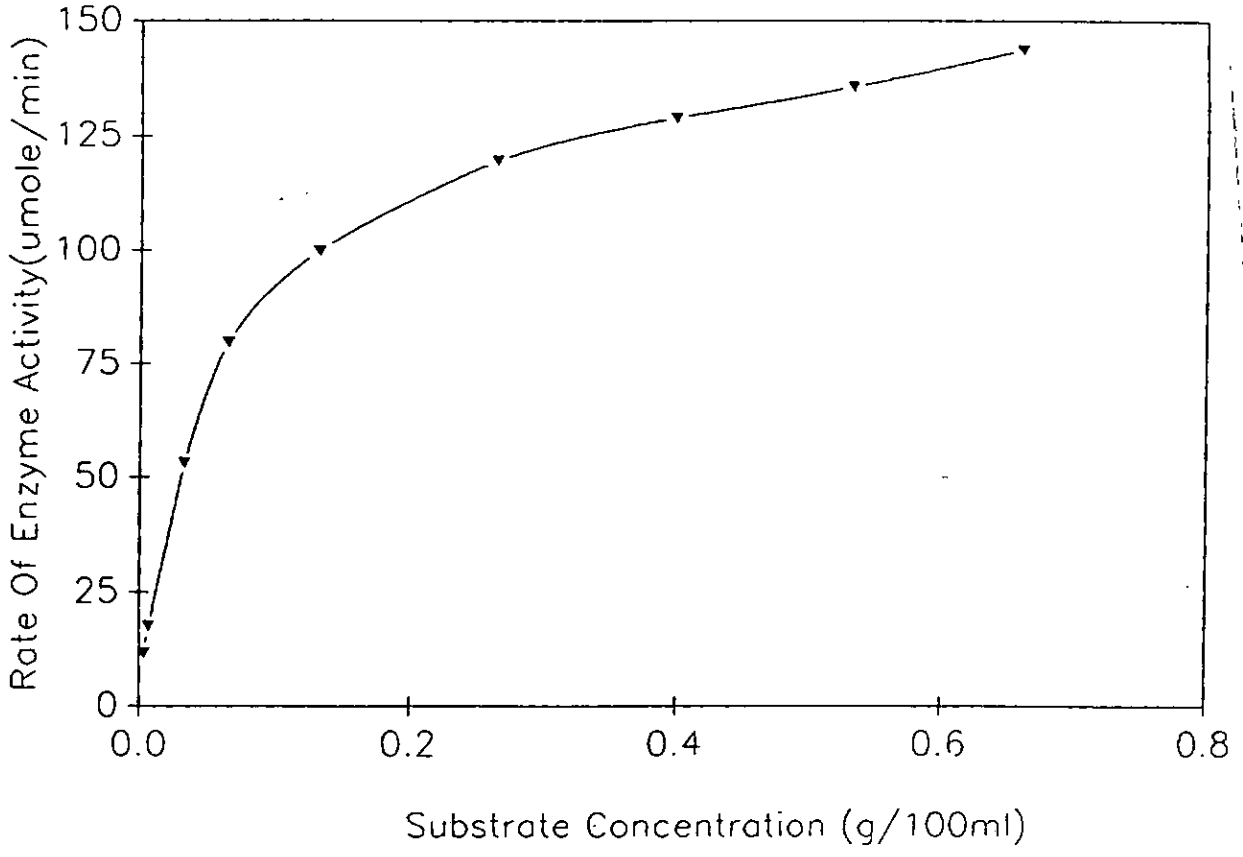

Reciprocal Rate (1/v) x 10^2
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
−1.5
−1.0
−0.5
0.0
0.5
1.0
Reciprocal Substrate Concentration [1/s]

Figure 3.22a

Dependence of rate
(μmole min^{-1} g^{-1} tissue) on substrate
concentration (mM) for cellobiase activity

Figure 3.22b

Double reciprocal plot
of reciprocal rate (μmole min^{-1} g^{-1})
against reciprocal substrate concentration
(mM) for cellobiase activity


Rate Of Enzyme Activity(umole/min)
150
125
100
75
50
25
0
0.0
0.2
0.4
0.6
0.8
Substrate Concentration (g/100ml)

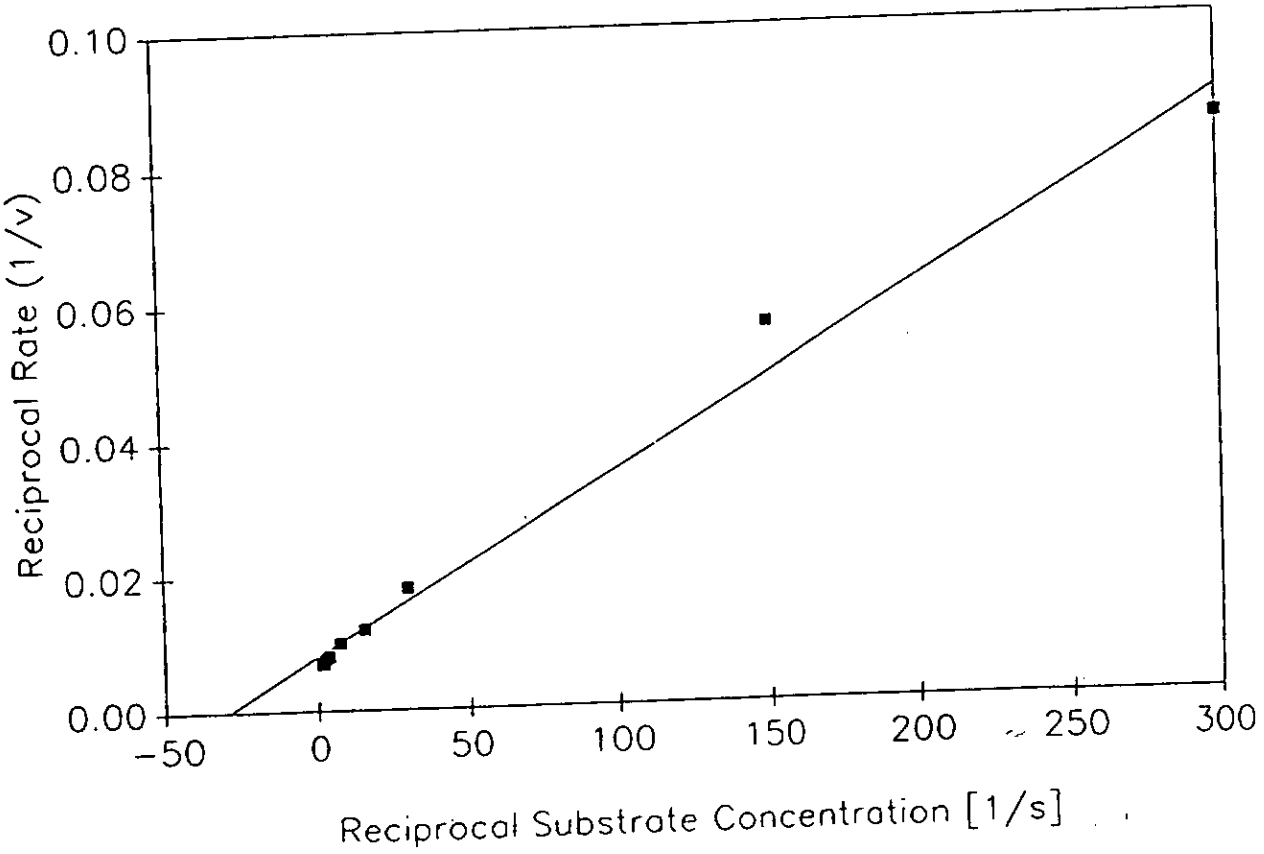
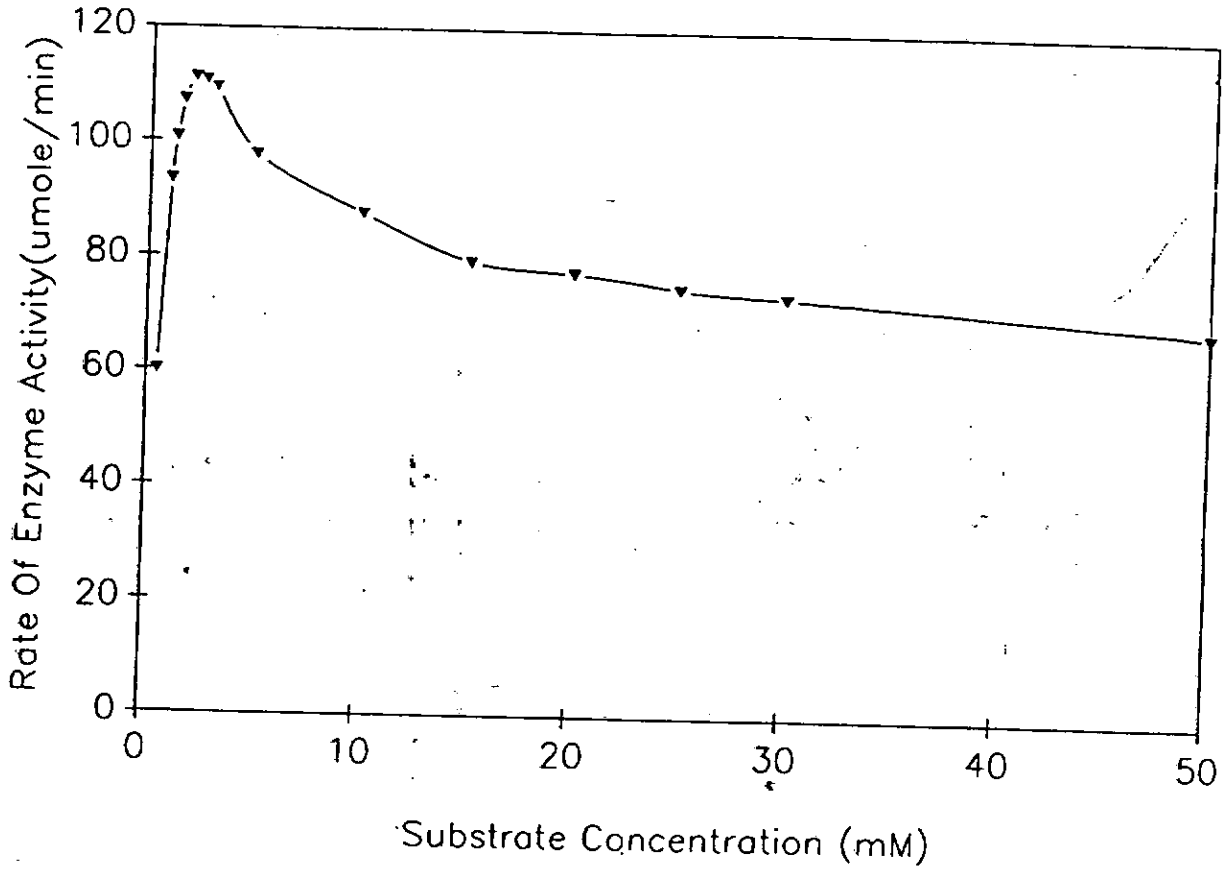

Reciprocal Rate (1/v)
0.10
0.08
0.06
0.04
0.02
0.00
-50
0
50
100
150
200
250
300
Reciprocal Substrate Concentration [1/s]

Figure 3.23a

Dependence of rate
(μmole min^{-1} g^{-1} tissue) on substrate
concentration (g/100ml) for amylase activity

Figure 3.23b

Double Reciprocal Plot of
Reciprocal Rate (μmole min^{-1} g^{-1})
Against Reciprocal Substrate
Concentration (g/100ml) for
Amylase Activity


Rate Of Enzyme Activity(umole/min)
120
100
80
60
40
20
0
0
10
20
30
40
50
Substrate Concentration (mM)

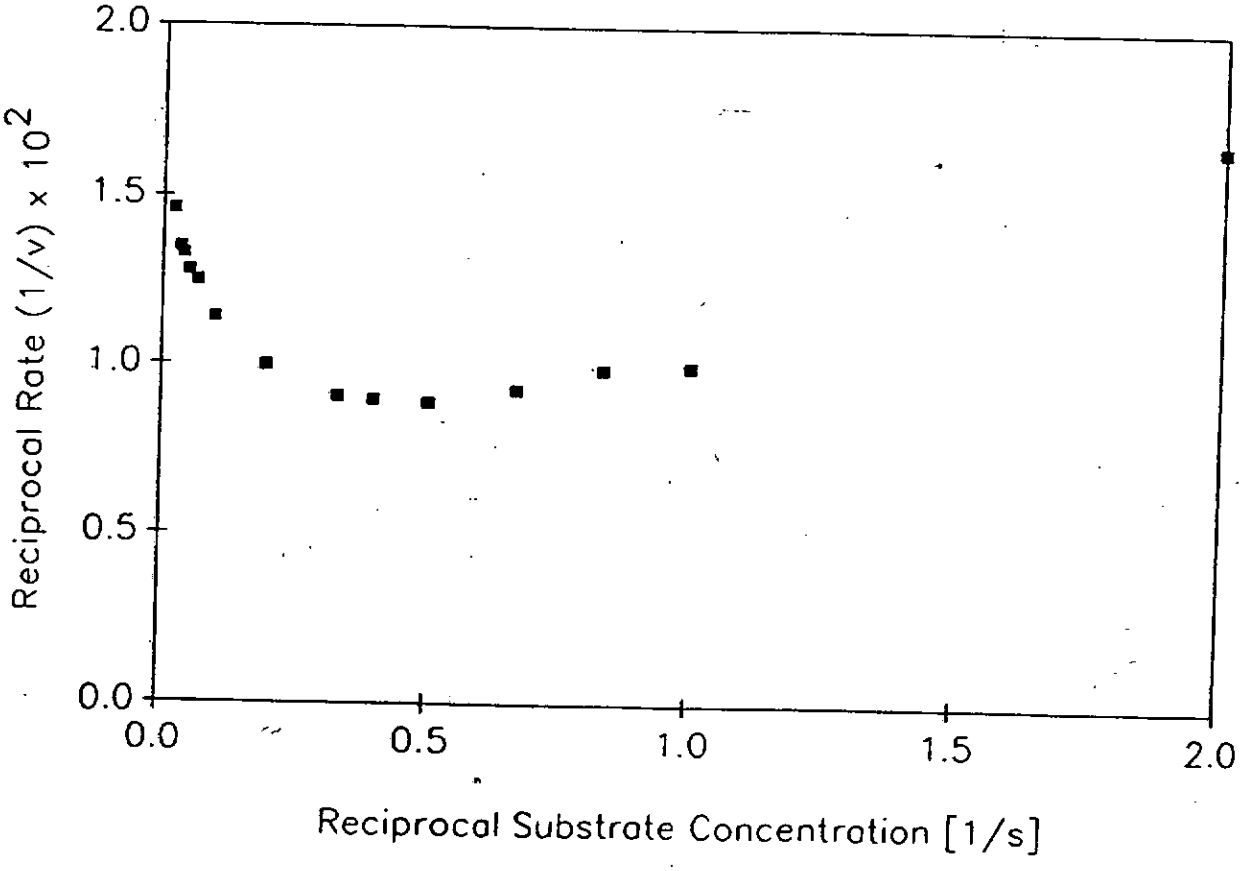

Reciprocal Rate (1/v) x 10^2
2.0
1.5
1.0
0.5
0.0
0.0
0.5
1.0
1.5
2.0
Reciprocal Substrate Concentration [1/s]

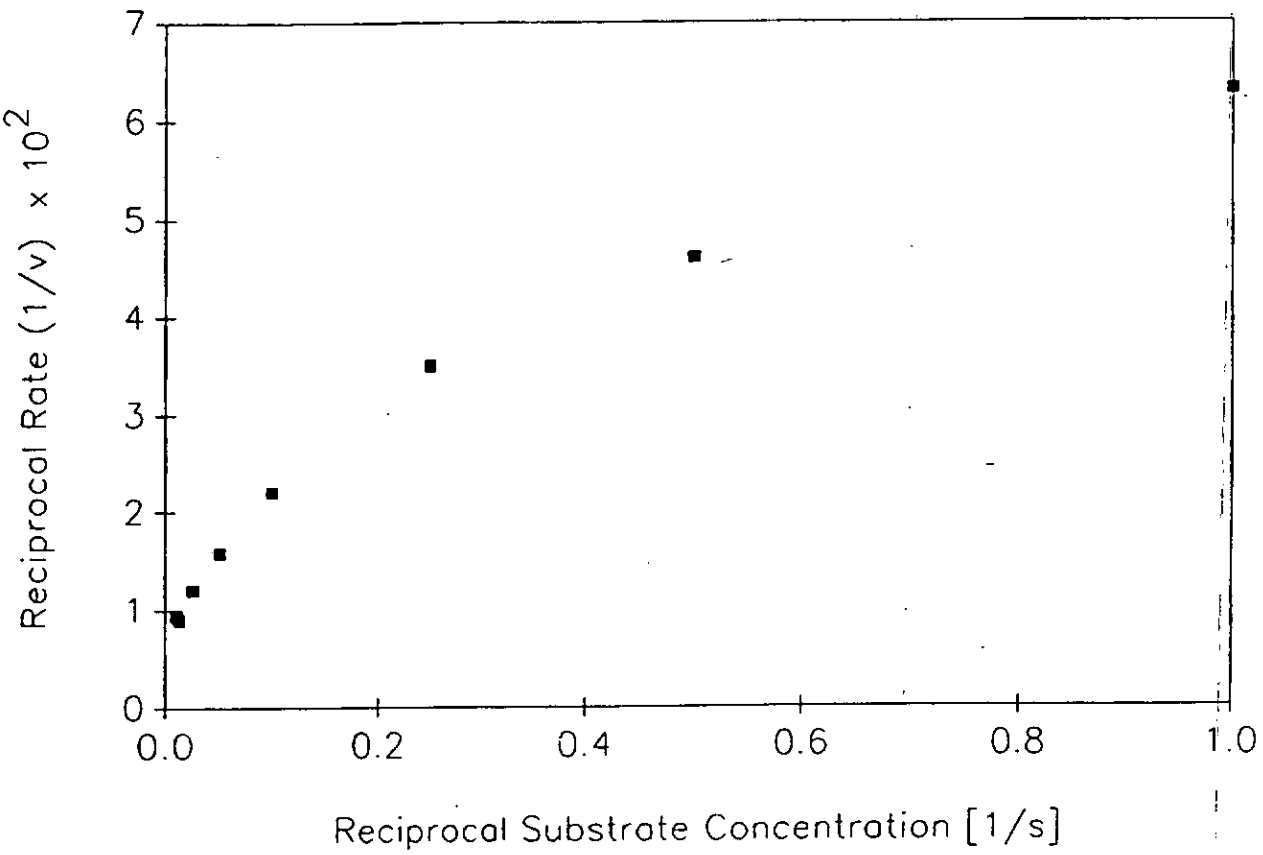
Figure 3.24a

Dependence of rate
(μmole min^{-1} g^{-1} tissue) on substrate
concentration (mM) for maltase activity

Figure 3.24b

Double reciprocal plot of
reciprocal rate (μmole min^{-1} g^{-1})
against reciprocal substrate concentration
(mM) for maltase activity

b


Reciprocal Rate (1/v) x 10^2
0
1
2
3
4
5
6
7
0.0
0.2
0.4
0.6
0.8
1.0
Reciprocal Substrate Concentration [1/s]

a

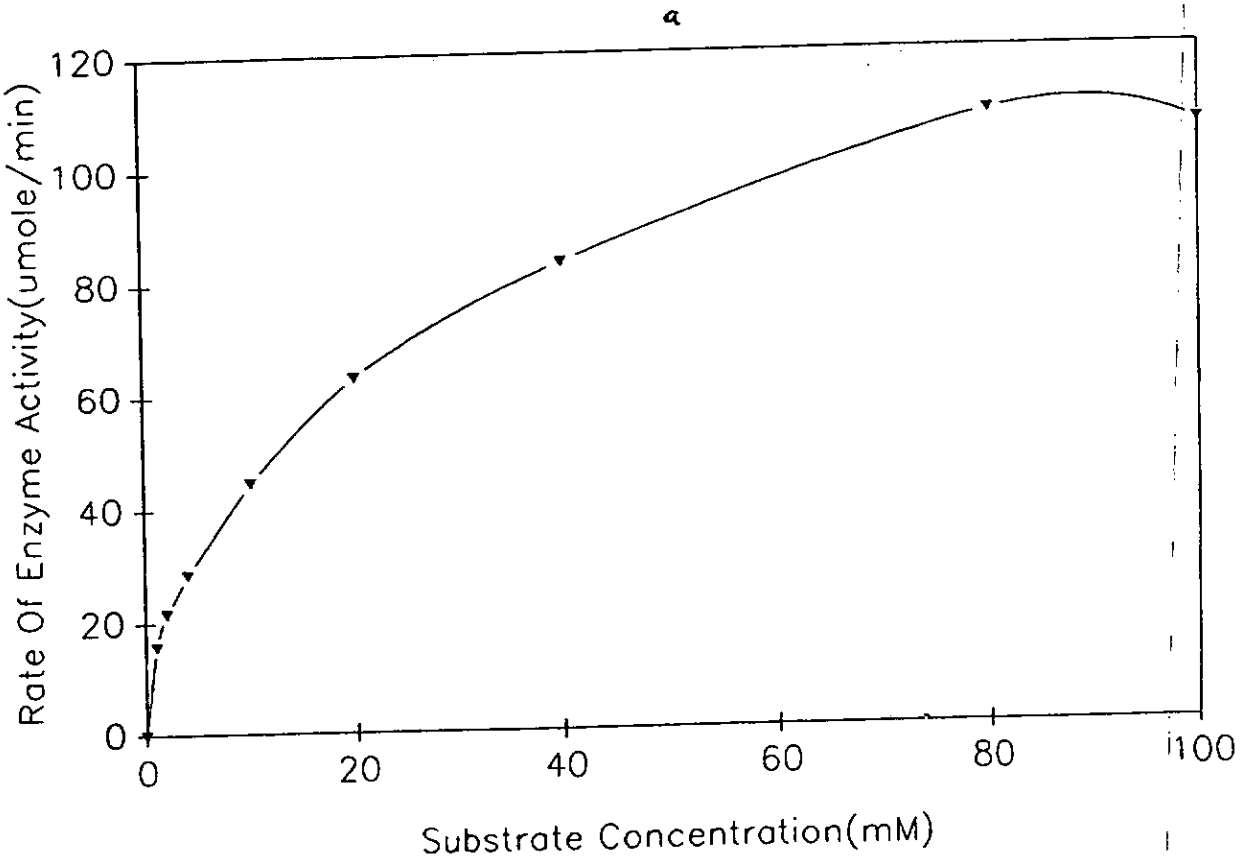
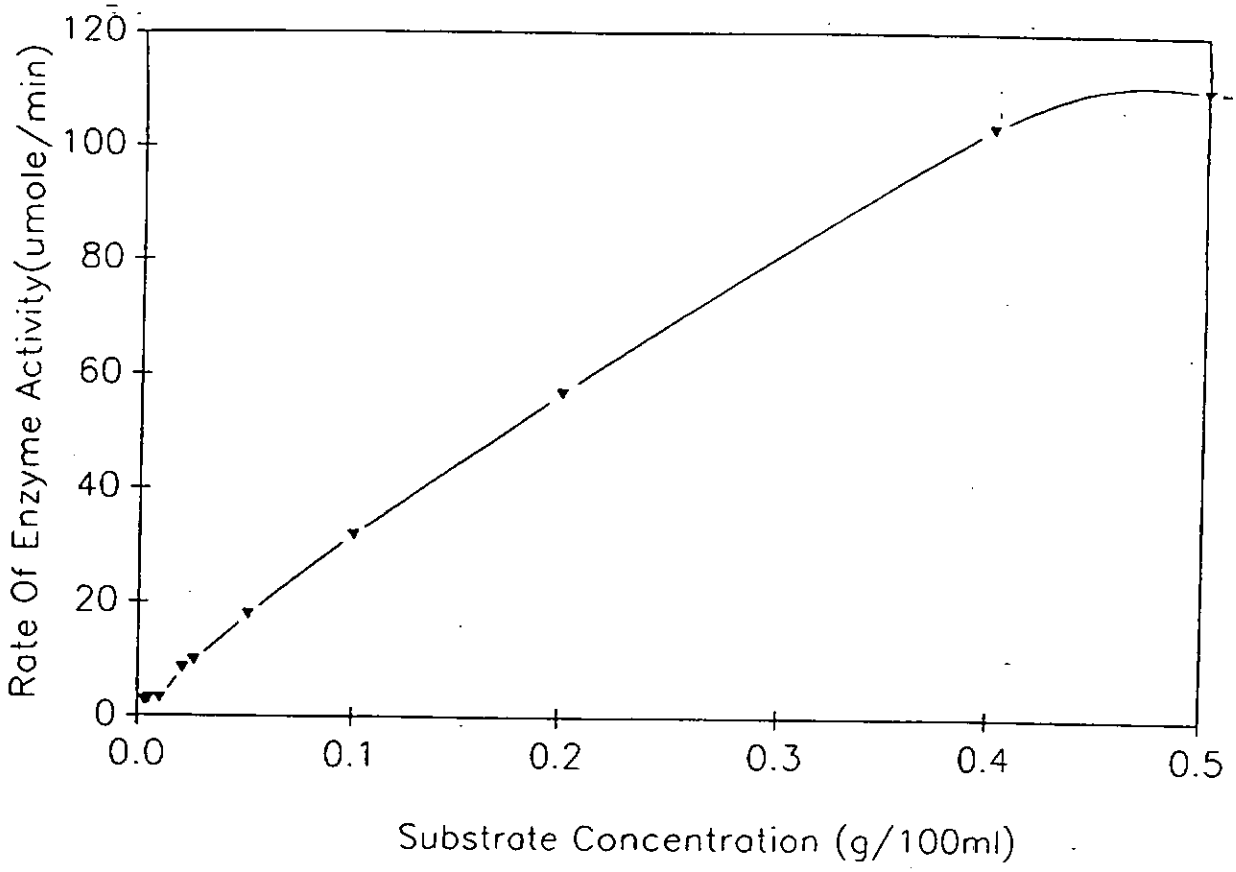

Rate Of Enzyme Activity(umole/min)
0
20
40
60
80
100
120
0
20
40
60
80
100
Substrate Concentration(mM)

Figure 3.25a

Dependence of rate
(μmole min^{-1} g^{-1} tissue) on
substrate concentration (mM) for
lactase activity

Figure 3.25b

Double reciprocal plot of
reciprocal rate (μmole min^{-1} g^{-1})
against reciprocal substrate concentration
(mM) for lactase activity


Rate Of Enzyme Activity(umole/min)
120
100
80
60
40
20
0
0.0
0.1
0.2
0.3
0.4
0.5
Substrate Concentration (g/100ml)

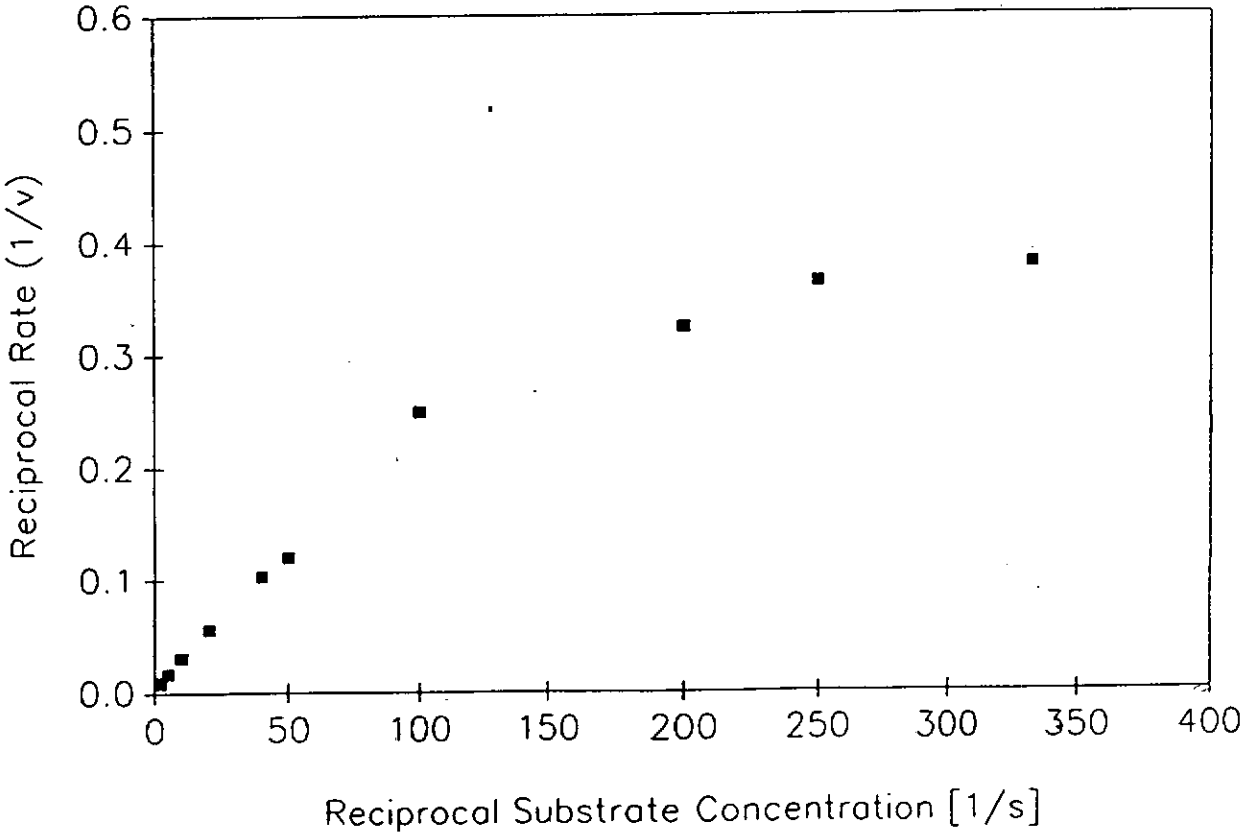

Reciprocal Rate (1/v)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
50
100
150
200
250
300
350
400
Reciprocal Substrate Concentration [1/s]

Figure 3.26a

Dependence of rate
(μmole min^{-1} g^{-1} tissue) on substrate
concentration (g/100ml) for
cellulase activity.

Figure 3.26b

Double reciprocal plot of
reciprocal rate (μmole min^{-1} g^{-1})
against reciprocal substrate concentration
(g/100ml) for cellulase acivity.

Table 3.6 Comparison of the Effect of Substrate Concentration on the Activity of the Crude Crop Juice of A. marginata

	Equation Used	Km(mM)	VMax umole min^{-1}	Ki(mM)
Cellobiose	Michaelis-Menten equation	0.86 ±0.1	311 ±9.2	
Lactose	Two-enzyme equation(1)	34.98 ±6.5	18.0 ±3	
	(2)	0.45 ±0.2	124 ±6.3	
Maltose	Substrate Inhibition equation	0.4 ±0.1	125.0. ±11.7	41.0 ±11.8
Amylase	Alichaelis-Mention equation	6.46 ± 0.1	453.32 ±26.8	-
Sucrose	Michaelis-Mention equation	17.07 ±1.9	4.712 ± 0.3	-

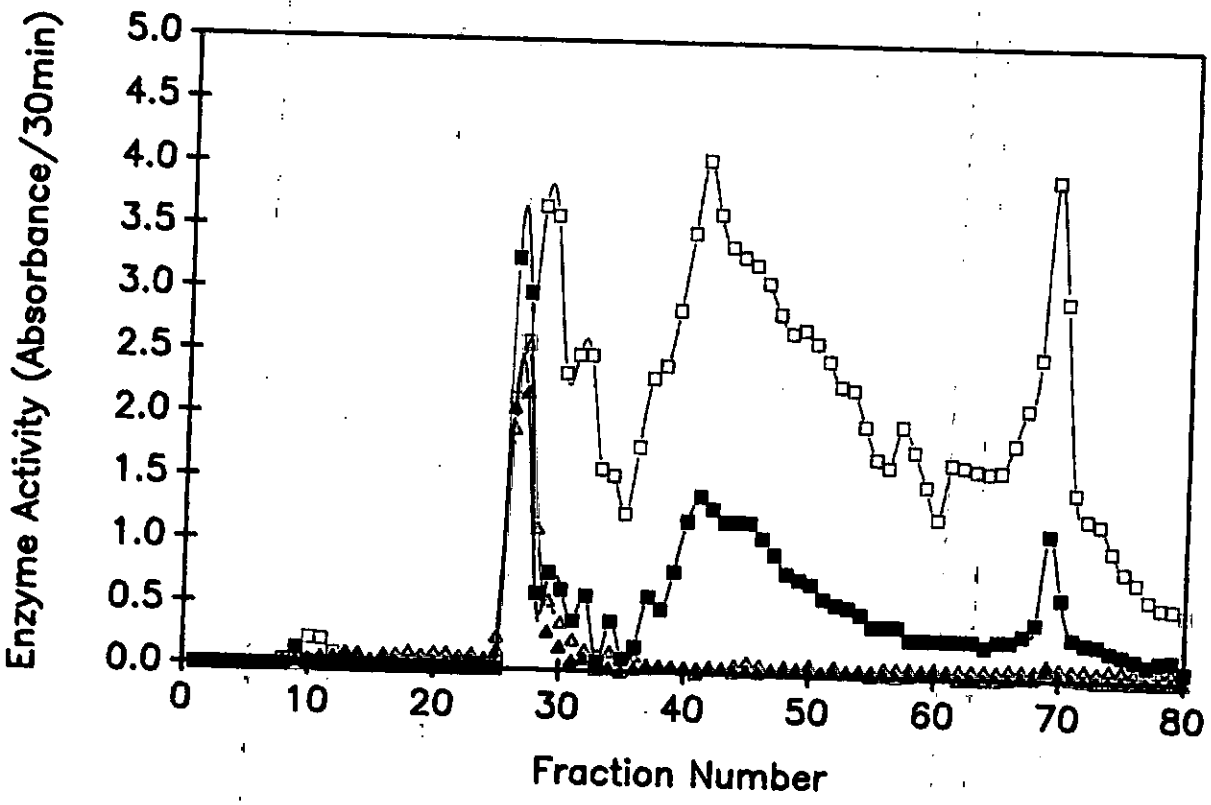
3.7 Separation of the Carbohydrases of the crop Juice of A. marginata

An attempt was made to separate the different types of enzyme present in the crop juice. Different separation methods were employed, based on differences in the size, net charge, hydrophobicity and the possession of specific binding sites of the enzyme proteins. Fractions were analysed by gel electrophoresis as well as enzyme assay.

3.7.1 Separation of Crude Crop Juice using Ion Exchange Chromatography on a DE-52 Column

When the crude crop juice preparation was applied to a DEAE-cellulose column, a significant amount of protein was eluted with the 10mM phosphate buffer pH6.8 wash (Figure 3.27a; fractions 8-14). Protein and enzyme activity was then eluted almost in a single peak contained in fractions 26-29, very early in the linear gradient of 10mM - 200mM phosphate buffer pH6.8. A third major protein peak in the elution profile was obtained when 100ml of 500mM phosphate buffer pH6.8 was added to the column as the final wash.

All the disaccharidases were initially bound to the column as no significant activity was detected in fractions 8-14 (Figure 3.27b) corresponding to the wash through. All the fractions from tubes 26-29 significantly hydrolysed sucrose, maltose, lactose,


Enzyme Activity (Absorbance/30min)
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
10
20
30
40
50
60
70
80
Fraction Number

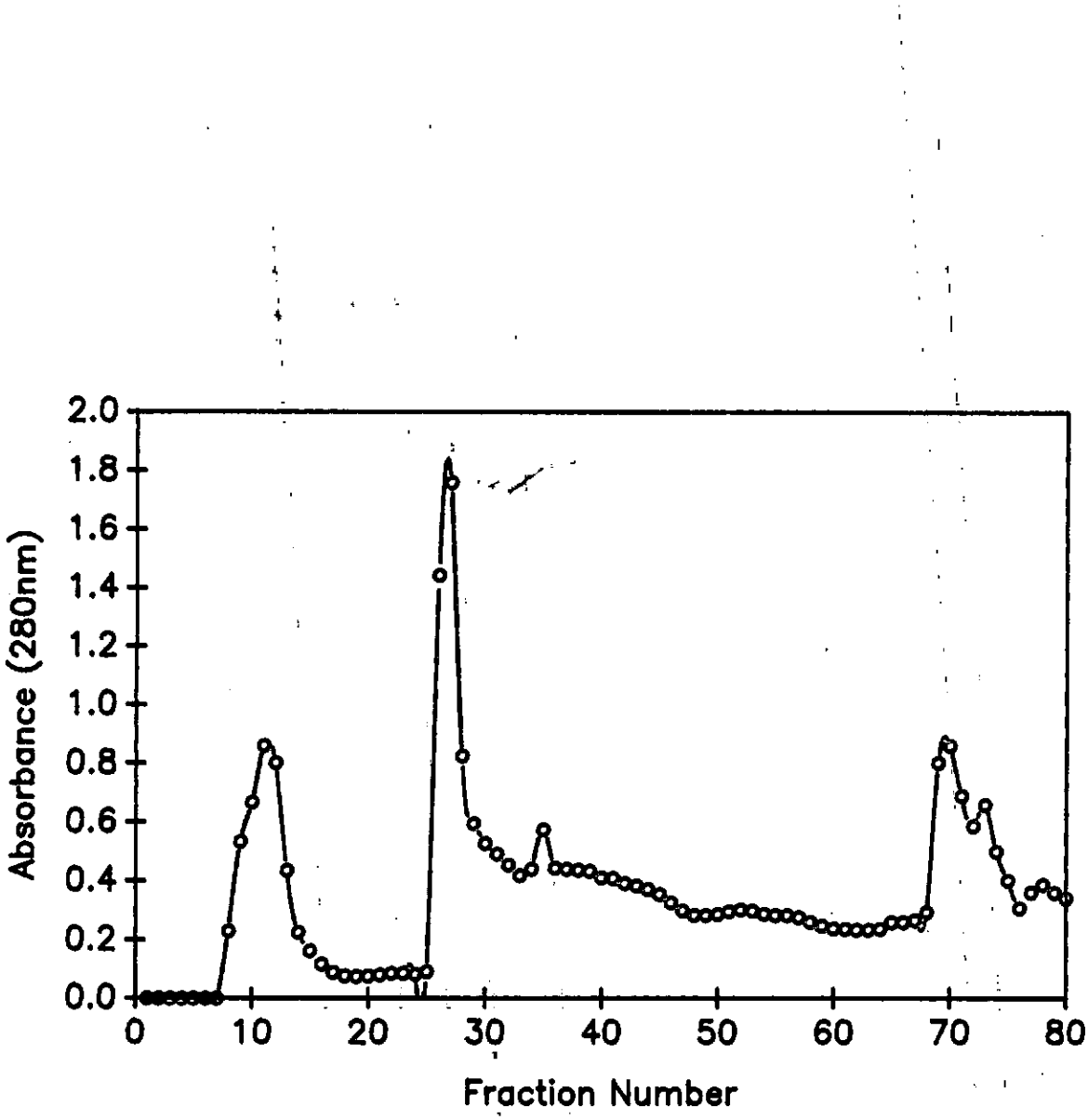

Absorbance (280nm)
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
40
50
60
70
80
Fraction Number

Figure 3.27a

Distribution of protein in the DE-52 column elution profile of crude crop juice by measurement of absorbance at 280nm

Protein (280nm) -o-

Figure 3.27b

Distribution of enzyme activity in the DE-52 column elution profile of crude crop juice

Key :

Sucrase activity -▲-

Maltase activity -△-

Lactase activity -●-

Cellobiase activity -□-

cellobiose, starch and cellofas B as shown in Figure 3.27b and Figure 3.28.

For the disaccharidases, sucrase and maltase eluted together as a very distinct peak early in the gradient. This peak also contained lactase activity, but both the lactase and cellobiase activities were distributed throughout the linear gradient of 10mM-200mM phosphase buffer pH6.8 in two major peaks as shown in Figure 3.1b. A third peak of lactase and cellobiase activities was eluted with the 500mM wash (Figure 3.27b; fraction 68-75).

Thus, the elution profile of the disaccharidases showed that there was a very clear difference between the distribution of maltase and sucrase, and that of lactase and cellobiase. The distribution of cellobiase was very similar to that of lactase, except that the first peak of cellobiase activity was slightly retarded on the column than that of lactase indicating that these two enzyme activities are not completely identical.

Cellulase activity showed a similar distribution to that of lactase and cellobiase activities (Figure 3.28) coming out in a series of peaks, except that significant activity was recovered in the wash fractions.

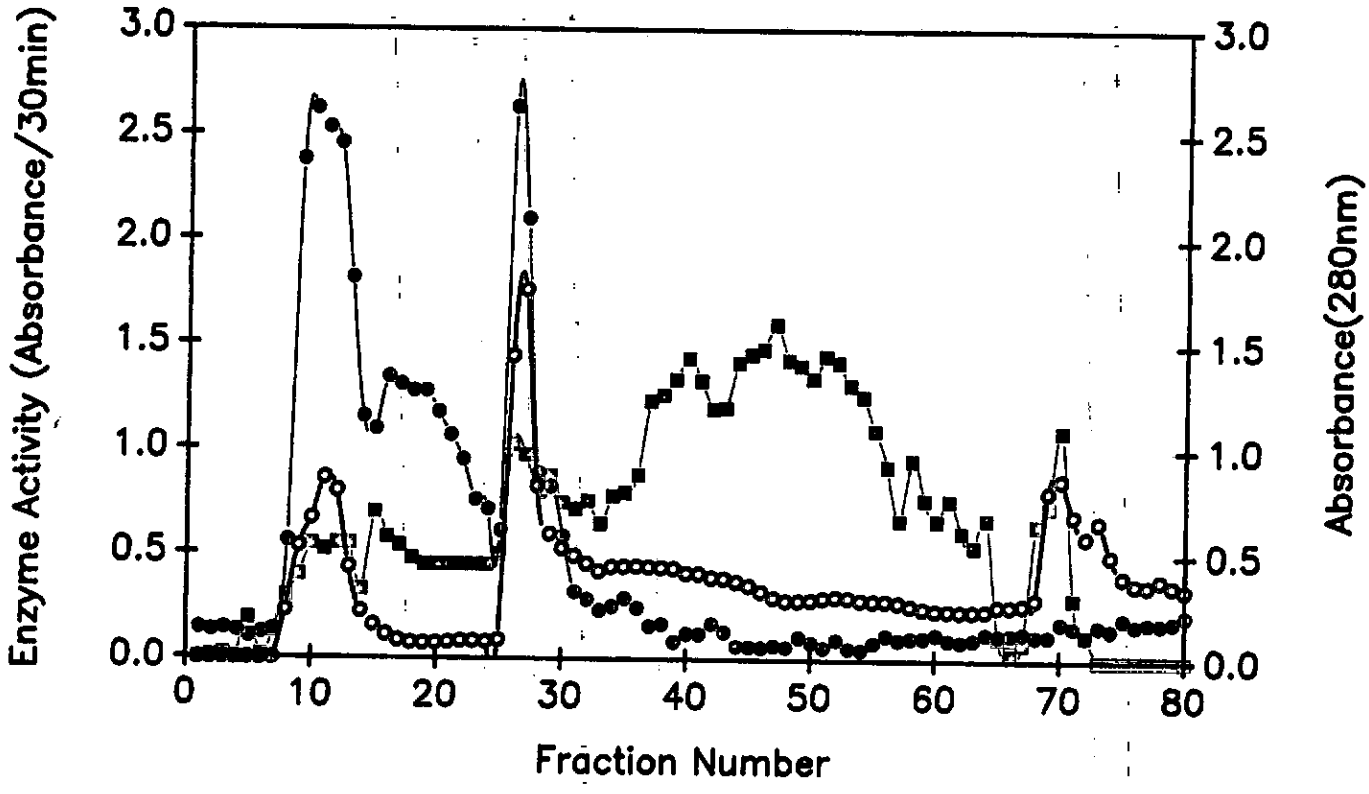

Enzyme Activity (Absorbance/30min)
Absorbance(280nm)
Fraction Number
0.0
0.5
1.0
1.5
2.0
2.5
3.0
0
10
20
30
40
50
60
70
80

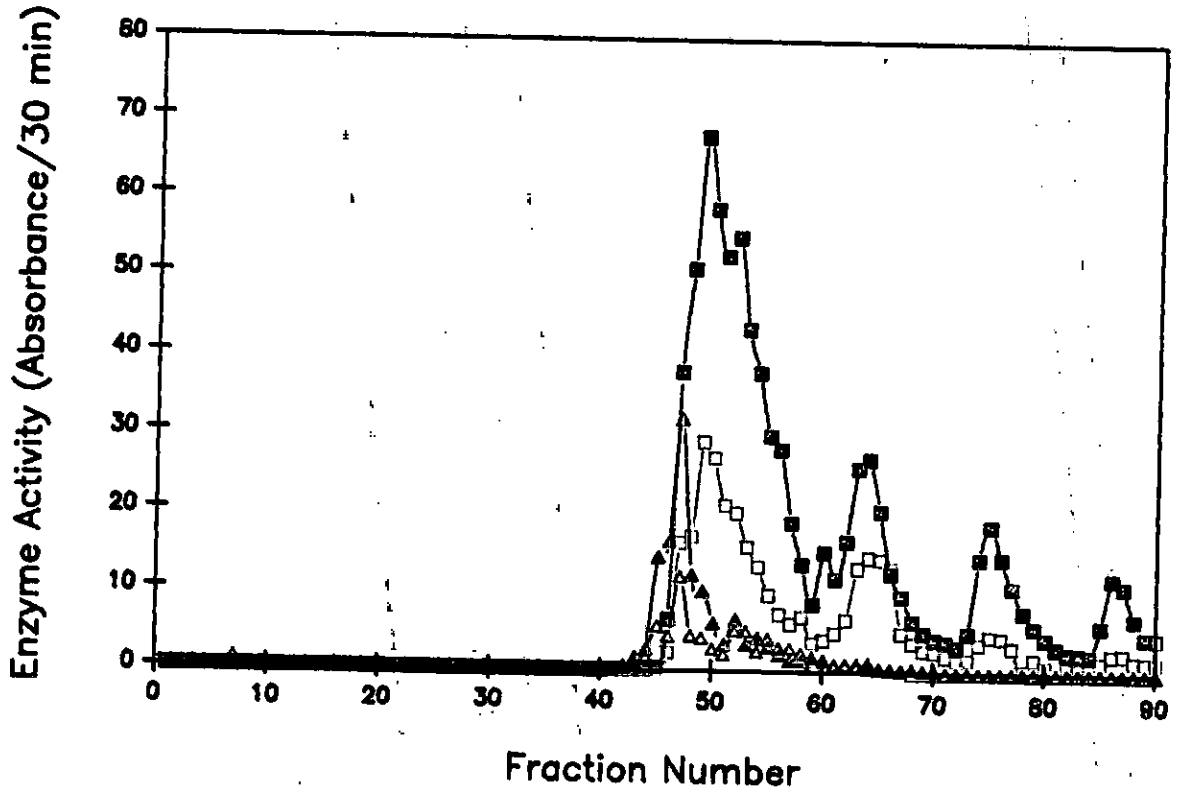
Figure 3.28

Distribution of protein with amylase and cellulose activity in the DE-52 column elution profile of crude crop juice

Protein (280nM) - o -

Amylase activity - ● -

Cellulase activity - ■ -


Enzyme Activity (Absorbance/30 min)
0
10
20
30
40
50
60
70
80
90
Fraction Number

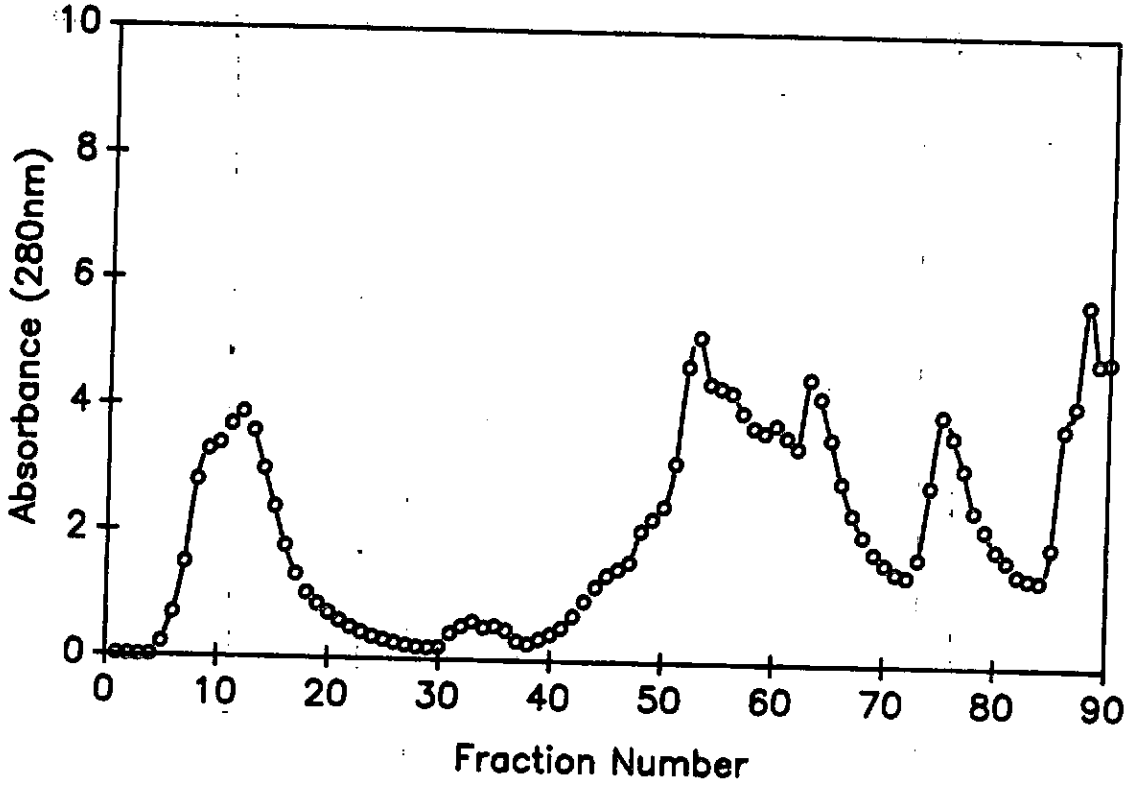

Absorbance (280nm)
10
8
6
4
2
0
0
10
20
30
40
50
60
70
80
90
Fraction Number

Figure 3.29a

Distribution of protein in the DE-52 column elution profile of crude crop juice using a linear gradient of 10 - 80mM phosphate buffer pH6.8, followed by a stepwise elution with 120mM, 200mM and 500mM phosphate buffer at the same pH.

Protein (280mM) -o-

Figure 3.29b

Distribution of disaccharidase activity in the DE-52 column elution profile of crude crop juice using a linear gradient of 10-80mM phosphate buffer pH6.8, followed by a stepwise elution with 120mM, 200mM and 500mM phosphate buffer at the same pH

Sucrase activity - Δ -

Maltase activity - ▲ -

Lactase activity - □ -

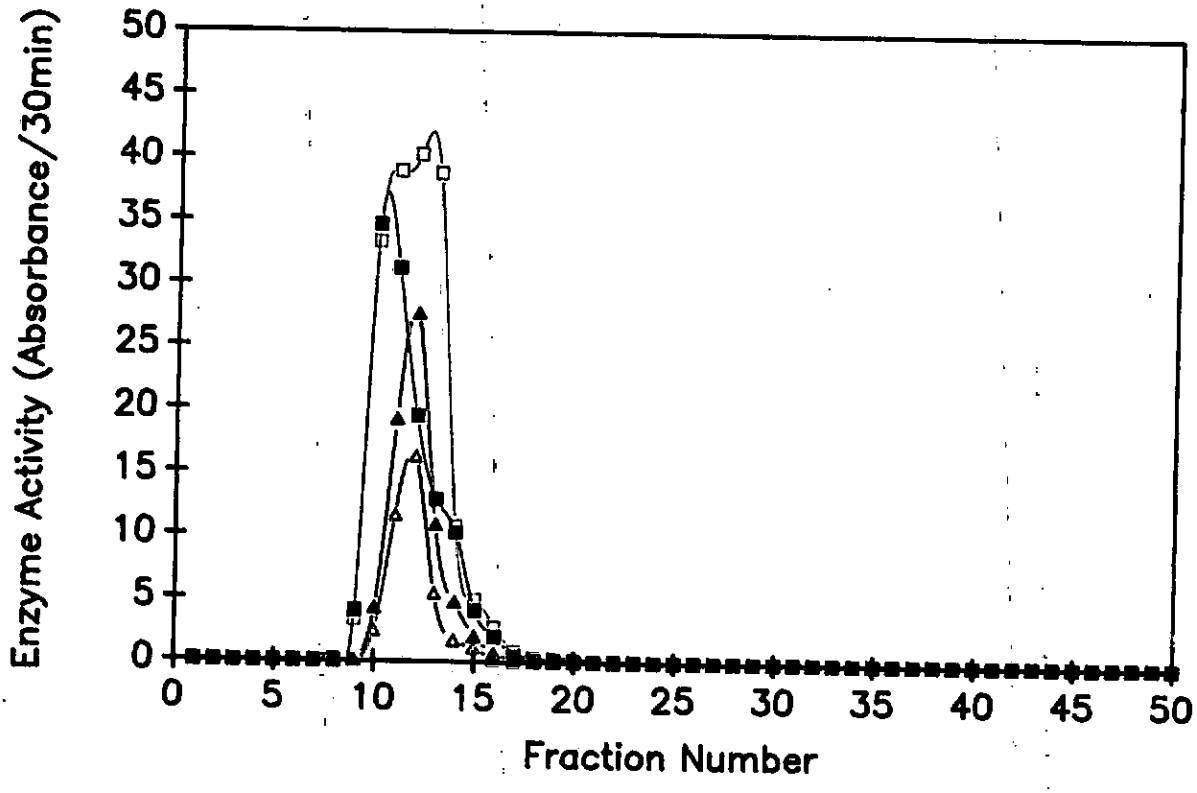
Cellobiase activity - ■ -

Amylase activity on the other hand, showed a quite different distribution in the elution profile, having two peaks in the wash region (fractions 8-13 and 14-23) and a third peak early in the gradient (fractions 25-30) which coincides with the maltase/sucrase peak.

The conditions under which the first DE-52 column was run, was varied, to ascertain if this change would resolve any of the carbohydrases.
A stepwise elution was employed such that the initial wash was with 10mM phophate buffer pH6.8, followed by a gradient which was decreased to 10mM-80mM in ionic strength, to elute the maltase/sucrase activity, followed by a stepwise elution with 120mM, 200mM and 500mM respectively. This still did not resolve any of the carbohydrases from each other, although the peaks were sharpened as shown in Figures 3.29a and b.

3.7.2 Gel Permeation Chromatography on a Bio-Gel P200 Column

The crude crop juice proteins were eluted in three main peaks with 100mM phosphate buffer pH6.8. The elution pattern of the protein, when absorbance was taken at 280nm is shown in Figure 3.30a. All the fractions were tested, and the disaccharidases were eluted as a peak of activities (Figure 3.30b; fractions 10-15). The pattern


Enzyme Activity (Absorbance/30min)
50
45
40
35
30
25
20
15
10
5
0
0
5
10
15
20
25
30
35
40
45
50
Fraction Number

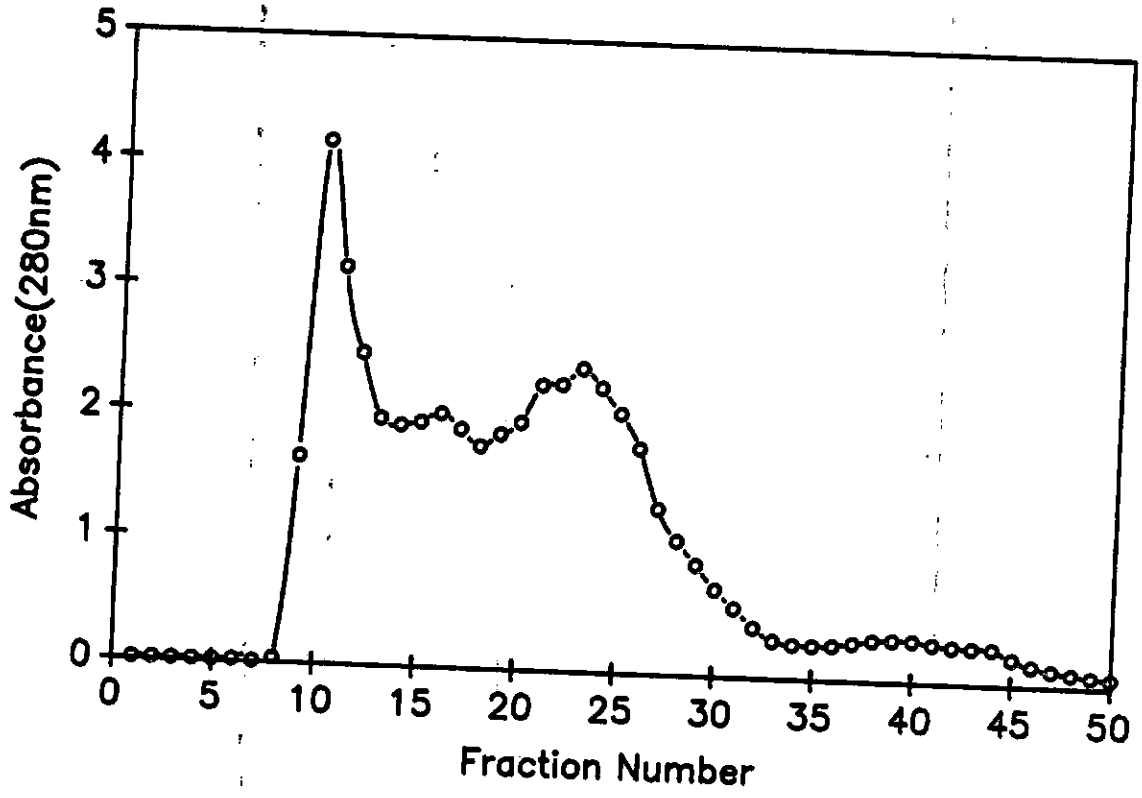

Absorbance(280nm)
0
1
2
3
4
5
0
5
10
15
20
25
30
35
40
45
50
Fraction Number

Figure 3.30a

Distribution of protein in the Bio-Gel P200 column elution profile of crude crop juice

Protein (280nm) -o-

Figure 3.30b

Distribution of disaccharidase activity in the Bio-Gel P200 column elution profile of crude crop juice

Sucrase activity - Δ -

Maltase activity - ▲ -

Lactase activity - ■ -

Cellobiase activity - □ -

of elution was such that maltase, sucrase, lactase and cellobiase activities were all eluted in the same region of the profile near the void volume. Maltase and sucrase showed identical distribution in a single represented peak. Lactase and cellobiase both showed evidence of two peaks of activity in similar positions but differing from each other in the proportion of activity in each peak.

Cellobiase activity seemed to be equally distributed in the two peaks while lactase activity appeared mostly in the higher molecular weight peak with a small shoulder in the lower molecular weight region. The enzymatically active fractions for the disaccharidases, coincided with the first protein peak (Figure 3.30a and b; fractions 10-15).

The distribution of activity for amylase and cellulase (Figure 3.31) was completely different from that of the disaccharidases, although there was an overlap of activities in fractions 10-15. The major activity peak for both polysaccharidases coincided with the third protein peak in fractions 17-25 (Figure 3.30a and 3.31). The distriution pattern of activity for amylase and cellulase were very similar, but not identical. Estimation of the molecular weight of "amylase" from the Ve/Vo ratio of elution volume over void volume on this column yeilded a value of Mr 30,000, while the value for cellulase was about Mr 36,000.

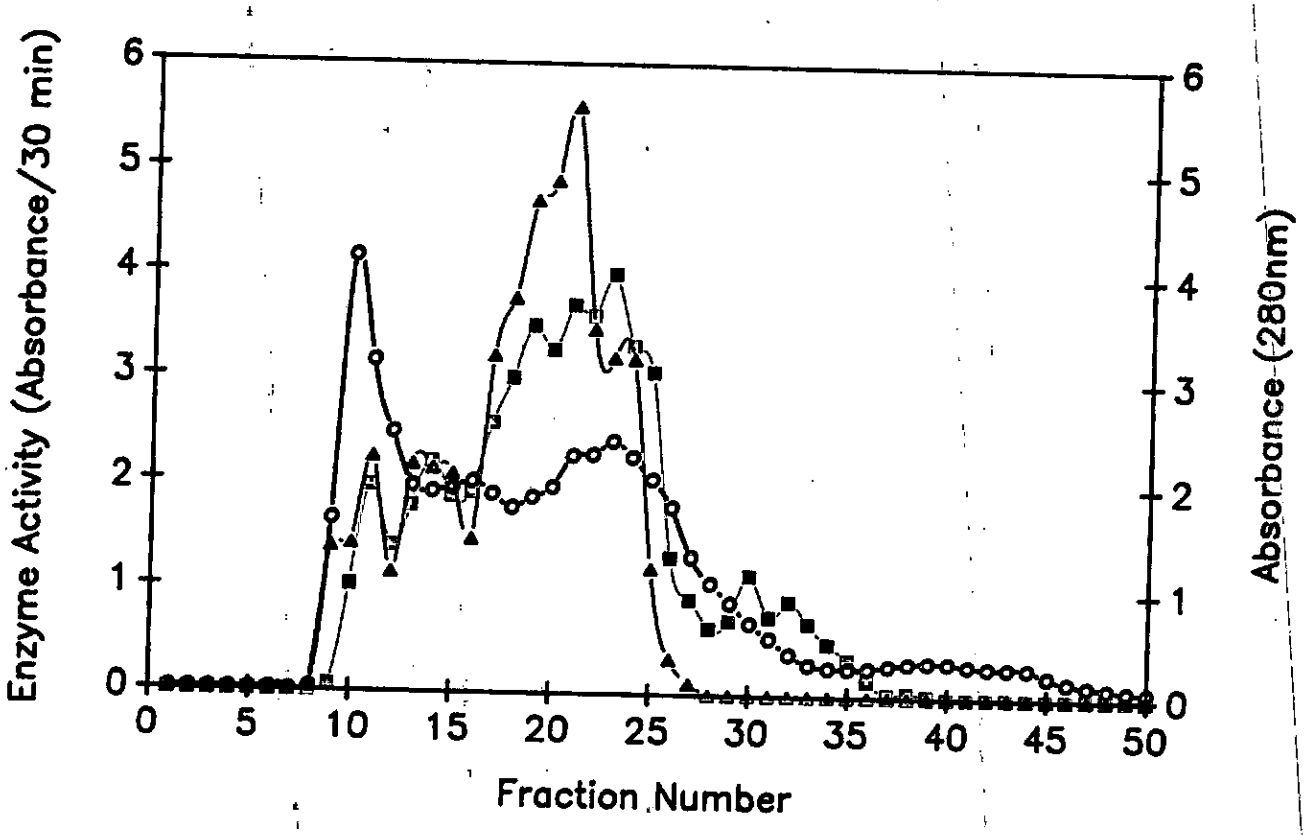

Enzyme Activity (Absorbance/30 min)
Absorbance (280nm)
Fraction Number
0
1
2
3
4
5
6
0
5
10
15
20
25
30
35
40
45
50

Figure 3.31

Distribution of polysaccharidase activity in the Bio-Gel P200 column elution profile of crude crop juice

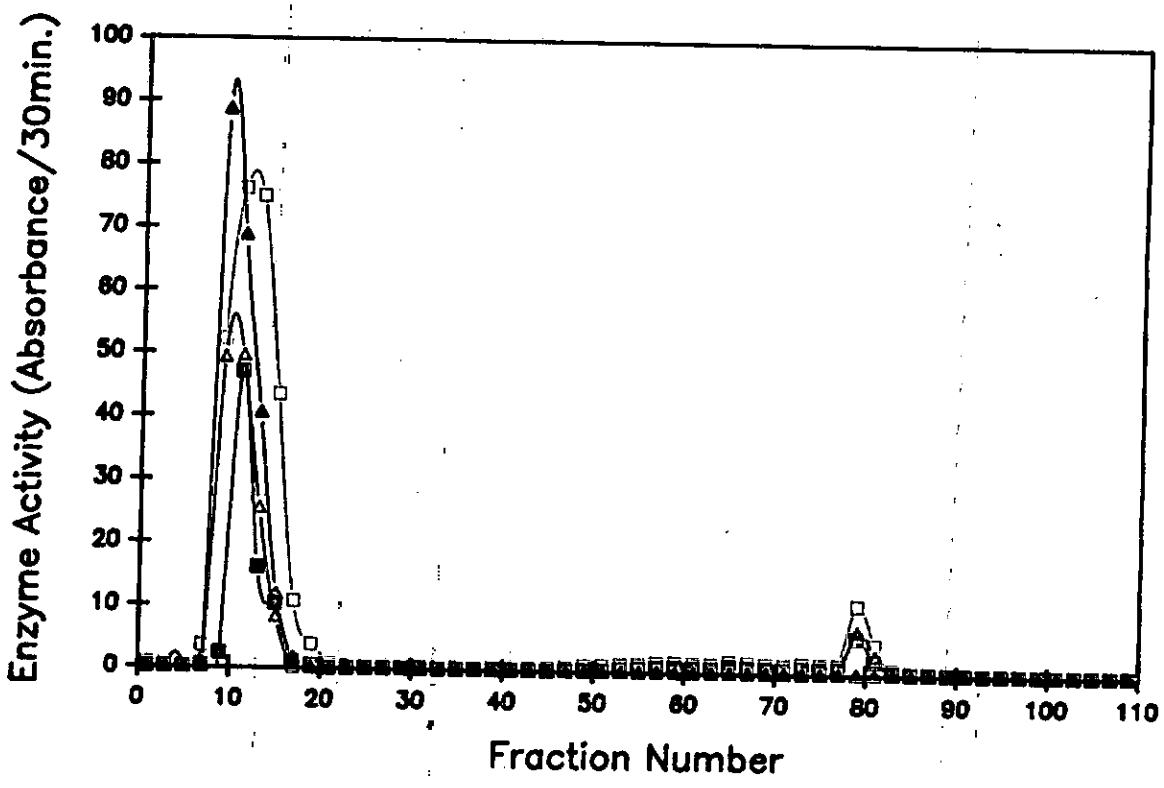
Amylase activity -▲-

Cellulase activity -●-

3.7.3 Hydrophobic Chromatography of Crude Crop Juice Proteins on Octyl CL 4B Sepharose Column

Crop juice concentrate was applied to the column after equilibrating with 1M ammonium sulphate in 10mM sodium acetate buffer pH5.7. The bulk of the protein was eluted in the wash with 1M ammonium sulphate in 10mM acetate buffer pH5.7 (Figure 3.32a; fractions 8-16). An inverse gradient of 1.0M-0.0M ammonium sulphate in 10mM phosphate buffer did not elute any protein (at 280nm absorbance reading). While very small amounts of protein was eluted with the final wash with 10mM acetate buffer pH5.7. All the carbohydrases tested for came straight through with the initial wash and none of the enzymes stuck to the column (Figure 3.32b and 3.33; fractions 8-16).

The experimental conditions under which the first hydrophobic column described above, was run was varied to see if this will bring about any seperation in the crop juice carbohydrases. Thus, the enzymatically active peak from the experiment reported above (Figure 3.32b and 3.33; fractions 8-16) was pooled and adjusted to 2M with ammonium sulphate and applied to a hydrophobic column which had been equilibrated with ammonium sulphate (2M) in 10mM acetate buffer pH5.7. Under this new experimental conditions, about 85% of the protein stuck to the column with only a small


Enzyme Activity (Absorbance/30min.)
100
90
80
70
60
50
40
30
20
10
0
0
10
20
30
40
50
60
70
80
90
100
110
Fraction Number

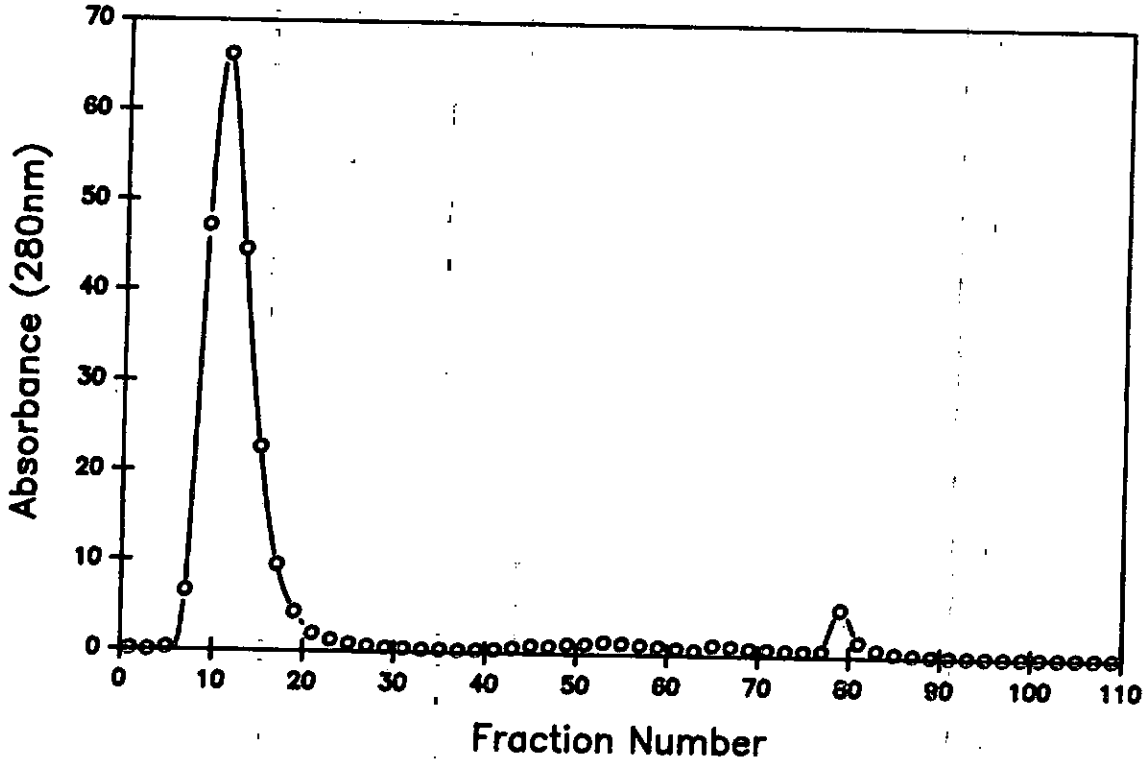

Absorbance (280nm)
70
60
50
40
30
20
10
0
0
10
20
30
40
50
60
70
80
90
100
110
Fraction Number

Figure 3.32a

Distribution of proteins in the Octyl sepharose 4B column elution profile of crude crop juice

Protein (280nm) -o-

Figure 3.32b

Distribution of disaccharidase activity in the Octyl sepharose 4B column elution profile of the crude crop juice

Sucrose activity - Δ -

Maltase activity - ▲ -

Lactase activity - ■ -

Cellobiase activity - □ -

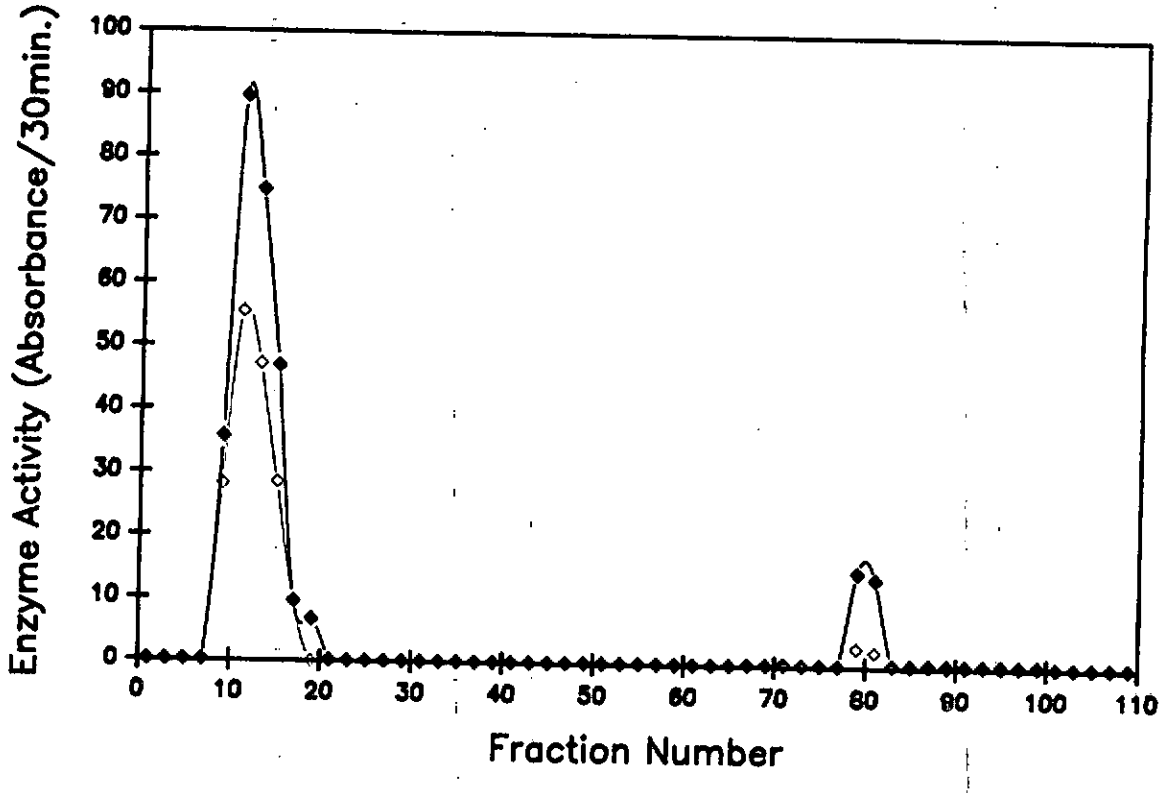

Enzyme Activity (Absorbance/30min.)
100
90
80
70
60
50
40
30
20
10
0
0
10
20
30
40
50
60
70
80
90
100
110
Fraction Number

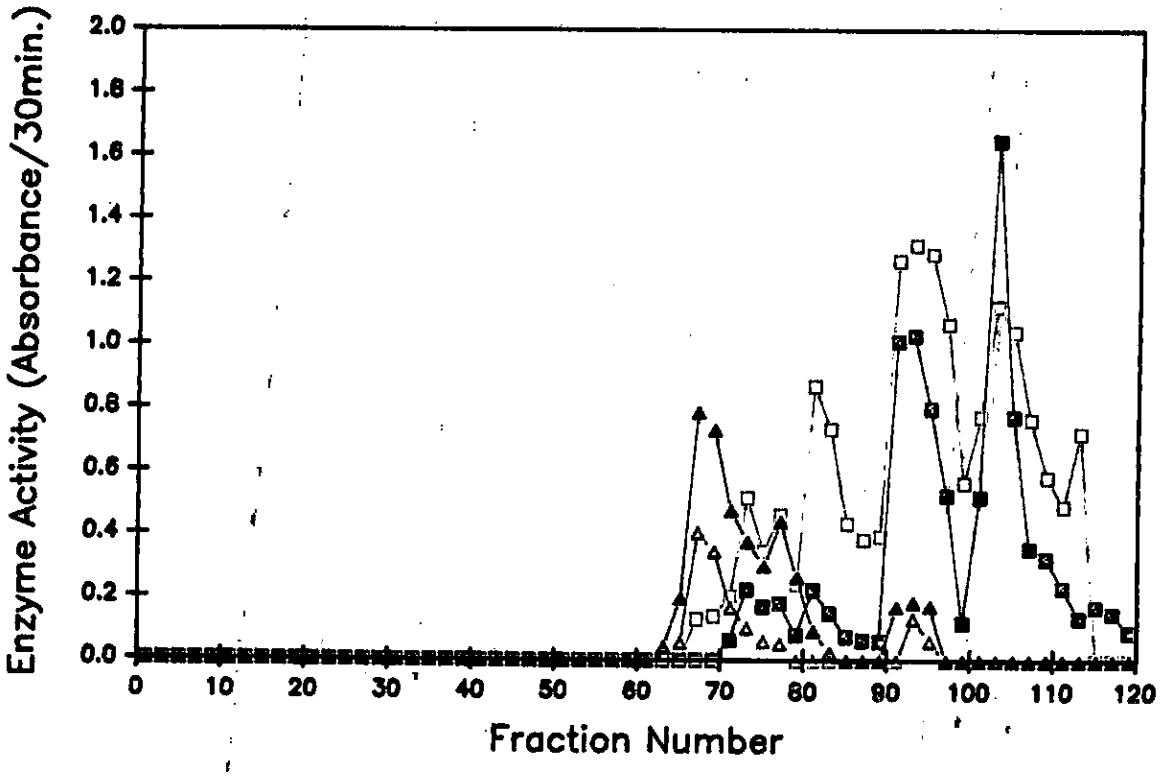
Figure 3.33

Distribution of polysaccharidase activity in the Octyl sepharose 4B column elution profile of crude crop juice

Amylase activity - ◆-

Cellulase activity - ◇-

amount coming out in the 2M ammonium sulphate in 10mM acetate buffer pH5.7. Consequently, when the column was developed with an inverse gradient of 2.0M-0.0M ammonium sulphate in 10mM phosphate buffer, a major broad protein band was eluted with about four poorly resolved peaks (Figure 3.34a; fractions 59-88). When these fractions were assayed for maltase and sucrase activities, fractions 65-75 and 91-95 (Figure 3.34b) were enzymatically active. Lactase and cellobiase exhibited a higher degree of hydrophobicity so were more retarded on the column, but they both came after the maltase and sucrase peak, with slight overlap in their activities (Figure 3.34b; fractions 73-80, 80-88 and 100-113). Lactase activity distribution was very similar to that of cellobiase, as shown in Figure 3.34b. Amylase activity eluted about midway in the gradient with a major sharp activity peak coincident with an equally sharp peak of aborbance at 280nm (Figure 3.35; fractions 75-83). There was also some scatter of amylase activity further down the gradient. There was overlap in the distribution pattern of cellulase with amylase (Figure 3.35; fractions 75-83) and also with the B-disaccharidases (Lactase and cellobiase) as evident in Figure 3.34a and 3.35; fractions 81-88, despite which, the enzyme "cellulase" still showed a distinct elution pattern from the other enzyme activities.


Enzyme Activity (Absorbance/30min.)
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
40
50
60
70
80
90
100
110
120
Fraction Number

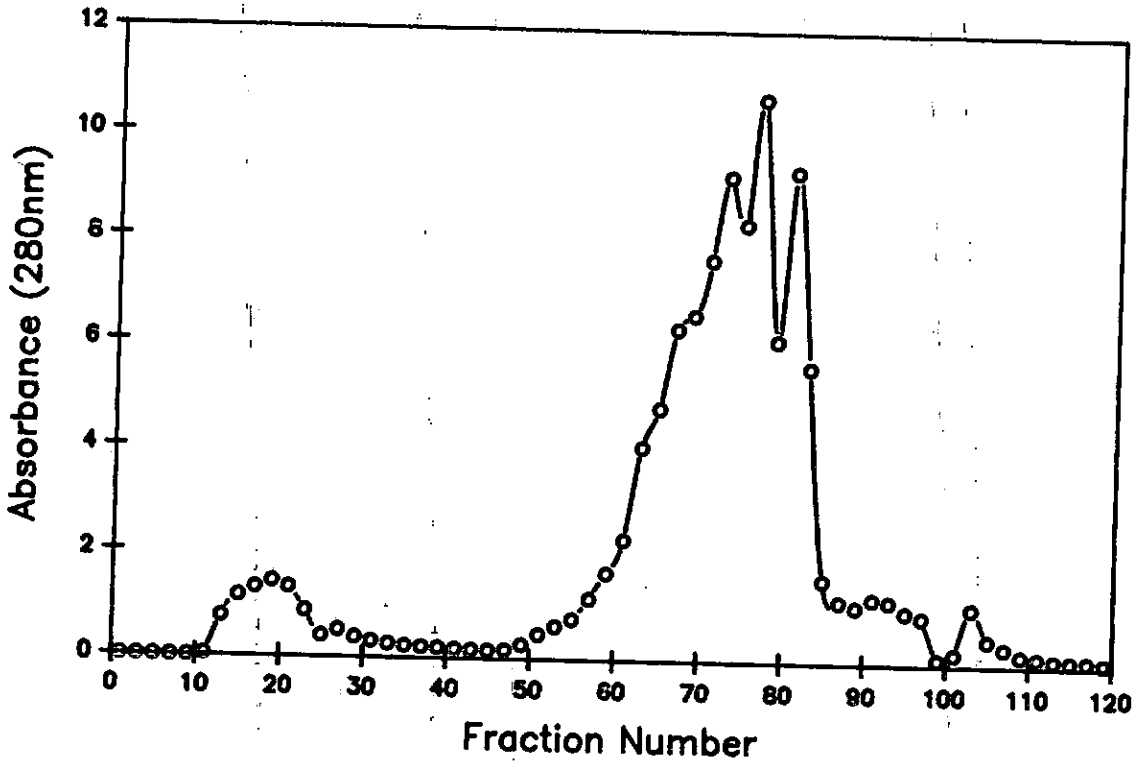

Absorbance (280nm)
12
10
6
6
4
2
0
0
10
20
30
40
50
60
70
80
90
100
110
120
Fraction Number

Figure 3.34a

Distribution of protein in the Octyl sepharose 4B column elution profile of partially purified crop juice (sample from previous hydrophobic column - Figure 3.32a; fractions 8-16)

Protein -o-

Figure 3.34b

Distribution of disaccharidase activity in the Octyl sepharose 4B column elution profile of partially purified crop juice (sample from a previous hydrophobic column, figure 3.32a; fractions 8-16)

Sucrase activity - Δ -

Maltase activity - ▲ -

Lactase activity - ■ -

Cellobiase activity - □ -

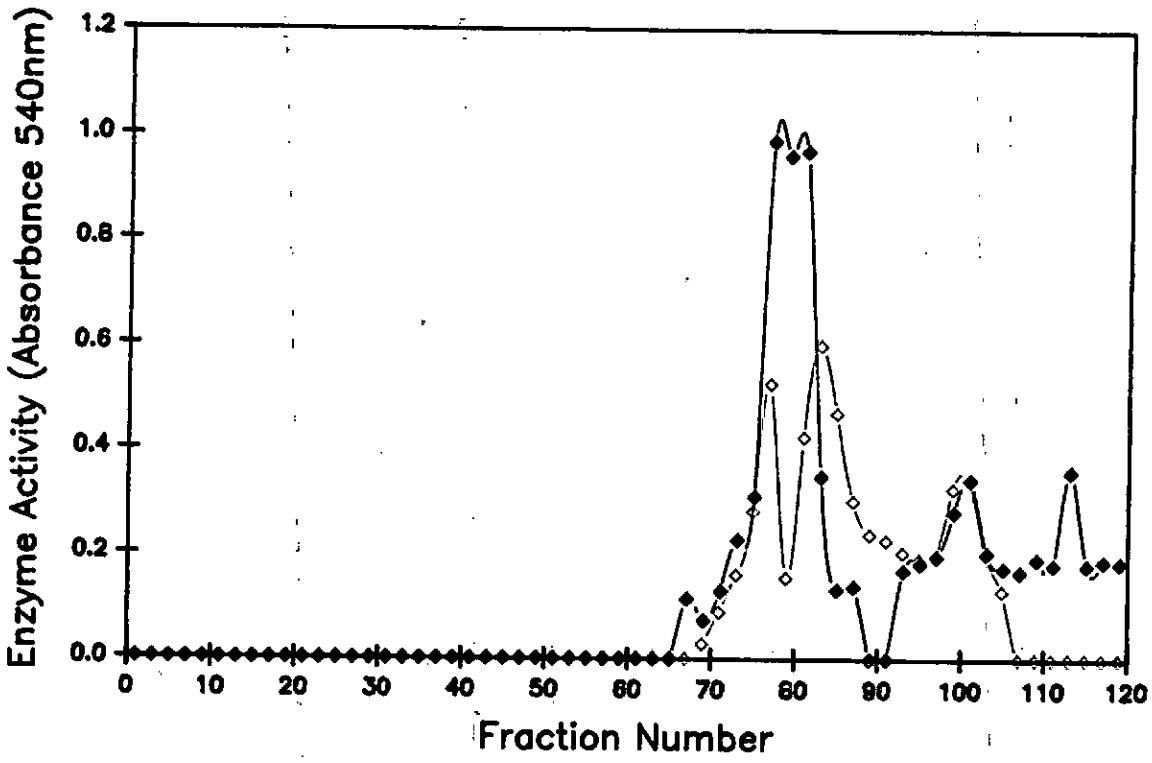

Enzyme Activity (Absorbance 540nm)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
40
50
60
70
80
90
100
110
120
Fraction Number

Figure 3.35

Distribution of polysaccharidase activity in the Octyl sepharose 4B column elution profile of partially purified crop juice (sample from a previous hydrophobic column as in Figure 3.33; fractions 8-16)

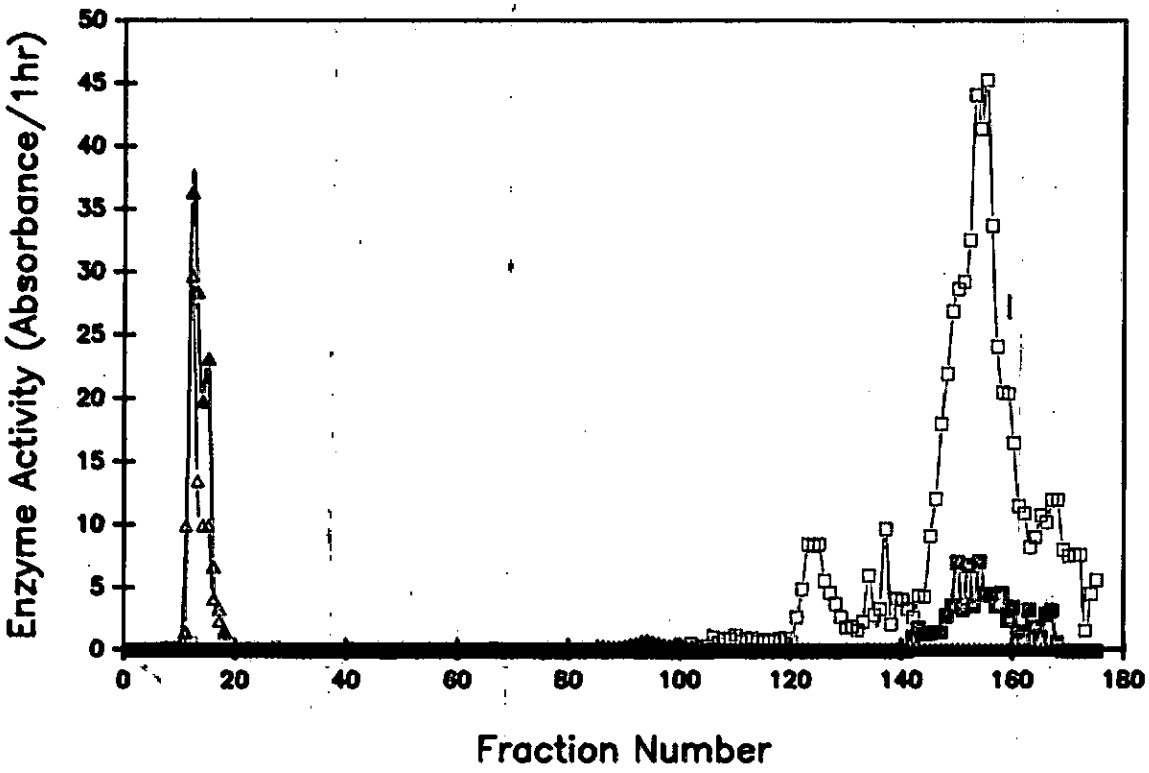
Protein (280nm) - ○ -

Amylase activity - ● -

Cellulase activity - ◆ -

A slightly different result was obtained on Octyl sepharose chromatography of a crop juice proteins which were first partially seperated on a DE-52 column. In this experiment, the elution buffer used was changed from sodium acetate buffer pH5.7 to 10mM sodium phosphate buffer pH6.8 in a gradient of 2.0M to 0.0M ammonium sulphate. One of the protein peaks active towards all the disaccharides (Figure 3.29b; fraction 46-58 from DE-52 column) was applied to an equilibrated hydrophobic column and washed with 2M ammonium sulphate in phosphate buffer pH6.8.

Maltase and sucrase failed to bind to the column and appeared in the wash through fractions from the column (Figure 3.36a and 3.36b; fractions 11-16). A smaller peak of cellobiase activity (which also hydrolysed lactose, but not to a significant extent) was eluted at the tail end of the 2.0M-0.0M ammonium sulphate inverse gradient in 10mM phophate buffer pH6.8. Continued application of 10mM phosphate buffer after the gradient, eluted a small protein peak coincident with a large peak of cellobiase and lactase, coming out together in a single peak of activity (Figure 3.36b). Thus, "maltase" and "sucrase" were completely seperated from lactase and cellobiase. The two groups of enzymes appeared to be very specific for their substrates, there being virtually no activity against lactose and cellobiose by "maltase/sucrase" and vice versa.


Enzyme Activity (Absorbance/1hr)
50
45
40
35
30
25
20
15
10
5
0
0
20
40
60
80
100
120
140
160
180
Fraction Number

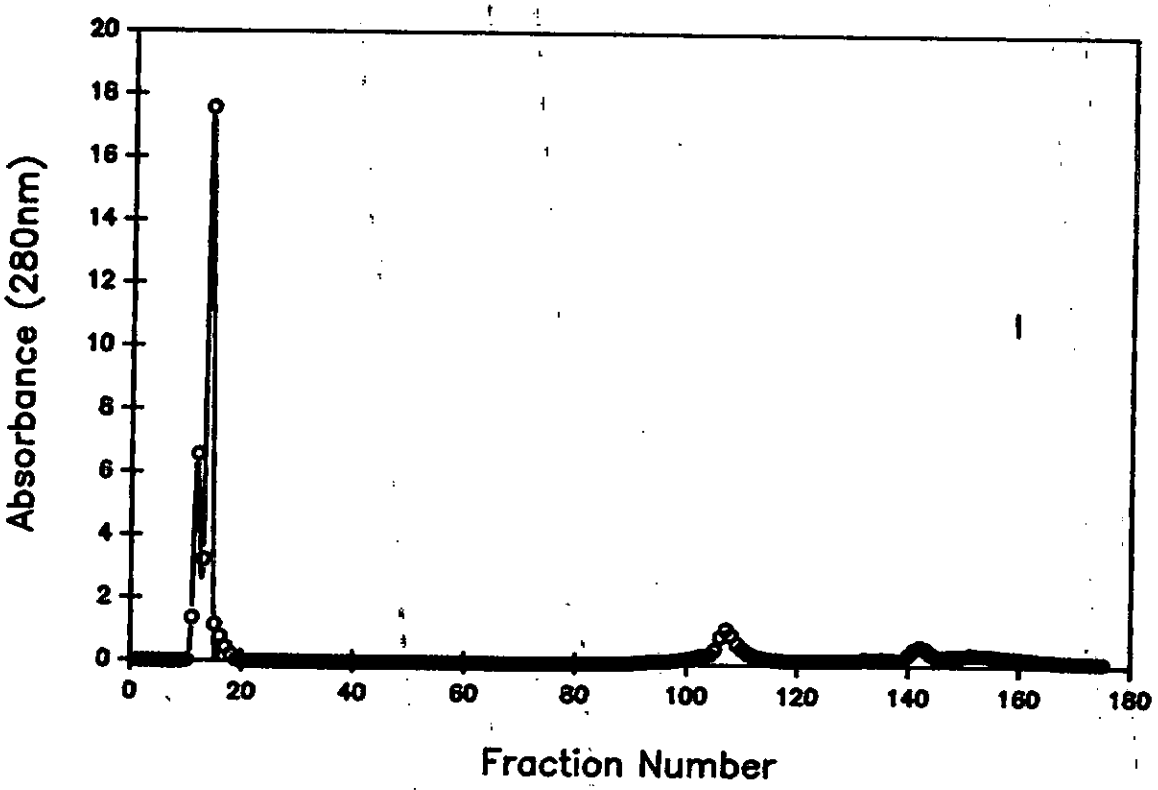

Absorbance (280nm)
20
18
16
14
12
10
8
6
4
2
0
0
20
40
60
80
100
120
140
160
180
Fraction Number

Figure 3.36a

Distribution of protein in the Octyl sepharose 4B column elution profile of partially purified crop juice (sample from a DE-52 column; figure 3.29b; fractions 46-58)

Protein (280nm) -o-

Figure 3.36b

Distribution of disaccharidase activity in the Octyl sepharose 4B column elution profile of partially purified crop juice (sample from a DE-52 column; figure 3.29b; fractions 46-58)

Sucrase activity - Δ -

Maltase activity - $\blacktriangle$ -

Lactase activity - $\blacksquare$ -

Cellobiase activity - $\square$ -

3.7.4 Inhibition of disaccharidases by Tris

Tris is a well known inhibitor of mammalian disaccharidases and has been used in affinity columns for their purification. Thus as an initial assessment of the verification of such a stage in the current work the inhibition of the snail's disaccharidases by Tris was compared. The degree of inhibition on maltase activity was least amongst all the enzyme activities. The percentage inhibition by 0.05M Tris of maltase activity was 23%, while the highest percentage inhibition at the same Tris concentration was 87%. The result is summerized in Figure 3.37.

3.7.5 Tris Sepharose 6B Affinity Chromatography of Crop Juice

Figure 3.38a and 3.38b show the elution profile of the four disaccharidases under investigation from Sepharos 6B Tris affinity columm. In an initial experiment when crude crop juice was applied to the Tris affinity column in 10mM phosphate pH6.8 and washed through with the same buffer, all the enzyme ativity was retained on the column. When eluted with 150mM Tris pH7.4, a protein peak active towards maltose, sucrose, lactose and cellabiose was released in a single peak. No seperation of activities was achieved except that cellobiase appeared to elute slightly earlier than the other three enzymes.

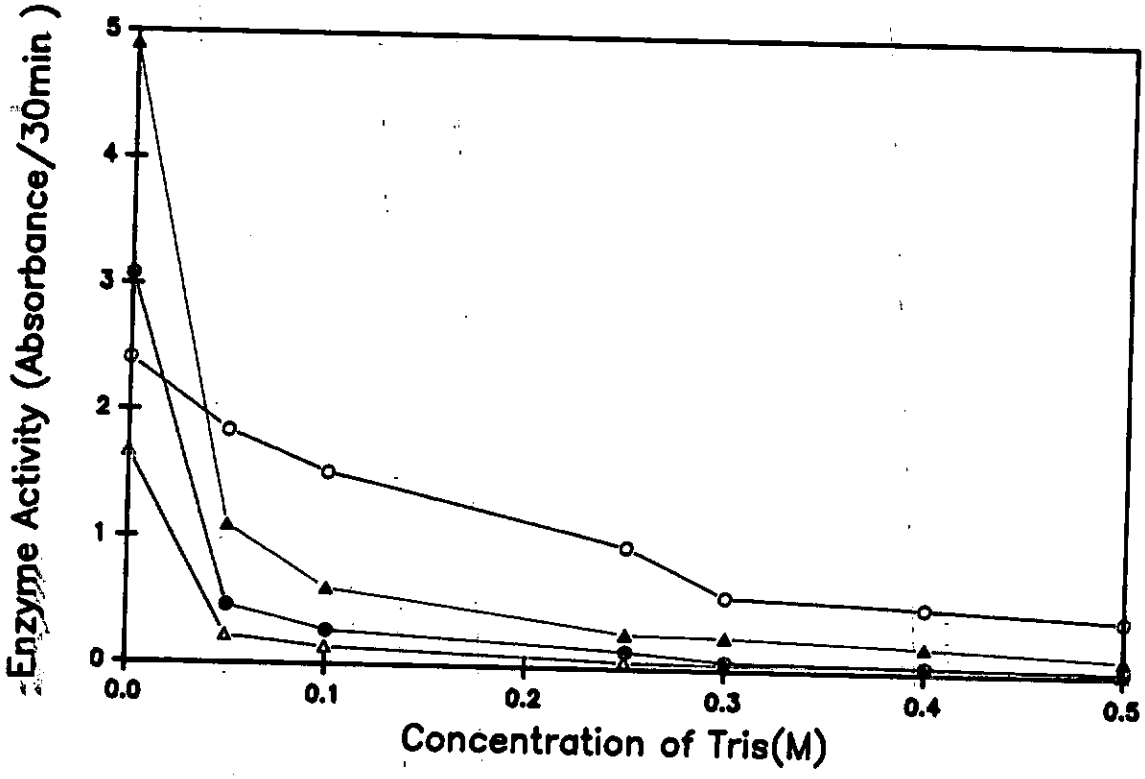

Enzyme Activity (Absorbance/30min)
5
4
3
2
1
0
0.0
0.1
0.2
0.3
0.4
0.5
Concentration of Tris(M)

Figure 3.37

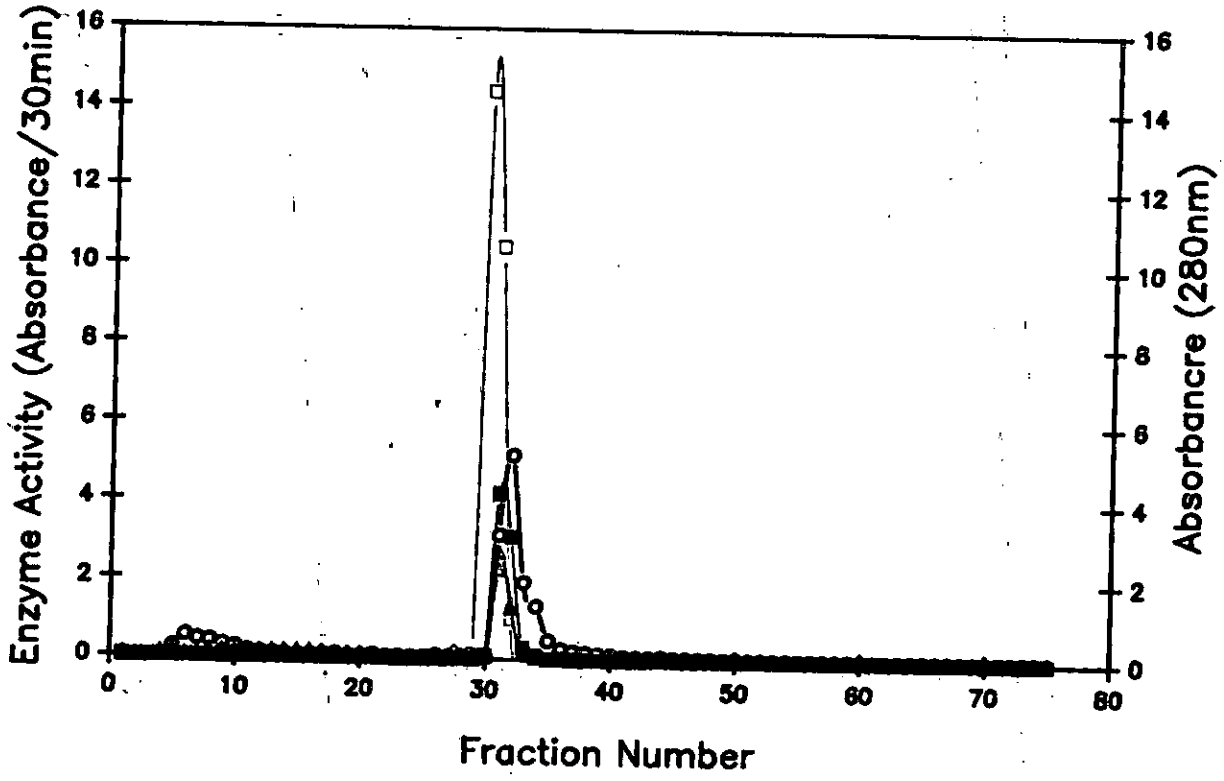
Tris inhibition of enzyme activities

Maltose activity - ■ -

Sucrase activity - ▲ -

Lactase activity - ● -

Cellobiase activity - ▲ -


Enzyme Activity (Absorbance/30min)
Absorbancre (280nm)
Fraction Number
0
2
4
6
8
10
12
14
16
0
10
20
30
40
50
60
70
80

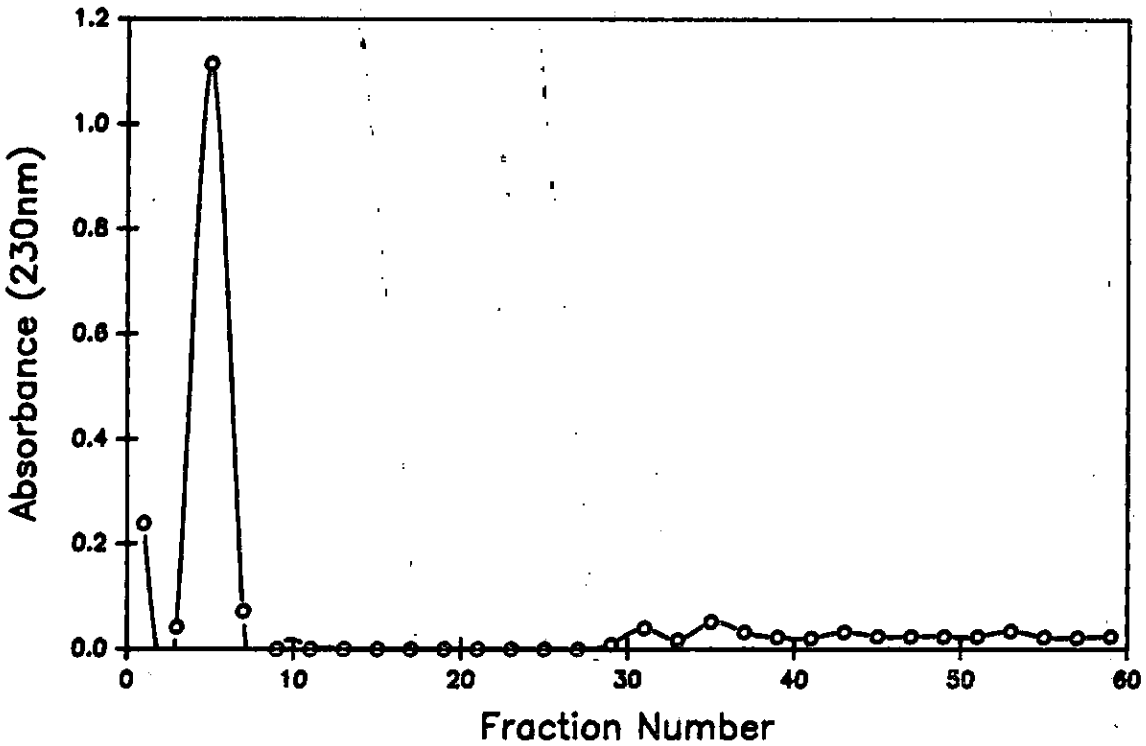

Absorbance (230nm)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
40
50
60
Fraction Number

Figure 3.38a

Distribution of protein in the Sepharose 6B Tris affinity column elution profile of crude crop juice

(The column was initially washed through with 10mM phosphate buffer then followed with 150mM Tris solution pH7.4)

Protein (280nm) -o-

Figure 3.38b

Distribution of disaccharidase activity in the Sepharose 6B Tris affinity column elution profile of crude crop juice.

(The column was initially washed through with 10mM phosphate buffer pH6.8 and followed with 150 mM Tris solution pH7.4)

Sucrase activity - ▲ -

Maltase activity - ▲ -

Lactase activity - ■ -

Cellobiase activity - □ -

It was subsequently found that 100mM phosphate pH6.8 buffer also completely eluted the disaccharidases activity together (Figures 3.39a and 3.39b), suggesting that the binding was not specific. Other eperiments were performed using a gradient of 10mM to 100mM phosphate buffer pH6.8 and finally with 150mM Tris solution pH7.4, in an attempt to resolve the different activities.

Unfortunately the activities were found across the gradient without separation as shown in Figures 3.40a and 3.40b. Although the enzymes were strongly inhibited by Tris in earlier experiments, we were unable to separate them on Tris affinity columns at this stage.

3.7.6 Simple Mixed Substrate Experiments

3.7.6.1 Does one Enzyme Hydrolyse both Lactose and Cellobiose?

The results of the sets of experiments carried out to investigate if there was one enzyme hydrolysing both lactose and cellobiose is summerized in Table 3.7a.

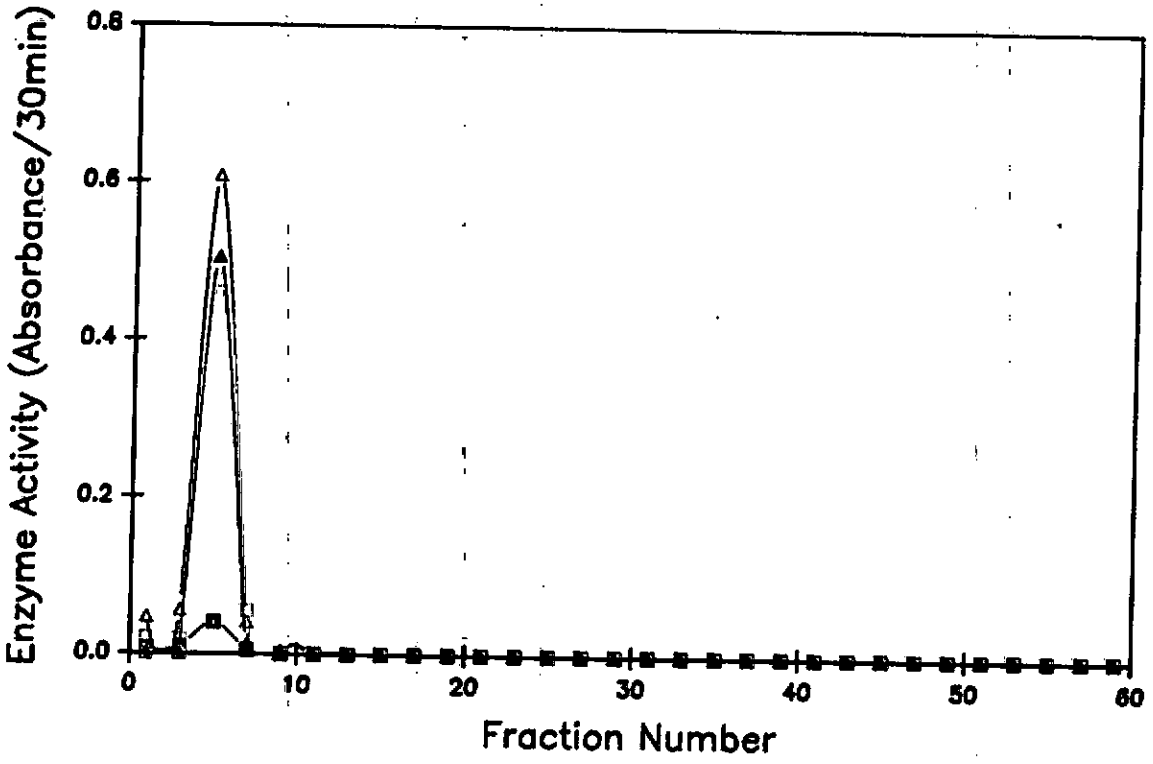

Enzyme Activity (Absorbance/30min)
0.8
0.6
0.4
0.2
0.0
0
10
20
30
40
50
60
Fraction Number

Figure 3.39a

Distribution of protein in the Sepharose 6B Tris affinity column elution profile of crude crop juice

(protein eluted with 100mM phosphate buffer pH6.8)

Protein (280nM) -o-

Figure 3.39b

Distribution of disaccharidase activity in the Sepharose 6B Tris affinity column elution profile of crude crop juice.

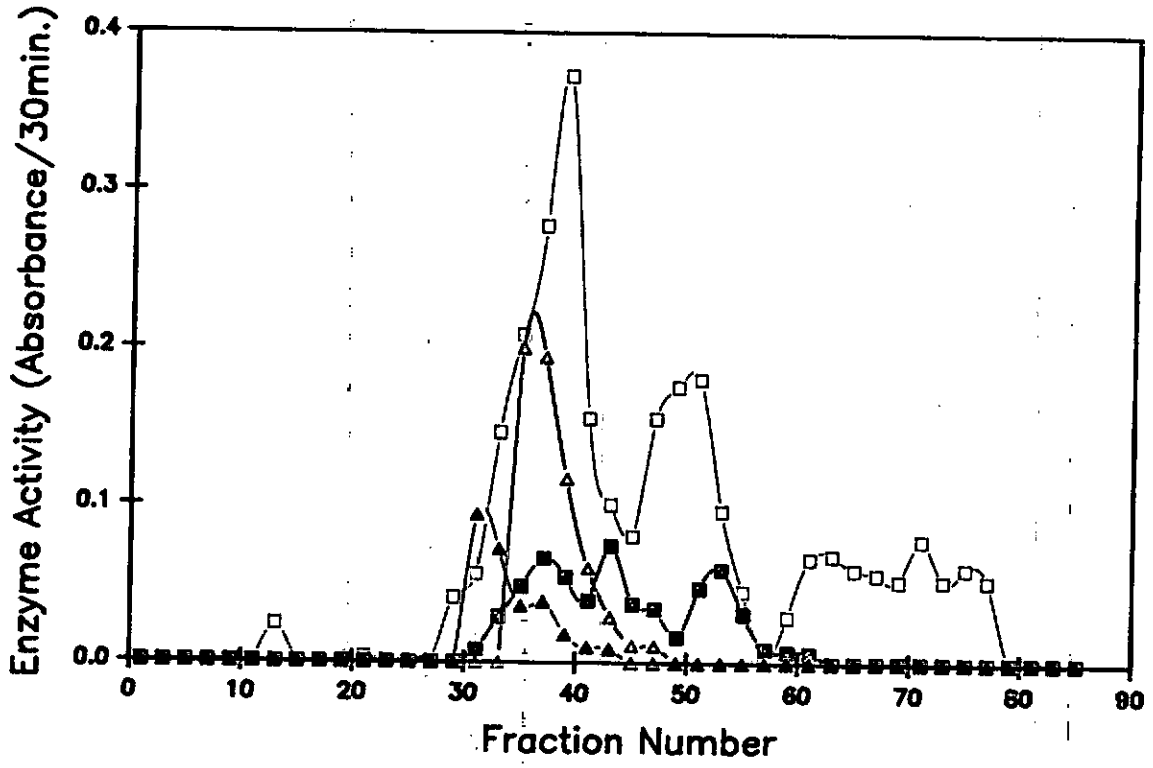
(Enzymes eluted with 100mM phosphate buffer pH6.8)

Sucrase activity - ▲ -

Maltase activity - △ -

Lactase activity - ■ -

Cellobiase activity - □ -


Enzyme Activity (Absorbance/30min.)
0.4
0.3
0.2
0.1
0.0
0
10
20
30
40
50
60
70
80
90
Fraction Number

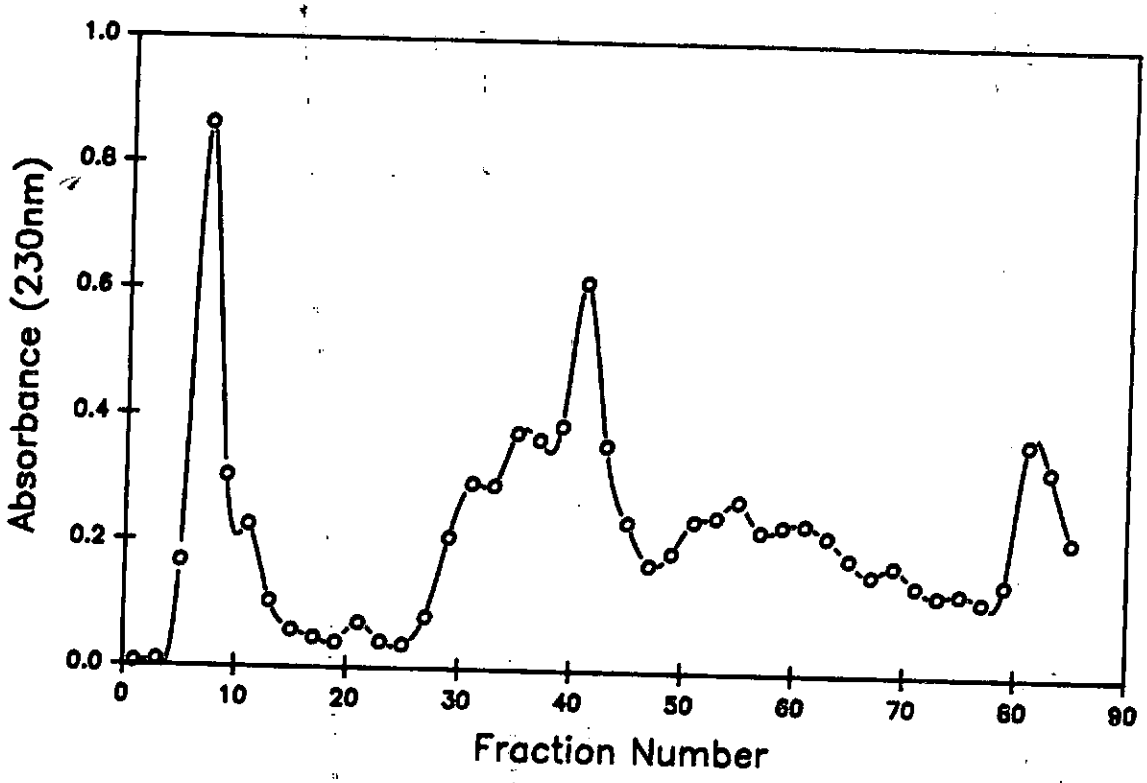

Absorbance (230nm)
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
40
50
60
70
80
90
Fraction Number

Figure 3.40a

Distribution of protein in the Sepharose 6B Tris affinity column elution profile of crude crop juice.

(Using a gradient of 10-100mM phosphate buffer pH6.8).

Protein (280nm) -o-

Figure 3.40b

Distribution of disaccharidase activity in the Sepharose 6B Tris affinity column elution profile of crude crop juice (using a gradient of 10-100mM phosphate buffer pH6.8)

Sucrase activity - ▲ -

Maltase activity - △ -

Lactase activity - ● -

Cellobiase activity - □ -

Table 3.7a **Hydrolysis of Lactose and Celliobiose using Crude Crop Juice as the Enzyme**

Substrate	Absorbances at 670nm with 1mM Substrate	Absorbances at 670nm with 10mM Substrate
Lactose (X)	0.136	0.574
Cellobiose (Y)	0.999	2.397
Lactose and Cellobiose (Z)	1.030	2.197
Calculated sum of Lactose and Cellobiose (X + Y)	1.135	2.971

The amount of glucose released from lactose (X) and Cellobiose (Y) separately, when added did exceed the amount of glucose released from both lactose and cellobiose (Z), when used together as substrates in the same enzyme assay as measured by the absorbance of the reaction mixture at 670nm.

Instead of using crude crop juice as the enzyme extract, partially purified enzyme, active towards cellobiose and lacose were pooled from a hydrophobic column (Figure 3.36b; fractions 140-170)) and was as the enzyme extract. Although Z was still less than (X + Y) in these sets of experiments with the partially purified enzyme at 10mM substrates concentration, at lower concentration (1mM), Z was more synegistic as reported in table 3.7b but the difference was too small to be significant.

Table 3.7b **Hydrolysis of Lactose and Celliobiose using Purified Enzyme Solution as the Enzyme Extract**

Substrate	**Absorbances at 670nm with 1mM Substrate**	**Absorbances at 670nm with 10mM Substrate**
Lactose (X)	0.400	1.928
Cellobiose (Y)	1.056	3.915
Lactose and Cellobiose (Z)	1.311	3.544
Calculated sum of Lactose and Cellobiose (X + Y)	1.456	5.843

10mM Z (3.544) < X + Y (5.843)

1mM Z (1.311) < X + Y (1.456)

3.7.6.2 Does one Enzyme Hydrolyse Both Maltose and Sucrose?

Similar sets of experiments were performed in the case of maltose and sucrose, to find out in this case if it was the same enzyme hydrolysing both maltose (X) and sucrose (Y) or not. The results were similar with that obtained when lactose (X) and cellobiose (Y) were used as substrates.

The result is summerized in Table 3.8a.

Table 3.8a **Hydrolysis of Sucrose and Maltose using Crude Crop Juice of A. marginata as the Enzyme Extract**

Substrate	Absorbance at 670nm with 1mM Substrate	Absorbance at 670nm with 10mM Substrate
Maltose (X)	0.868	0.938
Sucrose (Y)	0.087	0.633
Maltose and Sucrose (Z)	0.925	0.981
Calculated sum of Maltose and Sucrose (X + Y)	0.955	1.571

From Table 3.7b the result still shows that Z < (X + Y) both when the substrate concentration was 10mM and at a lower concentration of 1mM. Although at a lower concentration (1mM), Z could be seen to be more additive because X + Y (0.955) is more or less same as Z (0.925). But when partially purified enzyme solution, pulled from a sepharose 4B tris column, used as a third step of purification as shown in figure 3.39b, fractions 11-16 was used on maltose and sucrose in the same set of experiments described above for the crude crop juice, it was found that the result was not additive. That is, Z was not equal to or greater than (X + Y) as shown in table 3.8b)

Table 3.8b **Hydrolysis of Sucrose and Maltose using Partially Purified Enzyme Solution**

Substrate	**Absorbances at 670nm with 1mM Substrate**	**Absorbances at 670nm with 10mM Substrate**
Maltose (X)	0.136	0.574
Sucrose (Y)	0.999	2.397
Maltose and Sucrose (Z)	1.030	2.197
Calculated sum of Maltose and Sucrose (X + Y)	1.135	2.971

3.7.7. Polyacrylamide Gel Electrophoresis in SDS

As shown in Figure 3.41a, a fresh preparation of crude crop juice proteins from A. marginata migrated in SDS polyacrylamide gel to give several bands with molecular weight ranging from above Mr 205,000 to less than Mr 29,000.

When the peak of enzymatic activity with all the disaccharidases and polysaccharidases from a typical DE-52 cellulose column (Figure 3.29a and Figure 3.29b; polyacrylamide gel, the proteins in the enzyme solution migrated as shown in Figure 3.41b, which showed a slightly different pattern from the whole crop juice proteins, indicating little separation on DE52.

On the other hand electrophoresis of samples from the hydrophobic column showed that this technique was more effective in resolving the enzymes of the crop juice. Figure 3.42 shows the migratory pattern of the proteins from fractions 11-18, 120-140 and 141-170 respectively from the hydrophobic column which contained the bulk of the sucrase, maltase, lactase and cellobiase activities as shown in figure 3.36b. The peaks of enzymatic activities with cellobiase and lactase were also pooled from the same hydrophobic column (Figure 3.36 fractions 120-140 and 141-170 respectively). The protein present in these two separate pools migrated as a single band.

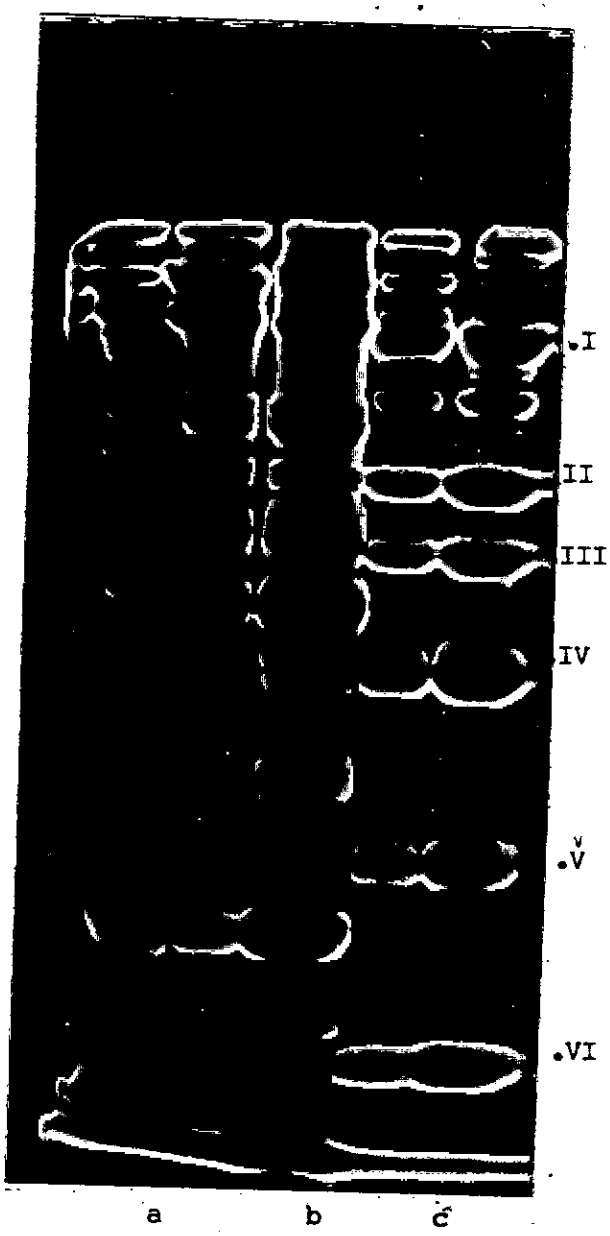

I
II
III
IV
V
VI
a
b
c

Figure 3.41

SDS polyacrylamide gel electrophoresis of crop juice preparation on 7.5% acrylamide gel

a) Whole crop juice

b) Crop juice proteins partially seperated on DE-52 chromatography

c) Protein standard

I - Mr 205,000

II - Mr 116,000

III - Mr 97,000

IV - Mr 66,000

V - Mr 45,000

VI - Mr 29,000

(Figure 3.44a and b) in each case, with an apparent molecular weight of 205,000. Similar results were obtained in several other similar experiments.

As shown in Figure 3.45a and b, the polysaccharidases pooled from fractions 75-80 and 81-88 from Figure 3.35 (overlapped with lactase and cellobiase activities), exhibited several protein bands with molecular weights between Mr 68,000 to just above Mr 29,000 respectively. "Cellobiase" and "lactase" seem to be of large molecular weight proteins while the "maltase" and "sucrase" fraction contained a considerable amount of protein in the region of Mr 300,000-97,000. The top limit value (Mr 300,000) corresponds to the estimated molecular weight from Bio-Gel P200 column.

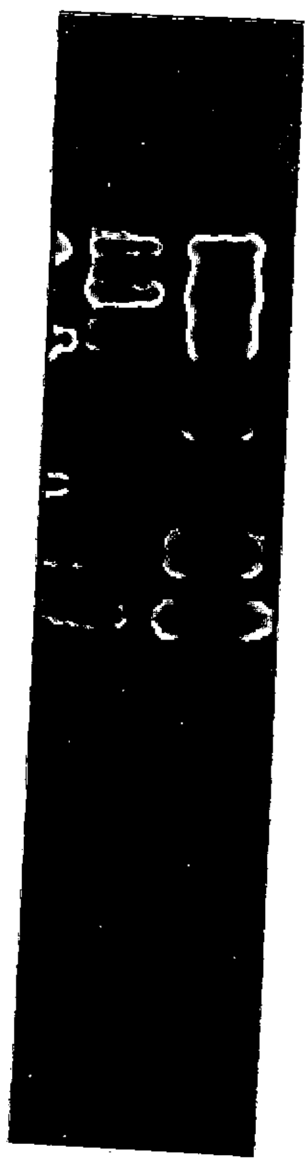


Figure 3.42

Migration of Proteins corresponding to the peak of enzymaic activities with maltase and sucrase from fractions 11-18; Figures 3.36b (On a hydrophobic column) of SDS get electrophoresis

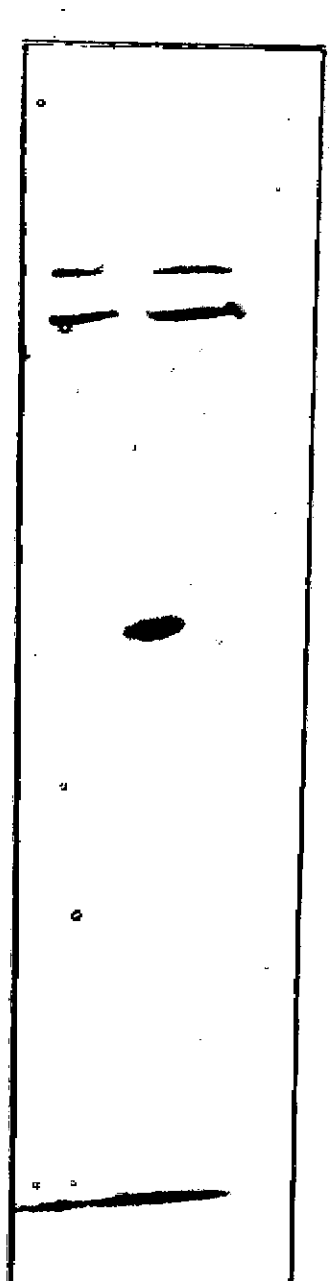


Figure 3.43

Migration of proteins corresponding
to maltase and sucrase activities
from factions 11-18;
figures 3.36b
on SDS gel electrophoresis

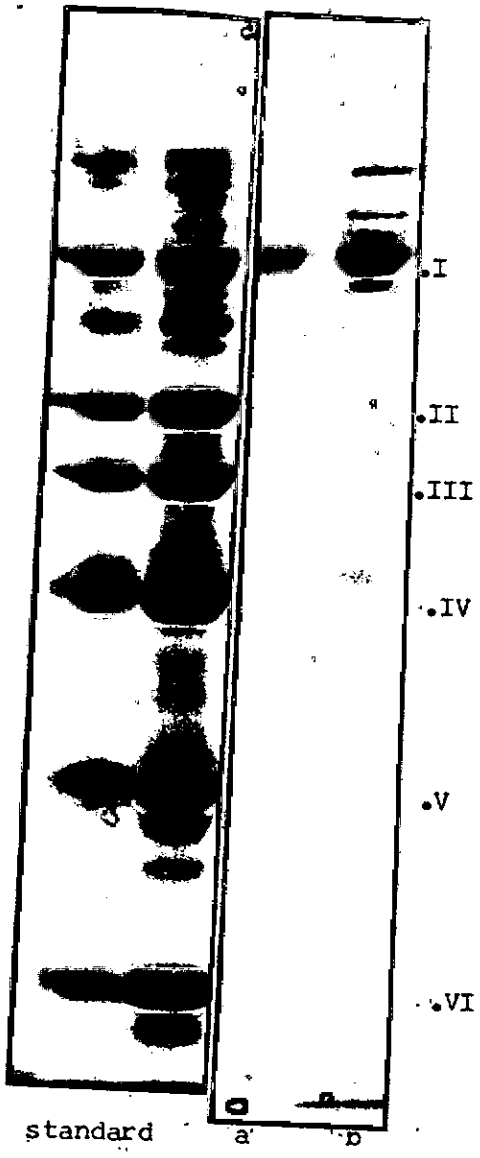

I
II
III
IV
V
VI
standard
a
b

Figure 3.44

Migration of Proteins from figure 3.36b

a) Fractions 120-140

b) Fractions 141-170

Corresponding to cellobiase and lactase activity pools respectively on SDS polyacrylamide gel

Protein Standard

I	-	Mr 205,000
II	-	Mr 116,000
III	-	Mr 97,400
IV	-	Mr 66,000
V	-	Mr 45,000
VI	-	Mr 29,000

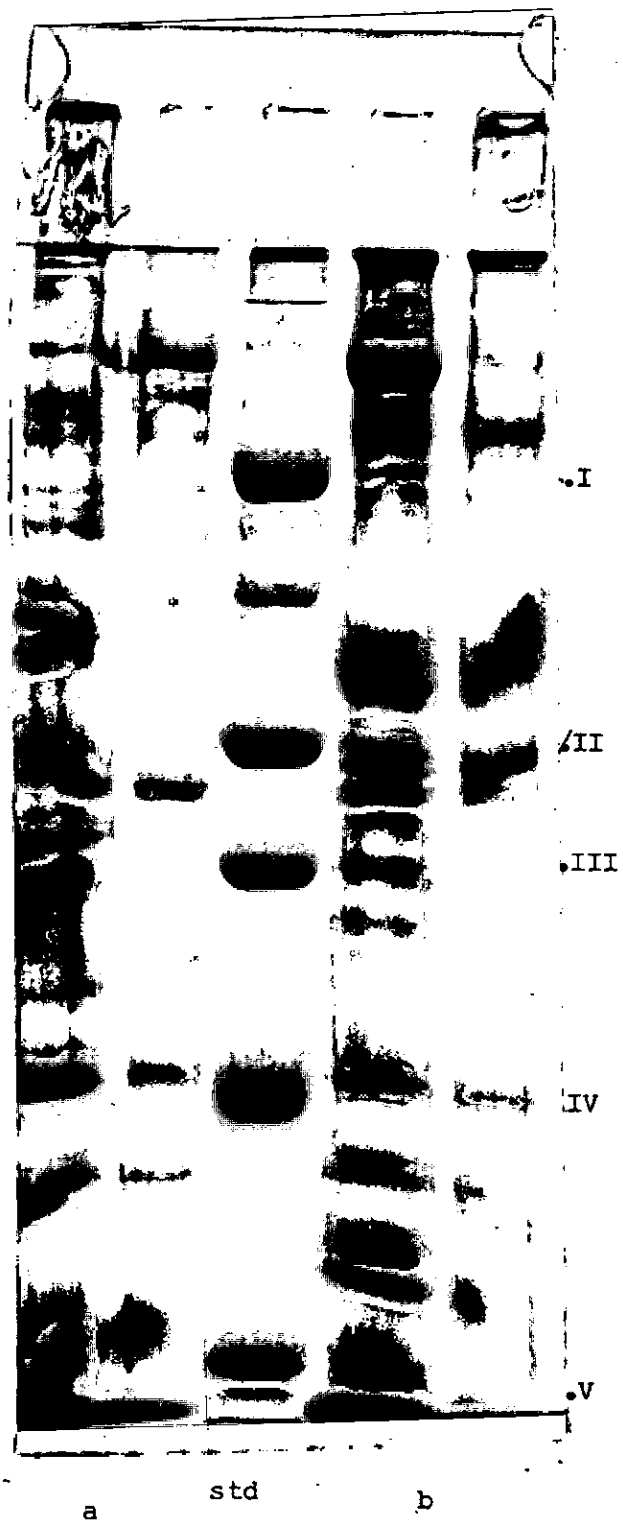

I
II
III
IV
V
a
std
b

Figure 3.45

Migration of partially separated polysaccharidases proteins (Figure 3.35) from a hydrophobic column

a) Fractions 75-80

b) Fractions 81-80

on SDS polycrylamide gel

Protein Standard

I	-	Mr 205,000
II	-	Mr 116,000
III	-	Mr 97,400
IV	-	Mr 66,000
V	-	Mr 45,000
VI	-	Mr 29,000

CHAPTER 4

DISCUSSION AND CONCLUSION

The main function of the saliva secreted is believed to be purely lubricatory during feeding, assisting with the removal of food from the radula and also helping with its passage through the oesophagus (Runham, 1975). In Archachatina this is also believed to be true since little or no activity was associated with the salivary gland extracts when used as enzyme source in assays.

The crop is the largest part of the fore-gut and the site of storage and initial digestion of food, although, in this work, it is suggested that it is main site of extracellular digestion. The crop leads directly into a very simple stomach.

Previous work has traced a simplification of the stomach in more advanced gastropods, involving the reduction or loss of the dorsal caecum, so that in many genera, as in Archachatina, the stomach is little more than a bag within which the food is mixed with digestive juices, in addition to serving as a passage to and from the digestive gland (Carriker, 1947 and Morton, 1955c). This is unlike the situation found in fresh water pulmonates, where the terminal portion of the crop or oesophagus is partly or wholly muscularized to form a gizzard in a place of a stomach (Morton, 1955c).

The intestine is a long thin-walled narrow tube. Unlike in carnivorous pulmonates such as conus in which the

intestine is often short and straight, it is common with herbivorous pulmonates as in Achatina fulica (Ghose, 1963) and Lymnaea (Carriker, 1946) in having long coiled intestines. This seems to be analogus to what is found in fish, where the herbivorous species feeding mainly on algae and higher plants, as in Tilapia, have a very long coiled up intestine, while the carnivorous fishes such as pike have short, straight intestines (Abadom, 1986).

The digestive tract undergoes a sharp bend at the stomach which receives ducts from the digestive gland. In all gastropods the digestive gland is enormous in size compared to the other parts of the alimentary system. Its secretion is taken to the other parts of the tract where required, either by a single digestive duct as in Littorina (Graham, 1949) or by two ducts as in Archachatina (Odiete and Akpata, 1983), Helix (Ghose, 1963), Pterocers (Young, 1932) or by 3 or more ducts as in Gastropteron and Gymnestoma.

In Archachatina both digestive ducts do not open into the stomach but one opens into the stomach and the other into the crop. This is similar to what is found in Achatina (Ghose, 1963), unlike in most molluscs, where most of these digestive ducts open into the stomach. The opening of one of the digestive gland ducts into the crop could be seen as part of the morphological adaptation to suit the new role of the

crop, in some land molluscs, of storage and digestion, instead of conduction as found in the more primitive molluscs.

Although the function of the digestive gland had been a controversial issue for some time, it has been discovered that the cells of the digestive gland are different for different groups of molluscs because of their different food habits and subsequent adaptation (Fretter, 1943 (Onchidella); Ghose, 1963 (Achatina); Creek, 1953 (Acicula)).

The digestive gland has been reported to act as a reservior of calcium, which helps to maintain a constant pH in the stomach (Robertson, 1941) and also a source of calcium required for repairs to damage to the shell (Wagge, 1951).

From the observations on the structure of the dissection of the alimentary canal, supported with the findings of Odiete and Akpata, (1983), it is evident that the digestive gland functions primarily as a secretory organ, sending secretions through the digestive ducts to the connected crop and stomach cavities respectively.

The enzyme distribution experiments reported show a relatively strong sucrose-spliting ability by A. marginata. This is not surprising because when the snails are fed with succulent items containing sucrose,

they fed very well on them and consumed them within a very short time. Sucrose is most probably hydrolysed by an α-glucosidase. This inference is made because raffinose was found not to be hydrolysed by the enzyme extracts from this snail, even though both sucrose and raffinose have a ß-fructofuranosidase bond within their molecule, but this does not seem to be cleaved during hydrolysis. And since sucrose could either be acted on by an enzyme (ß-fructofuranosidase) which cleaves the ß-fructofuranoside bond in its molecule or an α-glucosidase which breaks the α-glycosidic bond in its molecule, it is most likely to be the latter.

Hydrolysis of maltose was found in the crop juice with very weak activities in the other parts of the gut. Although maltose is found free in higher plants, the main digestive function of maltase in vivo is evidently to complete break-down of maltose liberated by the digestion of starch (Kristensen, 1972). The capacity of the crop juice of A. marginata and other parts of its digestive tract extract to hydrolyse lactose was relatively higher than for sucrose and maltose. This is unexpected since it is difficult to imagine the role of a specific enzyme which hydrolyses lactose in the alimentary tract of A. marginata, where lactose is an unlikely natural substrate. Milk of mammals is about the only lactose source known in animals, but it has occasionally been isolated in higher plants (Karrer,

1958). Thus, it is probable that this could be ß-galactosidases, capable of acting on lactose substrate.

The strong cellobiase activity of this snail's crop juice is interesting. Here as in lactose, hydrolysis is conspicuous, even about twice the capacity of lactose. Cellobiose is not accumulated as a free sugar, but used by plants in the biosynthesis of other carbohydrates (Karrer, 1958). Generally, the occurrence of cellobiose is only recognised as the last but one step of the decomposition of cellulose. Kristensen, (1972) suggested that the natural substrate is likely to have universal occurrence, since celiobiose has been demonstrated in other terrestrial, limnic, and marine invertebrates. He analysed that possible compounds would be glucans connected via ß-linked to an aglucon. Amygualin is a ß-glucoside of this type, and is hydrolysed by ß-glucosidase emulsin which also acts on cellobiose. The glucan of amygualin is gentobiose, which has the glucose molecules connected via a ß(1-6) linkage, indicating that the ß configuration at the anomeric carbon is more important for the action of the enzyme than the location of the carbon atom in the second sugar.

Emulsin is a plant enzyme and according to Gottschalk, (1958), it has been established that plant ß-glucosidase from different sources vary in specificity.

Some act on cellobiose, gentobiose and other 3-D-glycosides while others have more restricted activity.

The polysaccharide substrates investigated were broken down much more easily than any of the disaccharides tested. This was also observed by Myers and Northcote, (1958) in their experiments on the carbohydrases of Helix. In both living and decaying plant material, polysaccharides, are important constituents. Cellulose is the most common of the polysaccharides and would be encountered by A. marginata, as this forms its preferred diet.

In the present study, the carbohydrate-hydrolysing capacity of the land snail is highest for cellofas B. The activity of the enzyme extracts was quite low when used on natural cellulose substrate. This is similar to Myers and Northcote (1958)'s experience. They found that natural cellulose gave variable results as a substrate for enzyme assays since the material is heterogeneous in both its physical and chemical structure, and may differ from sample to sample. Kristensen (1972) found poor hydrolysis of native structure carbohydrates such as cellulose with few exceptions such as methylcellulose and alginic acid. Only glucose and carboxymethyl glucose were found from an enzyme hydrolysate of cellofas B, which is one of the Carboxylmethyl celluloses normally degraded in the same way as natural cellulose. As such, it is often

more convenient for substrate assays. However, the distribution pattern of cellulase activity with natural cellulose as substrate was the same when cellofas B was used in enzyme assays in the present work.

The digestion of cellulose by gastropods, and indeed by other animals in which a "cellulase" is secreted is a controversial subject. According to Evans and Jones, (1962), one of the chief difficulties is presented by the fact that many investigators have not indicated precisely the nature of the substrates they have used to demonstrate cellulase activity. And because a true cellulase, (that is an enzyme capable of hydrolysing native cellulose) has not been found in the enzyme preparations made from most pulmonate gastropods' digestive tract, most workers use degraded cellulose and others, soluble derivatives of this substrate (Myers and Northcote 1958; Evans and Jones 1962). How these groups of snails degrade native cellulose in plant cells in its gut naturally needs to be further investigated. Although Florkin and Lozet (1949) suggested that it is probable that native cellulose is partially degraded by the true cellulase of bacterial origin in H. pomatia, but then the hydrolysis is completed by the very active ß-1:4 polyglucosidase present in the crop juice. Yet Odiete and Akpata (1983) in their study on the origin of the crop juice, the enzymes and microflora of the alimentary tract of A. marginata, concluded that the exo-glucanase

necessary to digest native cellulose is produced by the snail itself, since the microflora they isolated were unable to digest native cellulose. The result of this work concurs with Florkin and Lozet's (1949) findings.

Chitinase was detected in the present work only in the crop juice preparation of A. marginata and not in any other part of the gut. The presence of chitinase in molluscs seems to be as controversial as for cellulase (Jeuniaux, 1963; Horiuchi and Lane, 1966. The chitinolytic activity found in gastropods has been attributed by some authors to the presence of bacteria in the digestive tract of the animals (Owens, 1966). Jeuniaux, (1954) observed variable chitinase activity in the crop juice of H. pomatia, which correlated with the concentration of bacteria leading to the conclusion that bacteria are the source of the chitanase in that snail.

Proteolytic activity was found in the different parts of the gut although the snail is largely herbivorous. Achatina, a similar herbivorous pulmonate, does select dead insects and snails from the soil. It has actually been found, as reported by Ghose (1963), that when pieces of meat were supplied to Achatina in the laboratory, they clearly scraped off the last traces of flesh from the bones. Though the level of protease production is low in

A. marginata (present work), it is not surprising since the snail is mainly herbivorous. The distribution of the proteolytic activity in the gut of A. marginata indicates that the greater protein digestion is associated with the digestive gland. This agrees with the result of Cockburn and Reid (1982) who examined protein digestion and specific activity of the isolated endopeptidases and found that the bulk of digestion occurs intracellularly in the digestive diverticula of aeolids.

Olive oil was digested by A. marginata's gut tissue extracts. Although the snail is herbivorous, it also needs lipases to digest plant fat, needed for growth and reproduction (Sio and Teo, 1975). However, Myers and Northcote (1958) were of the opinion that the significant lipase activity they found in the digestive tract extract of H. pomatia, is probably an important factor in its utility as a cytase, contributing to the breakdown of lipoprotein structures in cell walls. Both extracellular and intracellular lipase activity was exhibited by A. marginata. Further evidence of both extracellular and intracellular fat digestion in gastropods is given by Cockburn (1976) who found that intracellular esterases are able to digest longer chained fatty acids molecules than extracellular esterases. He found that in the digestive gland, both acetate and

butyrate substrates are hydrolysed while in the stomach only the former was digested.

The results obtained from the enzyme distribution survey in the present work, show that the crop juice is undoubtedly the most important site for the digestion of fats and dissaccharides tested. The crop tissue extracts may be ranked second in position, while very little enzymes are found in the salivary gland, stomach, and intestine. This is not surprising considering their functions as described earlier. There is a slight difference for the site of polysaccharides digestion. While the crop juice still has the highest hydrolysing capacity to digest the complex sugars, the intestine ranks second in position to the crop juice as an important source of polysaccharidases. This is as expected. For the digestion of more complex types of foods like cellulose, starch and protein, ideally the appropriate enzymes are produced in the anterior region of the gut, so that there is adequate time for appreciable amounts of preliminary digestion, since several different kinds of enzymes could be involved at different stages of the digestion of these food substrates. Polysaccharides are complex sugars that

need to be acted on for longer periods by more than one enzyme, thus the digestion could take place in stages, starting from the salivary gland with the bulk of it taking place in the crop by crop juice, and the uncompleted digestion is completed in the posterior part of the gut, especially in the intestine (which is considerably long in A. marginata) and the digestive gland. This agrees with Kristensen (1972)'s finding that enzymes acting on polysaccharides need long exposure time of the substrates for complete digestion. Generally, when food materials enter the digestive tract of herbivorous snails, they are temporarily stored in the crop and oesophagus before they proceed to the stomach. During this period of temporary storage Teo (1980) suggested that it would be advantageous to the animal to have food digestion initiated in the crop and oesophagus, since the stomach of the snail is relatively small and so the period of time for food to remain will not likely be long.

From observations in the structure of Archachatina's alimentary canal and the results on the distribution of the various enzymes assayed, it is evident that the digestive gland functions as the main site of secretion among various other functions that has been reported by earlier workers, (Odiete and Akpata 1983), except for the completion of protein digestion. Thus, the crop could be the main site of digestion while the digestive gland secretes the bulk of these enzymes, which are

conveyed to the crop and stomach for extracellular food digestion. This is similar to the situation in Helix (Myers and Northcote, 1958) where their proteinases were found to be of the intracellular digestive type. The results of the present study also agrees with that of Boucaud-Camon (1982). He found a strong proteolytic activity in the digestive gland as soon as feeding began in Sepia but a lesser one in the caecum. Cockburn and Reid (1972) reported that the differences between extracellular and intracellular activity in aeolids indicates that the greater portion digestion is intracellular rather than extracellular.

The crop juice, which is found to contain over 90% of the digestive enzyme activity orgniates from the digestive gland (Evan and Jones, 1962b, Ghose 1963). However, Stone and Morton (1958) suggested that the fluid in the gut is often associated with an active bacterial flora which may be responsible for the whole or part of the activity measured. There are contrasting results such as that of Odiete and Akpata, (1983) on Archachatina,, and Payne et al. (1972) on Scrobicularia which show that the snails and bivalve mollusc produced their own extracellular enzymes.

On the whole, the hydrolysis of the carbohydrates in this snail is shared by the salivary gland, crop, stomach, intestinal tissue extracts, the crop juice preparation and the digestive gland, with over 90% of activity with the crop juice. This is analogous with what is found in

mammals where polysaccharidases occur in pancreatic secretion with disaccharidases as well as Polysaccharidases completing the digestion in the small intestine of the gut. It will be interesting to follow up this difference by investigating the molecular genetics of the different vertebrate groups.

The results obtained from the present enzyme survey on carbohydrates show that the digestive system of Archachatina secretes enzymes which are capable of hydrolysing sucrose, maltose, lactose, cellobiose, starch and cellofas B. The ability of the crop juice preparation to digest a wide range of carbohydrates presents the problem of identifying the enzymes concerned.

It is generally believed that a carbohydrase is usually specific to a considerable degree with reference both to the linkage and to monosaccharide constituents of the polymer it splits. As most of these carbohydrases are not absolutely specific, it is likely that the crop juice preparations of Archachatina contain relatively few enzymes each of which can usually hydrolyse a range of substrates of similar chemical constitution. Myers and Northcote (1958) made a similar suggestion, that although, most of the carbohydrate substrates they used in their experiments were degraded by what seemed to be discrete enzyme systems, it is likely that a number of substrates were attacked by the same enzyme.

It was with this in mind, that further investigation into the properties of crop juice enzymes as well as their isolation was carried out.

The hydrolysis of -linked saccharides, viz. sucrose, maltose and starch could be accounted for by the presence of one or more -glucosidase with an optimum pH5.7. In close agreement with these data are those of Myers and Northcote (1958) who observed a low optimum pH5.8 for the hydrolysis of the -glucosides in Helix. The shape of the pH activity curve for the hydrolysis of starch in this work is similar to those of the other -linked saccharides (sucrose and maltose). They all have definitive pH optimal region. Lactose and

cellobiose, which are most likely acted upon by ß(1,4) polysaccharidase and ß-glucosidase respectively have broad pH optima region, with pH optimum of 5.7. This pH value is similar to that obtained by Kesler (1984) for Physa columella. This value is also close to the pH optima of 5.5 recorded for Helix pomatia by Myers and Northcote (1958).

The pH of the crop juice of Archachatina was about pH5.8 which is about the same as the pH value for the activity of all the enzymes tested. This is because under the correct natural conditions of time and temperature, the optimum hydrogen ion concentration as Graham (1931) found experimentally for the enzymes is the actual concentration encountered in those parts of the alimentary canal of the animal where that enzyme is normally effective.

While the enzymes all had very similar pH optima, their behaviour was very different with variation in substrate concentration. Large differences in affinity for different substrates were observed. There was evidence of two enzyme activities against one substrate (lactose). While with matose, there was substrate inhibition. These results suggest that the snail produces a complex system of carbohydrate-digesting enzymes. Initial attempts at separating these enzymes succeeded in separating at least two distinct types of disaccharidases as evident in figure 3.36b, on the hydrophobic column.

"Maltase and sucrase" were distinct from all the other enzymes and eluted as a single peak on a hydrophobic column suggesting that the enzyme that hydrolyses maltose and sucrose is a different protein from "lactase", "cellobiase", "amylase" and "cellulase". This is further confirmed by the result from the SDS gel electrophoresis where the protein migrated in at least two bands with a molecular weight of Mr 300,000 - Mr 97,000 after it has been reduced and denatured for 3-4mins at 100°C. The estimated molecular weight of this protein from the elution pattern on the Bio-Gel P200 column gave a value higher than Mr 200,000. This is in line with the other results discussed earlier, considering the fact that this protein could dissociate to form lower molecular weight proteins when heated in SDS. Similar results have been reported for maltase and sucrase from rat by papain digestion (Flanagan and Fostner, 1978; Lee et. al., 1980), from pigeon (Prakash et. al., 1983), man (Kelly and Alpers, 1973), rabbit (Sivakami and Radhakrishan, 1973) and pig (Sorensen et. al., 1982). Although in all these cases intestinal extracts were used as enzyme sources, and the molecular weight obtained in some cases higher than those obtained in Archachatina, they all behaved in a similar way, when heated in SDS at 100°C.

The results of the chromatography of "maltase" and "sucrase" on the basis of size, net charge, molecular

weight and affinity to specific binding site, indicates the presence of a single enzyme hydrolysing both sucrose and maltose substrates. This is further confirmed by the evidence from the mixed substrate experiment carried out to find if it was the enzyme hydrolysing sucrose and maltose. The result showed that the amount of enzyme activity obtained when both sucrose and maltose were (mixed together and) used as a single substrate was less than the amount of activity obtained when they were used seperately as respective substrates (that is, the addition of the amount of enzyme recorded when maltose was the only substrate plus the amount of enzyme activity recorded when sucrose was used). The result as reported in section 3.7.6 suggest that there is only one enzyme hydrolysing both maltose and sucrose.

"Lactase" and "cellobiase" were eluted together in all the different chromatographic techniques used in separation procedures. Nevertheless, there is still a slight difference in their distribution in most of the different column elution profiles. On the DE-52 column for example, although they came out together within the same protein region at the beginning of the 10mM-20mM phosphate buffer pH6.8 gradient as evident in figure 3.27b, and throughout the gradient in a series of peaks, "cellobiase" was a little retarded on the column, coming out later than lactase in the first activity peak (Figure 3.27b; fractions 26-30).

Further evidence, that there are two different proteins involved in hydrolysing these two ß-glucosides is shown in their distribution in the hydrophobic column elution profile, (Figure 3.34b and 3.35) with a small peak of "cellobiase" (not absolutely specific to cellobiose, since it hydrolysed lactose to a very small degree) coming off first before the large single activity peak for both "lactase" and "cellobiase".

When these two separate activity peaks were pooled and electrophoresed, the proteins in each pool migrated as a high molecular weight band corresponding to a molecular weight of Mr 205,000. This is in line with the estimated value (Mr > 200,000) obtained from the Bio-Gel P200 elution profile. Thus, there are three possible explanations. The first one is that this could be just one enzyme acting on both lactose and cellobiose and the small "cellobiase" peak that comes off first on the hydrophobic column is just a tail of activity coming off at the end of the ammonium sulphate inverse gradient. A second possibility is that, there are two enzymes hydrolysing lactose and cellobiose seperately.

This second possibility agrees with the result of the effect of substrate concentration on rate of "lactase" activity. The data for the double reciprocal plot of reciprocal lactase activity against reciprocal substrate (lactose) concentration gave a fit with the two-enzyme equation, indicating that there could be two

enzymes present in which one has a high Km and V_{max} and the other with a low Km and V_{max}. The simple saturation experiment performed to investigate further the possibility of the presence of two enzymes hydrolysing lactose and cellobiose gave results indicating that it is just one enzyme acting on lactose and cellobiose. The results show that there is competition for the binding site, even at low substrate concentrations, the calculated value of enzyme activity using lactose and cellobiose separately as substrates was more than the actual (experimented) amount of enzyme activity using a mixture of the substrates as shown in table 3.7a and b. This suggests that there is just one enzyme present, and that the two different substrates (lactose and cellobiose) were competing for the binding site, such that one has inhibitory effect on the other substrate. At the higher substrate concentration, there was a clear inhibition of cellobiose hydrolysis by lactose, which means that lactose could be binding the same enzyme site as cellobiose.

The only other explanation for the results from the different chromatography elution profiles and the kinetic studies (indicating there are at least two enzymes acting on each substrate independently) is that of negative co-operativity in which case the enzyme has two or more sub-units. This is most probably (if the protein is split) the case as evident from the series of

"cellobiase/lactase" activity peaks in the DE-52 and hydrophobic column elution profiles. It will be interesting to compare the relatedness of the various components of this ß-galactosidase on the two above mentioned columns, in terms of pH optima and substrate specificity. This has not been investigated at this stage because the amount of respective enzyme solutions from each fraction were just about enough for the various enzymes under investigation, and as such little or nothing was left for further investigation.

In most of the separation procedures, cellulase activity eluted in the same series of activity peaks with "cellobiase" and "lactase" but still showed a different distribution. On the DE-52 column, there are three activity peaks coinciding with those of the ß-galactosidases, but differing in their degree of specificity. However, the largest (broad) peak, which is the third cellulase activity peak, although hydrolysed lactose and cellobiose to a certain extent, appears to be a ß(1-4) polyglucosidase, which also seems to elute from the hydrophobic column (figure 3.35) as fractions 70-80 and 81-90. The difference in movement of these proteins active towards cellofas B in the different columns elution profile indicates a possibility of Archachatina having at least three different types of cellulolytic enzymes in the crop juice, possibly differing in their specificities towards the length of glucose chain attacked and the mechanism

of the degradation. From the ratio of activity of the three main peaks on the DE-52 and hydrophobic column, it seems more likely that there are two different "cellulases" active towards cellobiose and lactose only and a third type which is capable of hydrolysing starch as well.

Whittaker and Merler (1956) reported that the cellulolytic enzyme they purified also attacked many ß(1-4) linked glucosans in an essentially random manner. The elution pattern of cellulase activities on the different columns used compares favourably with that of Helix in giving three fractions of cellulase activity (Myers and Northcote, 1958). Florkin and Lozet (1949) reported that the cellulase of Helix pomatia is of bacteria origin. As suggested by Tracey (1951) in earthworms and later by Myers and Northcote (1959) in Helix, the cellulases of the respective animals are of both bacterial and animal origin. Similar conclusions could not be reached in this work on the cellulolytic enzymes separated on the various columns, since the situation is complex, involving at least three enzymes at a time.

The molecular weight of "cellulase" was estimated to be approximately between Mr 29,000 and Mr 68,000 which is close to that of H. pomatia given as 63,000 ± 15,000 (Whitaker, et al, 1954).

"Amylase" shows a unique elution behaviour on the DE-52 column, which none of the other enzymes exhibited. Thus

this may well be a different enzyme. Amylase activity was eluted in two peaks, with the major peak coming straight through the column without being retarded at all. This peak also hydrolysed cellofas B (a cellulose) to a small extent suggesting that this is possibly a type of amylase that could also act on ß(1,4) polyglucoside bond, that is, a ß-amylase. The second amylase peak coincided with the disaccharidases figure 3.1b and 3.2) peak of activity. It could be a protein complex containing different groups of dissaccharidases, which are also active on starch or it could be that there is really a type of amylase, probably an α-amylase which overlaps with the disaccharidases.

In conclusion, there appears to be at least three types of enzymes digesting the different sugars represented in the natural diet of Archachatina. There seems to be a general α-glucosidase hydrolysing several substrates containing α-glucosidic bonds (maltose, sucrose and starch). While the ß-linked polysaccharidases and ß-linked glucosides are acted upon by a ß-glucosidase (cellobiase/lactose) with a third enzyme, which appears to be an active ß(1-4) polyglucosidase completing the hydrolysis of the polysaccharides. Although each of these enzymes appears to be made up of more than one component, not much can be said about this until a more

detailed investigation is carried out on each of these groups of enzymes separately.

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