This is to certify that the

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α-GALACTOSIDASES OF COWPEAS

(Vigna unguiculata)

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BY

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ABSTRACT

α-GALACTOSIDASES OF COWPEAS

(Vigna unguiculata)

By

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Cowpeas (Vigna unguiculata) are the main source of protein for many people in the world. However, like other legume seeds cowpeas contain α-galactosides which cannot be hydrolyzed and absorbed because of the lack of α-galactosidase activity in the small intestine.

The objectives of this research were to (a) purify and study the properties of cowpea α-galactosidases, (b) study the activity of α-galactosidases in dormant and germinating cowpeas and (c) determine the changes in oligosaccharides during the germination of seeds and the incubation of a paste from the seeds.

Three forms of α-galactosidases, I, II^1 and II^2 were isolated from dormant cowpeas by means of gel filtration and ion exchange chromatography. These forms exhibited different values of optimum activity, pH 5.0, 5.9 and 5.3, respectively. Their optimum activity temperature, 50° C, was the same. The molecular weights (MW) obtained by gel filtration chromatography were approximately 110,720 ± 3780, 29,000 ± 355 and 30,000 ± 368, respectively. SDS-PAGE of these enzymes yielded the following MW values: 30,916 ±

1,042, 29,875 ± 413 and 30,350 ± 550 daltons, respectively. Based on the results obtained (Enzyme I and II^2 were eluted at the same pH and NaCl gradients from ion exchange chromatography columns), enzyme I appears to be a tetramer of enzyme II^2. The K_m and V_{max} were determined using artificial and natural substrates. The stability of enzymes I, II^1 and II^2 during storage for 24 hrs at four different temperatures and at various pH values were studied.

Germination of cowpeas resulted in a significant increase in α-galactosidase activity. The oligosaccharide changes during the germination of cowpeas and the incubation of their paste were studied. Germination did not have a significant effect on the oligosaccharide content during 24 hr. Incubation of a cowpea paste made from germinated seeds resulted in a considerable reduction of the flatus-related oligosaccharides. It appears that the most efficient way to decrease the flatus-related sugar of cowpeas, especially stachyose, is to form a cowpea flour paste and let it stand overnight in a warm place (30° C).

IN THE NAME OF ALLAH
THE MERCIFUL THE COMPASSIONATE

TO

My Father Rashid and My Mother Sabriah

ACKNOWLEDGMENTS

All praise and thanks are due to Allah, Lord of the universe, for His merciful divine direction throughout my study.

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INTRODUCTION

Almost a hundred years ago, an enzyme was isolated from yeast which hydrolyzed melibiose, 6-α-D-galactopyranosyl-D-glucose. This enzyme was first called melibiase and later renamed α-galactosidase (α-D-galactoside galactohydrolase, E.C.3.2.1.22). The new name is due to the discovery that the enzyme hydrolyzes α-galactosides in general. However, it was also noticed that α-galactosidases catalyze trans-α-D- galactosylation reactions.

These enzymes have multiple significance. They are used in molecular structure studies in biochemistry. In plant, animal and microbial physiology they serve to trace the fate of the many α-galactosides present in nature. In applied enzymology, they have been suggested as tools for:

a) Removing the flatus-generating galactosides, namely raffinose, stachyose and verbascose present in legume seeds (beans, peas, cowpeas, soybeans), Figure 1.
b) Facilitating the extraction of sucrose from molasses or increasing the sucrose yield by eliminating raffinose which interferes with the crystallization of sucrose.
c) Converting type-B human erythrocytes to type -o- by removing the α-galactoside residues.

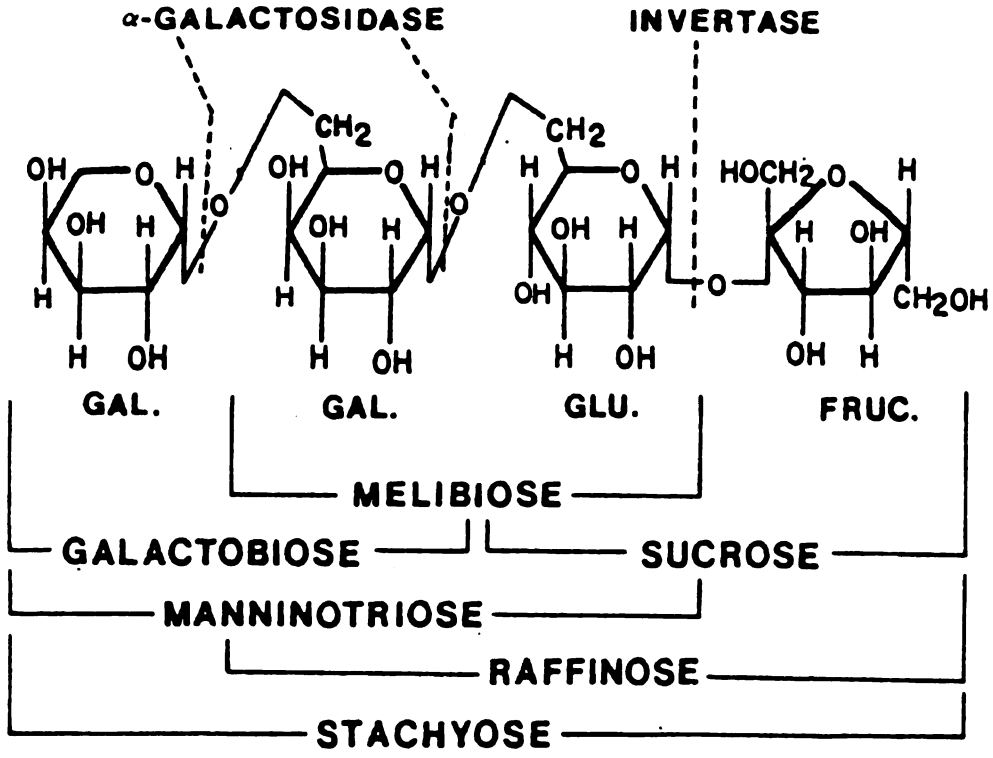


Figure 1. Structure of stachyose and related sugars

Several factors motivated the choice of cowpeas in our study. Cowpeas (Vigna unguiculata) are the main source of protein for many people in Africa. The nutritional value of cowpeas lies in their high protein content, which is 20-25% and is double the protein value of most cereals (Appendix 1).The practice of eating cowpea dishes in combination with cereal foods enhance their protein value (Dovlo et al., 1976). Nevertheless, cowpeas like other legume seeds contain α-galactosides which cannot be hydrolyzed and absorbed in the gut for lack of α-galactosidase activity in the small intestine. In the large intestine,however, these galactosides are fermented by microorganisms and may cause flatulence, distention, cramps and diarrhea. These symptoms can be more severe in young children.

The objectives of this research were to study:

a) The purification and properties of cowpea α-galactosidases.
b) The activity of α- galactosidases in dormant and germinating cowpeas.
c) The oligosaccharide changes during the germination of seeds and the incubation of a seed paste.

LITERATURE REVIEW

OCCURRENCE AND LOCALIZATION

α-Galactosidases (α-D-galactoside galactohydrolase ,E.C.3.2.1.22) have been shown to be widely distributed in plants, animals and microorganisms. As a rule, these enzymes are present in all tissues containing α-D-galactosides (Barham et al., 1971; French,1954; Wollenfels and Malhotra,1961). The galactosyl oligosaccharides rank next to sucrose in their abundance in the plant kingdom (Dey, 1980).

α-Galactosidases in most cases are found intracellularly in various organelles (mitochondria, chloroplasts, microsomes).In E. coli (Burstein and Kepes,1974; Gabriel and Bonner,1957; Budiger,1968) and Aerobacter aerogenes, α-galactosidase is not constitutive but can be induced by the introduction of α-D-galactosidases in the culture media (Bailey and Howard, 1963).

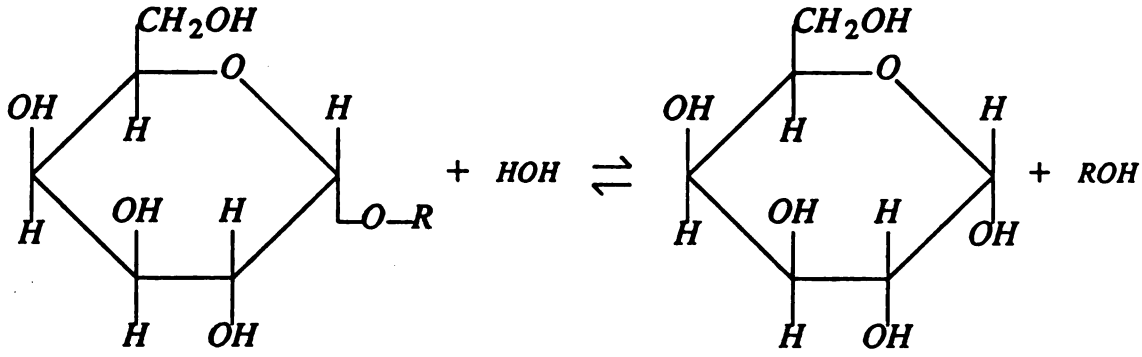
Multiple forms of the enzyme have been found in various parts of seeds (e.g., cotyledon, endosperm and embryo) and root tips (Dey and Campillo, 1984). The α-galactosidase activity is generally located in the water soluble fraction of an extract. However, in several cases the activity was

cell-wall bound (Klis et al., 1974; Thomas and Webb, 1979).The bound enzymes can be extracted by salt solution .

The presence of α-galactosidase has been shown in the protein bodies isolated from seeds (Plant and Moor, 1982; Hankins and Shannon, 1978). Protein bodies are generally the sites of storage proteins and other hydrolytic enzymes (Van der Wilden et al. 1980; Harris and Chrispeels, 1975).

PROPERTIES OF α-GALACTOSIDASES

α-Galactosidases catalyze the following reversible reaction:



where R represents alkyl, aryl, monoglycosyl or polyglycosyl groups.

Dey and Pridham (1969a) reported that most forms of the enzyme are not absolutely specific for the glycone residue and will hydrolyze the structurally related α-D-fucopyranosides and β-L-arabinopyranosides. Water can be replaced by a number of organic galactose acceptors. In

this way simple galactosides and oligosaccharides have been synthesized. De novo synthesis of oligosaccharides is also possible when high concentrations of galactose are incubated with the enzyme (Dey and Pridham,1972). Tanner and Kandler (1968) observed that for an α-galactosidase isolated from Phaseolus vulgaris, the transgalactosylation from galactinol to raffinose (with the formation of stachyose) occurs more readily than hydrolysis.

MULTIMOLECULAR FORMS

Petek and Dong (1961) were first to report multimolecular forms of α-galactosidases. Using alumina column chromatography, they isolated tow separate forms of this enzyme. Later Dey and Pridham (1969a) showed that dormant seeds of V. faba have tow forms of α-galactosidase with different molecular weights. In later work the tow forms of the enzyme (I and II), obtained from Sephadex columns, were re-examined by ion exchange (CM-cellulose) chromatography. Enzyme II was eluted with tow active fractions (II^1 and II^2) (Dey and Pridham, 1972).

Some dormant seeds appear to contain α-galactosidase of only one molecular size. The number of forms of α-galactosidase may be related to the physiological state of the seed (Barham et al.,1971). Forms of α-galactosidases showed different pH optima and Km and Vmax values with

artificial and natural substrates, and also differed in their thermal stabilities (Dey and Pridham, 1969b).

MOLECULAR WEIGHTS

Many dormant seeds contain two forms of the enzyme, one form apparently having a substantially higher molecular weight (MW) than the other. Dey and Pridham (1969b) showed that α-galactosidase I, the high MW form from V. faba, has a subunit structure. This enzyme dissociated into six inactive protein fractions when passed through a Sephadex G-100 column in the presence of 6 M-urea. The α-galactosidases isolated from various sources have different molecular weight. Most of these were estimated by gel filtration and SDS-PAGE and ranged between 25,000-200,000.

SPECIFICITY

A. Hydrolase Activity

In general, change of configuration of hydrogen and hydroxyl groups on any single carbon atom of a glycoside substrate is sufficient to reduce the rate or completely inhibit the hydrolytic action of the corresponding hydrolase. Two main factors govern the rate of hydrolysis of the substrate by α-galactosidases. First, the ring structure must be pyranoid, and second, the configuration of -H and -OH on carbon atoms 1,2,3, and 4 must be similar to that of α-D-galactose. Changes at C-6 of the glycosyl

moiety of the substrate can be tolerated by α-galactosidases as in the case of other glycosidases, such as, β-galactosidase, β-glycosidase and α-mannosidase (Adams et al., 1943; Helferich and Appel, 1932). Hence the glycosides indicated in Figure 2 can be hydrolyzed by the α-galactosidases (Dey and Pridham, 1972). The affinity of the enzyme (1/Km) for the substrate seems to depend largely upon the structural changes in the sugar moiety and follows the order :α-D-galactoside > α-D-fucoside > β-L-arabinoside. This suggests that one of the specific points of attachment of the substrate to the enzyme is through the primary alcohol group of the galactose structure (Dey and Pridham, 1972).

The aglycone group of a substrate may or may not have a marked effect on hydrolysis by glycosidases. Normally, the group does not completely inhibit hydrolysis. Various α-galactosidases are known which hydrolyze the following naturally occurring and synthetic α-D-galactosides :

1) Galactosides: methyl-, ethyl-, n-propyl-, phenyl, o-nitrophenyl-, m-nitrophenyl-, p-nitrophenyl-, m-chloro-phenyl-, and 6-bromo-2-naphthyl α-D-galactosides (Sheimin and Crockar, 1961; Tsou and Su, 1964).

2) Oligosaccharides: melibiose, O-α-D-gal-(1-> 4)-D-gal, melibionic acid, raffinose, stachyose, verbascose, and

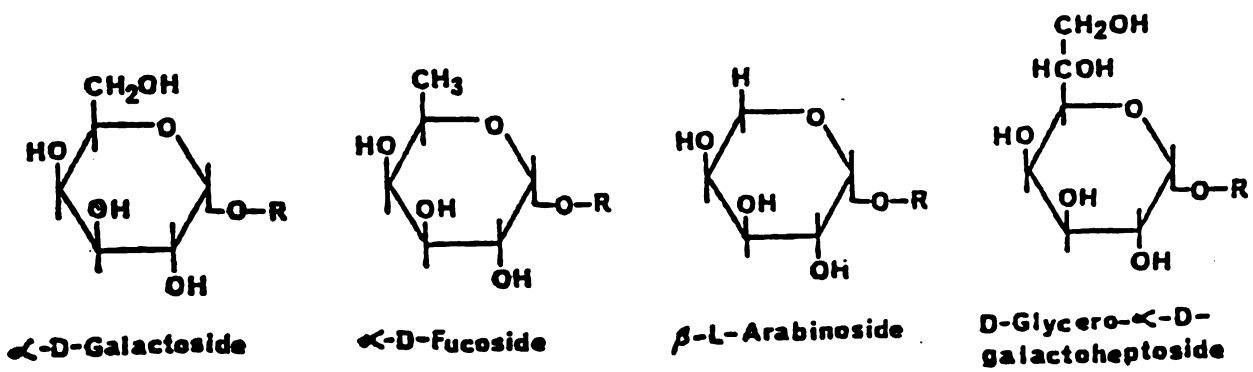


Figure 2. Glycosides hydrolyzed by α-galactosidase

higher homologues (Suzuki et al., 1970; Bailey and Howard, 1963; Bailey, 1962).

3) Polysaccharides: galactomannans are attacked by α-galactosidases from various sources (Bahl and Agrawal, 1969; Courtois and Petek, 1966). These polysaccharides normally have a basic structure cosisting of a backbone of β-1,4-linked D-mannosyl residues to which D-galactosyl residues are attached by α-1,6-linkages. The galactose content varies depending on the plant source. Not all α-galactosidases hydrolyze galactomannans; those that do, appear to remove terminal galactose residues only. Some α-galactosidase can also remove terminal α-D-galactosyl residues from blood group B substance (Zarmitz and Kabat, 1960; Watkins, 1966). In general, aryl α-D-galactosides are better substrates than alkyl derivatives or disaccharides (Dey and Pridham, 1972; Wallenfels and Malhotra, 1961).

High affinity of the enzyme for the substrate does not necessarily correlate with high V_{max} values (Dey and Pridham, 1972). Dey and Pridham (1969a) reported that the Vmax of the enzymes from V. faba is not influenced by the nature of the substituent on the phenyl ring of aromatic galactosides. The affinity (1\Km) shows some tendency to increase when electron-attracting substituents are present (Bailey, 1962; Bailey and Howard, 1963). The conversion of melibiose to melibionic acid, oxidation of the reducing

group, does not appear to affect the rate of hydrolysis (Dey and Pridham, 1972). The rate of hydrolysis decreased with an increase in the chain length, in a homologous series of α-D-galactosides, but in two microbial enzymes the reverse was reported to occur (Petek et al.,1969; Baily, 1963).

French et al.(1953) reported that almond α-galactosidase was able to split the internal galactosidic linkage of stachyose, forming galactobiose and sucrose. Coffee α-galactosidase can only cleave stachyose in a stepwise fashion starting from the nonreducing end. α-Galactosidase of Streptococcus bovis hydrolyzes verbascose in a similar stepwise mode (Bailey and Howard, 1963).

B. Transgalactosylation

Blanchard and Albon (1950) were first to observe the transgalactosylase activity of yeast α-galactosidase. They showed that galactose could be transferred from melibiose to a second melibiose acceptor molecule with the formation of manninotriose. Transgalactosylation reactions to aliphatic hydroxyl groups are normally accompanied by a pronounced hydrolytic activity. An α-galactosidase from wheat bran with a high transfer/hydrolysis ratio has also been described by Wohnlich (1963). Li and Shetlar (1964) have shown with pneumococcal α-galactosidase that phosphorylation, reduction or oxidation of either C-1 or C-

6 of D-glucose or D-galactose destroyed the acceptor properties of these hexoses. They have further reported that the incubation of α-galactosidase from Calvatia cyanthiformis with o- or p-nitrophenyl α-D-galactosides gave rise to two oligosaccharides containing only D-galactose.

In general, it appears that α-galactosidases preferentially transfer galactosyl groups to the primary alcoholic groups of the acceptor (Dey and Pridham, 1972). Galactosidases are known to synthesize oligosaccharides when incubated with monosaccharides, and this procedure has been used for the preparation of several glucose and galactose derivatives (Helferich and Leete, 1955; Peat et al., 1952; Stehen et al., 1962). Yeast α-galactosidase, for example, in a solution of ≈ 17% D-galactose polymerizes 7.5% of the sugar and about 60% of this appears as 6-O-α-D-galactosyl-D-galactose (Clancy and Whelan, 1960; 1967a). By allowing a mixture of D-galactose and N-acetyl-D-glucosamine to react in the presence of yeast α-galactosidase, Clancy and Whelan (1967b) have also produced 3-O- and 6-O-α-D-galactosyl-N-acetyl-D-glucosamine.

KINETIC PROPERTIES

A. Effect of Substrate Concentration

p-Nitrophenyl α-D-galactoside has been shown to be inhibitory at high concentrations with both molecular forms

(I and II) of α-galactosidase from V. faba (Dey and Pridham, 1972). In contrast, galactose-containing oligosaccharides, such as melibiose and raffinose, do not show any inhibitory effect (Dey and Pridham, 1969b; Malhotra and Dey, 1967). Competitive inhibition of the enzyme has generally been observed with D-galactose (Dey and Pridham, 1972).

B. Effect of Temperature

α-Galactosidases display varying degrees of stability depending on their origin (Dey and Campillo, 1984). Crude enzyme from E. coli is extremely unstable, whereas the purified enzyme can be lyophilized and stored at 4° C for up to 2 months without loss of activity (Jones and Kosman, 1980). The ionic strength of α-galactosidase solutions may also be an important factor in stability (Carnie and Porteons, 1963). Most α-galactosidases behave normally in the sense that the rate of the enzyme-catalyzed reaction increases to an optimum value with increasing temperature until inactivation of the enzyme occurs (Dey and Campillo, 1984).

C. Effect of pH

The pH optima of α-galactosidases vary. Most α-galactosidases show single broad pH optima, but in several cases the enzymes exhibit two peaks. The tetrameric enzyme from mung bean was converted predominantly into the

monomeric form on storage in pH 7.0 buffer at -10° C for 2 years. However, on dialysis of the stored enzyme at pH 4.0 reconversion to the tetramer occurred (delCampillo et al., 1981).

D. Effect of Inhibitors

1. Group specific reagents. α-Galactosidase from Aerobacter aerogenes can be inhibited by sulphydryl reagents such as N-ethyl malcimide and iodoacetamide (Flowers and Sharon, 1979). On the other hand, α-galactosidases from spinach leaves, sweet almond, and V. faba are not specifically inhibited by such reagents. Thus not all α-galactosidases require -SH groups for activity (Somogyi, 1945; 1952; Fielding and Hough, 1971). Photooxidation in the presence of methylene blue inactivated α-galactosidases from sweet almond and V. faba (Somogyi, 1945; 1952; Dey and Pridham, 1972).

2. Metal ions. The Ag^{+} inhibition of V. faba enzyme I may be attributed to combination with carboxyl and /or histidine residues in the active site (Dixon and Webb, 1964). Ag^{+} and Hg^{++} ions strongly inhibited α-galactosidases. α-Galactosidase I from V. faba, which inactivated by Ag^{+}, regained most its activity on dialysis against McIlvaine buffer (pH 4.0 or 6.0), particularly in the presence of cysteine. The inhibitory effect of Ag^{+}

decreased with low concentrations of galactose. Such protection was observed only when galactose was added before the addition of Ag+. Thus, Ag+ is a competitive inhibitor (Dey and Pridham, 1969a). Hg^{++} is a potent inhibitor of several α-galactosidases, which suggests reaction with thiol groups.

3. Sugars and their derivatives. Suzuki et al. (1966) showed that D-galactose is a powerful and competitive inhibitor of α-galactosidases. L-Arabinose and D-fucose also inhibited the enzymes as structural analogues of D-galactose, whereas their enantiomers were ineffective (Dey and Pridham, 1969a). It has been established that 2-deoxy-D-galactose, D-glucose, D-mannose, D-xylose, and D-ribose do not inhabit α-galactosidases (Suzuki at el., 1970; Dey and Pridham, 1969a). Dey and Pridham (1969a; 1969b) have shown that various α-D-galactosides were competitive inhibitors of α-galactosidases.

MECHANISM OF ACTION

Because of insufficient knowledge of the chemistry and kinetics of the enzymes from most sources, few facts are available regarding the mechanism of action of α-galactosidases (Dey and Pridham, 1969a; 1969b; Dey, 1969).

So far there have been no studies on bond fission by α-galactosidases, although by analogy with other glycosidases it is likely that the galactose-oxygen bonds of substrates

are cleared. Nuclear magnetic resonance and polarimetry studies have clearly shown that the liberated galactosyl residues possess the same anomeric configuration as the substrate in sweet almond α-galactosidase reactions.

Specificity studies on sweet almond α-galactosidase with aryl α-D-galactosides show that the charges of the hydroxyl groups of the aglycon has a noticeable influence on the rate of enzymic hydrolysis. Thus, the hydrolytic reaction may be attributed to the presence of basic and acidic groups at the active site. These groups were identified to be the carboxyl (deprotonated) and imidazolium (protonated) groups, respectively (Dey and Pridham, 1972). A similar behavior is also observed with the V. faba enzyme I (Dey and Pridham, 1969a; 1969b; Nath and Rydon, 1954). It was further shown with the almond enzyme that binding of p-nitrophenyl α-D-galactoside to the active site lowers the pK of the aglycon dissociating on the acidic side and raises the value of the aglycon dissociating on the alkaline side of the pH optimum (Dey and Pridham, 1969b).

A "two-step" mechanism has been postulated for the action of sweet almond α-galactosidase, based on previous results (Dey, 1969). The aglycon is cleaved by the concerted action of carboxyl and imidazolium groups. This is followed by a reaction with an acceptor molecule (R'OH),

which may be water or an aliphatic alcohol, resulting in hydrolysis or transfer products. It is possible that the electrophilic attack of the imidazolium group alone is sufficient to cleave the glycosyl-oxygen bond with the formation of a carbonium ion at C-1 of the galactose moiety. In the complete two-step mechanism, two Walden inversions probably occur, resulting in the retention of the anomeric configuration in the final product (Dey and Campillo, 1984).

A "one-step" mechanism of action also has been suggested (Bahl and Agrawal, 1969). This involves the formation of a ternary complex of the enzyme, substrate, and acceptor with the carboxyl and the imidazolium groups playing a similar role, as in the two-step mechanism.

Recently, Mathew and Balasubramaniam (1987) proposed another mechanism of action of coconut α-galactosidase. The presence of two carboxyl groups, a tryptophan and a tyrosine at or near the active site of α-galactosidase was indicated. Based on this a new mechanism of action for α-galactosidase was suggested in which the ionizing group with a pK_a of 3.5 is a carboxyl group involved in stabilizing a carbonium ion intermediate. The ionizing group with a pK_a of 6.5 is a carboxyl group perturbed due to the presence of a hydrophobic residues in its vicinity which donates a H^+ ion in catalysis (Figure 3).

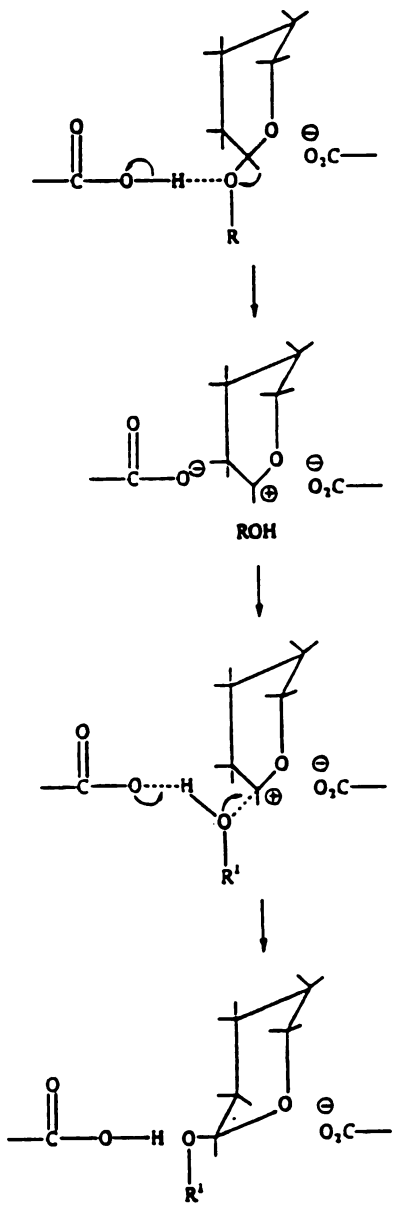


Figure 3. Mechanism of action of α-galactosidase

DETECTION AND METHODS OF ASSAY

The reducing disaccharide melibiose, or the non reducing trisaccharide raffinose, are the natural substrates of the plant enzyme and are often used for the enzyme assay (Delente and Ladenburg, 1972; Mital et al., 1974). The liberated hexoses can be measured by monitoring the increase in reducing power. Alternatively, the liberated D-galactose can be measured using D-galactose dehydrogenase (Dey and Kauss, 1981; Kurz and Wallenfels, 1970); in the case of melibiose, free glucose can be measured using glucose oxidase (Lioyd, 1966).

Synthetic substrates, such as phenyl-, ortho-, or para-substituted nitrophenyl α-D-galactosides are commonly used for routine enzyme assays. The liberated phenol and nitrophenols are estimated using the Folin reagent (Layne, 1957) and the absorbance of the yellow compound at 400-420 nm under alkaline condition, respectively. Plant α-galactosidases do not have cofactor requirements for activity (Dey and Campillo, 1984). However, in Escherichia coli, the cell-free α-galactosidase extract requires Mn^{++} and NAD for activity (Schmitt and Rotman, 1966).

The enzyme can be located on gels by 4-methylumbelliferyl α-D-galactoside. The liberated aglycone, which is water-insoluble, fluoresces under UV light (Dey and Wallenfels, 1974). Other substrates used for this

purpose are 1-naphthyl α-D-galactoside or 6-bromo-2-naphthyl α-D-galactoside (Hadacov'a and Benes, 1977; Benes and Hadacov'a, 1980; Williams et al., 1977; 1978; McCleary, 1979). The liberated water-insoluble aglycons are coupled with Fast Blue BB, resulting in the formation of a colored zone.

The enzymatic hydrolysis of PNPG by α-galactosidase liberates p-nitrophenol, which has a pK_a of 7.2. p-Nitrophenol in alkaline environment (1 to 2 pH units above its pK_a value) develops a deep yellow color suitable for quantitative measurement of the enzyme activity. Advantages of this method include simplicity, accuracy, sensitivity and fast results (Hough and Jones, 1962). The alkaline compounds, used to develop the color of p-nitrophenol, also stop the enzymatic reaction by raising the pH (5 ml of 0.1 M Na_2CO_3 were used). Figure 4 shows the mechanism of the chemical reactions occurring during the enzymatic hydrolysis of PNPG by α-galactosidases and the color development in the alkaline environment.

PURIFICATION

The separation of the multiple forms of plant α-galactosidases have been achieved by applying methods that resolve macromolecules according to their molecular weight (gel filtration) and ionic characteristics (ion-exchange chromatography, electrophoresis, and isoelectric focusing)

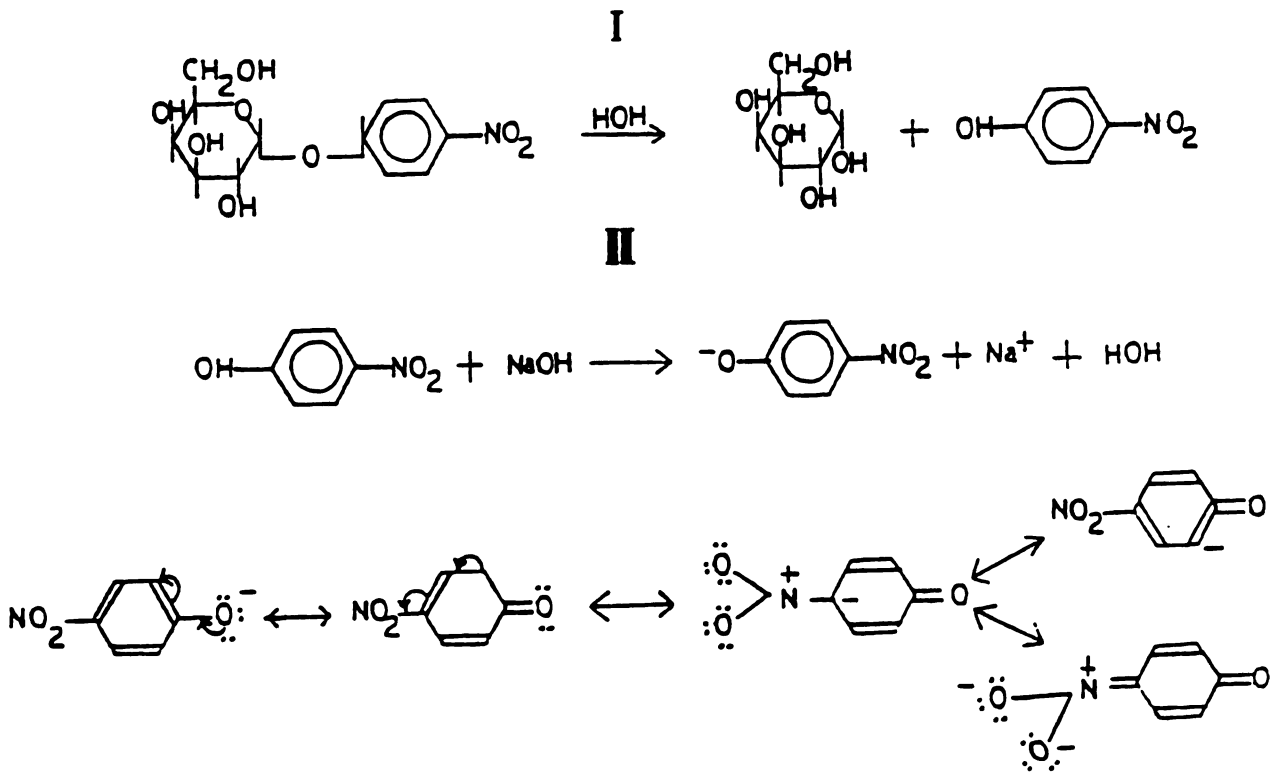


Figure 4. Enzymatic release of p-nitrophenyl from PNPG (I). Color development by increasing the pH of the p-nitrophenol solution (II)

(Hadacov'a and Benes, 1977; Williams et al., 1977; 1978). The procedure for extracting the enzyme from tissues determines the level of the total enzyme as well as the multiple-form composition.

The usual techniques of isolation include ammonium sulfate and organic solvent fractionations, heat treatment, acidification, ion exchange, gel chromatography and isoelectric focusing. Affinity chromatography has been used to separate several α-galactosidases. Melibiose-sepharose (delCampillo and Shannon, 1982; Mapes and Sweeley, 1972) and melibiose-Agarose affinity columns were used. Highly purified and apparently homogeneous α-galactosidases have only been isolated in a few cases (Dey and Pridham, 1969; Lee and Wacek, 1970).

ROLE OF THE ENZYME

The primary role of α-galactosidases in the storage organs of plants is to mobilize the reserve α-galactosyl-containing oligo- and polysaccharides (Dey and Pridham, 1972; Pridham and Dey, 1974; Dey, 1978; 1980). A low level of the enzyme exists in the early stage of germination of fenugreek seeds. At later stages, increase in the enzyme level coincides with galactomannan degradation in the endosperm. The embryo has a relatively high level of the enzyme, which was found to remain constant during germination and hydrolyze the raffinose family of sugars

(Reid and Meyer, 1973; McCleary and Matheson, 1974; Sciler, 1977).

The role of α-galactosidase in fruit is not clear; it is probably involved in the degradation of oligosaccharides. This enzyme participates in the control of internal osmotic pressure in the unicellular alga Ochromonas (Kreuzer and Kauss, 1980; Dey and Kauss, 1981).

CHANGES IN α-GALACTOSIDASE DURING SEED MATURATION

Pridham and Dey (1974) reported that examination of the elution patterns from Sephadex gels and total activity of α-galactosidases in immature seeds has shown that the small, green beans of V. faba contain only a low level of enzyme II and that this increased with maturation. Following this stage, a marked increase in the specific activity occurred which can be related to the appearance of enzyme I and a decrease in the activity of enzyme II.

OLIGOSACCHARIDES AND α-GALACTOSIDASE

It is well known that consumption of legume seeds by humans may result in gastrointestinal distress and flatulence. Nausea, cramps, and diarrhea may also occur in varying degrees (Rackis et al., 1970a), depending upon the legume consumed. Although the presence of raffinose and stachyose in the active gas-producing fraction of beans has been demonstrated, positive identification of all

flatulence-causing factors has not been made (Rackis et al., 1970b; Murphy et al., 1972; Steggerda et al., 1966).

The α-galactoside sugars raffinose and stachyose are not hydrolyzed or absorbed by the mammalian digestive system (Taeufel et al., 1965). Mammalian digestive juices lack the α-galactosidase (EC. 3.2.1.22) required to hydrolyze these low molecular weight oligosaccharides. The sugars pass into the lower bowel, ileum and colon, where they may be fermented by various resident bacteria with the production of gas. Major production of hydrogen, carbon dioxide, and methane gases by normal intestinal microflora takes place (Richards et al., 1968).

In attempt to reduce or eliminate the flatus activity of legumes, several approaches have been taken. Induced seed germination (Hsu et al., 1973) ethanolic and aqueous extraction (Rackis et al., 1970a; Kim et al., 1973), and adjustment of pH, temperature, and moisture content (Becker et al., 1974) have been reported to decrease raffinose and stachyose levels in beans. Antibiotics have been successfully employed to suppress microbial activity in the lower ileum and thereby reduce flatus (Murphy and Calloway, 1972; Richards and Steggerda, 1966).

GERMINATING LEGUME SEEDS

There is little doubt that seed α-galactosidases play an important role in the early stages of germination by hydrolyzing galactos-containing oligpsaccharides and, hence, provide metabolites for the developing seedling (Pridham and Dey, 1974; Courtois and Percheron, 1971; Dey and Campillo, 1984; Dey et al., 1986a). It is not known how the enzymes and their natural substrates are prevented from interacting during the maturation of seeds. The glycoprotien/lectin nature of the enzyme offers various possibilities of in vivo compartmentation /inactivation of the enzymes (Dey and Del, 1984; Dey and Pridham, 1982; Dey et al., 1986b) and the carbohydrate chains may be involved in the transport of these enzymes between compartments (Chrispeels, 1983; Dey and Campillo, 1984).

McCleary and Matheson (1974) reported that germinating seeds of lucerne, guar, carob and soybean initially depleted raffinose series oligosaccharides and then galactomannan. This depletion was accompanied by a rapid increase and then a decrease in α-galactosidases level.

CHANGES IN α-GALACTOSIDASES DURING SEED GERMINATION

Over the first 12 hr of V. faba germination there is rapid fall in total and specific α-galactosidase activities followed by an increase to about 50% the original value. After 24 hr, no marked change in activities occur up to 6

days (Pridham and Dey, 1974). The Sephadex gel elution patterns over this period show first a rapid decline in enzyme I and then a slow increase in the level of enzyme II (Pridham and Dey, 1974). The total activity after 6 days is still much lower than the original activity of the mature seeds in V. faba. It is possible that at this time starch takes over as the major reserve of the cotyledons (Pridham and Dey, 1974).

PHYSIOLOGICAL SIGNIFICANCE OF α-GALACTOSIDASES

In higher plant tissues, galactose-containing oligo- and poly-saccharides are common carbohydrate reserves, particularly in seeds. There is now little doubt that α-galactosidases function as hydrolytic agents in the utilization of these compounds as sources of energy and cell metabolites. (Pridham and Dey, 1974). The enzymes may also play an important role in the metabolism of galactolipids (Sastry and Kates, 1964) and in the function of chloroplast membranes (Bomberger and Park, 1966; Gatt and Baker, 1970). Few observations appear to have been made on the role of α-galactosidase in the animal kingdom. The enzyme does occur in brain tissues; in vivo it may be involved in the hydrolysis of digalactosyl diglycerides (Subba and Pieringer, 1970).

POSSIBLE APPLICATIONS

Sugar Factory

Crystallization of beet sugar may be further improved and the yield of the sugar increased if raffinose in the beet juice or molasses can be removed or decomposed by some method. Many workers successfully removed raffinose from molasses by incorporating crude preparations of α-galactosidase isolated from various microorganisms.

Suzuki et al. (1969) studied the hydrolysis of raffinose in beet molasses. They found that 80% of the raffinose was hydrolyzed by the α-galactosidase obtained from the mold Mortierella vinacea. The α-galactosidase formation by the mold was induced by sugars such as galactose, melibiose, raffinose and lactose.

Soymilk

Many attempts have been made to eliminate the oligosaccharides from soybean and its products by soaking, soaking and germination, fermentative processes, water extraction and ratio and by ultrafiltration (Kim et al., 1973; Mital et al., 1974). Enzyme treatment by microbial α-galactosidase offers a promising solution for the elimination of oligosaccharides from soymilk (Sugimoto and Van, 1970; Smiley et al., 1976; McGhee et al., 1978).

Cruz and Park (1982) reported that the production of extracellular α-galactosidase by Aspergillus oryzae was

induced by the addition of soybean carbohydrates to the culture medium. Addition of only stachyose or raffinose induced slight production of α-galactosidase. Treatment of soymilk with α-galactosidase for 2 hr resulted in complete despairing sucrose and a significance decrease in raffinose and stachyose content. Sugimoto and Van (1970) removed all the galactosaccharides from soymilk in three hours by treating it with α-galactosidase from A. satoi.

Blood-group-B

α-D-Galactosidases may be useful in studies of blood-group substances or other glycoconjugated cell surface (Harpaz et al., 1975). Hydrolysis of the terminal α-D-galactopyranosyl moieties of blood-group-B substances have been reported using α-galactosidases from different sources (Watkins, 1956; Hakomori et al., 1971; Oishi and Aida, 1972). Harpaz et al. (1975) isolated a coffee bean α-D-galactosidase which converted human type-B erythrocytes to type O. Pressey (1984) concluded that α-galactosidases isolated from tomatoes may be employed to convert group B erythrocytes to type O.

EXPERIMENTAL PROCEDURE

MATERIALS

The materials used in the experiments and their sources are listed below.

1. Cowpeas or Black-eye peas were purchased from California.

2. p-Nitrophenyl-α-D-galactoside (PNPG) free of p-nitrophenol (Sigma Chemical Co, St. Louis MO.).

3. Molecular weights standards used in SDS-PAGE and Sephacryl S-200 (Sigma).

4. Sucrose, raffinose and stachyose (Sigma).

5. β-D-galactose dehydrogenase was obtained from recombinant E. coli, using Pseudomonas fluorescent gene, suspended in 3.2 M $(NH_4)_2SO_4$, pH approximately 6.0, 58 units/mg of protein (Sigma).

6. Buffer solutions: MacIlvain (0.1 M citric acid and 0.2 M Na_2HPO_4), potassium phosphate and Tris buffers were prepared according to the Biochemists Handbook (1961) and adjusted to the desired pH level electrometrically.

7. Sephadex G-100 and G-200 (Pharmacia, Uppsala, Sweden).

8. CM-cellulose was purchased from Whatman (England), Accl-CM-cation (Waters Assoc. cationic resin).

9. SE 600 Series Vertical Slab Unit Electrophoresis Apparatus (Hoeffer Scientific Instruments, San Francisco, California).

10. Amicon pressure cell to concentrate the enzymes (Amicon Division, W.R. Danvers, MA.).

11. Beckman DU single beam absorption spectrophotometer.

12. HPLC system from Waters Assoc. Milford, MA.

METHODS

In order to achieve the objectives of the research, several methods were used. All the plots in the results were drawn using a computer program (Plotit).

ENZYME ASSAY

α-Galactosidase was assayed at 30° C by addition of enzyme solution (0.1 ml) to a mixture of McIlvaine buffer, pH 5.5 (0.25ml), and 12 mM p-nitrophenyl α-D-galactoside (0.05ml). After 15 min the reaction was stopped with 5 ml of 0.1M Na_2CO_3. The released p-nitrophenol was measured at 405 nm. The blank was prepared by adding either the enzyme solution or the substrate solution to the mixture after adding 5 ml 0.1 M Na_2CO_3.

α-Galactosidase activity was expressed either as A_{405}/g dry wt. or katals (Kat). One katal is the amount of enzyme activity that transforms 1 mole of substrate per

second. Specific activities were expressed as nkat/mg of protein. Protein determinations were made by the method of Lowry (1951) as modified by Cooper (1977) with crystalline bovine serum albumin as standard.

p-NITROPHENOL STANDARD CURVE

A standard curve was prepared by measuring the absorbance at 405 nm of p-nitrophenol concentrations ranging from 0.005-0.5 umoles/ml. The following mixture was used for each measurement: 0.35 ml MacIlvain buffer pH 5.5, 0.05 ml p-nitrophenol of correct dilution and 5.00 ml 0.1 M Na_2CO_3 for color development. All measurements were done in triplicate. The buffer solution was used as blank.

ENZYME ISOLATION AND PURIFICATION

Cowpeas were ground in a Wiley-Mill and extracted with McIlvaine buffer, pH 5.5, (1g seed powder / 2ml buffer) for 1 hr at 4° C. The slurry was centrifuged at 20,000 x g for 40min and the residue discarded. The pH of the supernatant was lowered to 3.5 by gradual addition of 1 M citric acid. The precipitated protein was discarded after centrifugation at 16,000 x g for 40 min. The pH of the supernatant was raised to 5.5 with Na_2HPO_4 solution and brought to 25% saturation with $(NH4)_2SO_4$. The precipitated protein was discarded and the supernatant was made 55% saturated with $(NH_4)_2SO_4$. The precipitated protein was collected by

centrifugation, resuspended (volumes; Tables 1 and 2) in McIlvaine buffer, pH 5.5, and dialysed overnight against the same buffer (2 l). This dialysed fraction was applied to a 1.9 x 70 cm Sephadex G-100 column and eluted with McIlvaine buffer, pH 5.5 , containing 0.1 M KCl. The fractions for each peak were pooled and concentrated separately by ultrafiltration under N_2 using an Amicon Y_{m10} membrane. Two different purification procedures were used after this step.

Procedure A

Enzyme I and II were dialysed against McIlvain buffer, pH 4.0 and 3.0 respectively. The dialysed samples were applied to a 0.9 x 10 cm Accel-CM-cation exchange column and eluted with McIlvaine buffer containing 1 M NaCl at a flow rate of 20 ml/hr. The pH gradient used to elute Enzymes I and II ranged between 4.0 to 7.0, and 3.0 to 6.0, respectively. The elution resulted in resolving α-galactosidase II in two enzyme fractions, II^1 and II^2.

Procedure B

Enzymes I and II from the gel filtration on G-100 were dialysed against McIlvain buffer, pH 3.5 and applied to a 0.9 x 14 cm, CM-cellulose column equilibrated with the same buffer. The flow rate was 25ml/hr. Following sample application, the column was washed with the buffer and elution of protein was carried out in a linear NaCl

gradient (0.05 to 0.4 M) in the same buffer. The elution resulted in resolving α-galactosidase II in two enzyme fractions, II^1 and II^2. All three enzymes I, II^1 and II^2 were recycled through the CM-cellulose column. The samples were stored at -20° C in 1 ml fractions until used. An outline of the purification scheme is shown in Figure 5.

MOLECULAR WEIGHT DETERMINATION

Gel-filtration and SDS-PAGE were used to determine the molecular weight of the enzymes.

Gel Filtration Chromatography (Sephacryl S-200)

The advantages of using gel chromatography to determine the molecular weight of the enzymes (Freifelder, 1976) are:

1. Enzymes are not affected by chromatography, because there is virtually no adsorption.

2. The zone spreading is less than other chromatographic techniques.

3. The elution volume is related in a simple manner to molecular weight.

4. The MW of an impure protein may be determined using this procedure if a specific detection test is available.

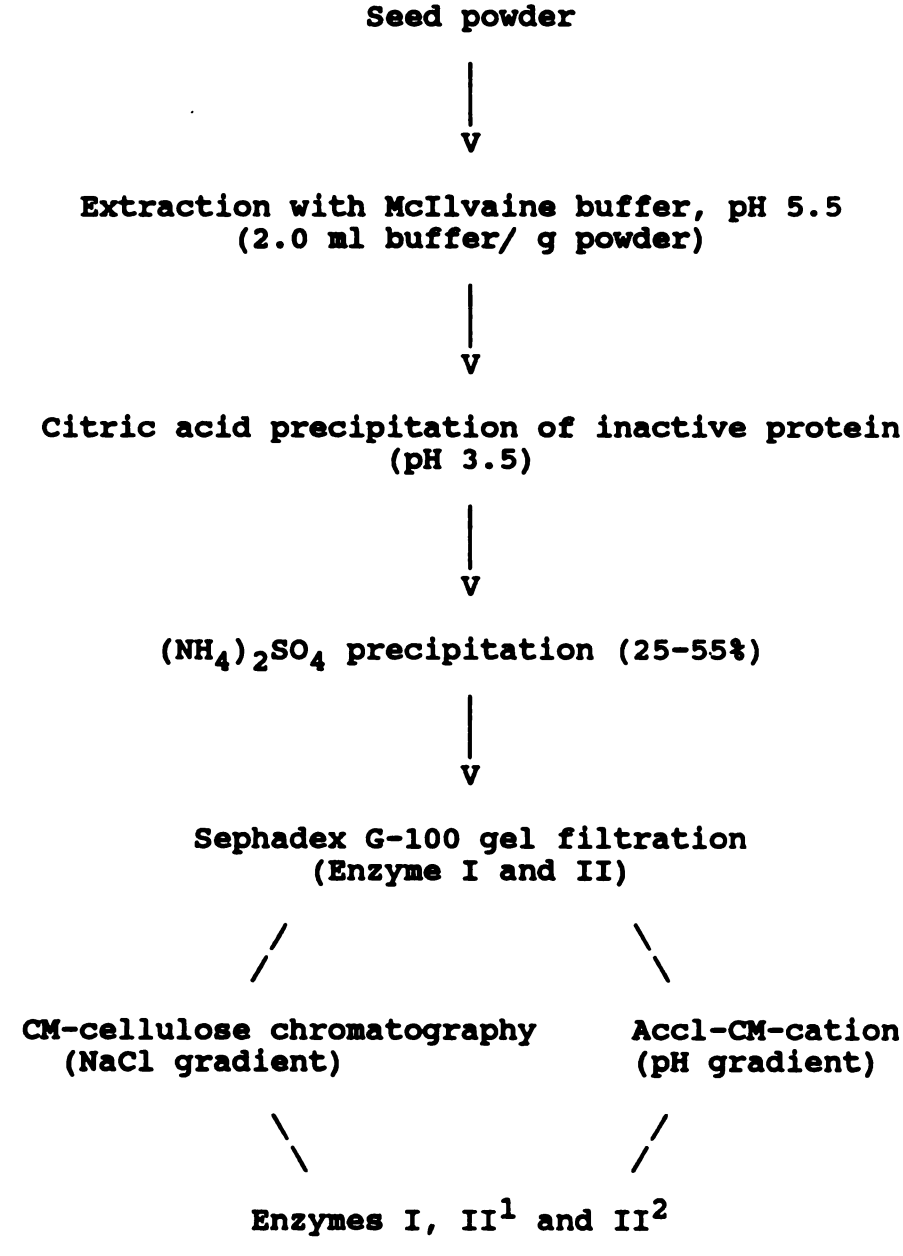


Figure 5. Outline of the purification scheme for α-galactosidases from cowpea

Disadvantages of gel chromatography

1. V_e/V_0 (V_e is the elution volume and V_0 is the void volume) may be temperature dependent.

2. Unreliable MW may be obtained if protein forms a complex with the gel, contains a large amount of carbohydrate, aggregates to larger complexes, or dissociates into subunits (Sigma, 1986).

3. In determining void volume, it is inadvisable to mix Blue Dextran with standards or sample protein because many proteins bind to Blue Dextran (Sigma, 1986).

The following steps were followed to determine the molecular weight of the enzymes using gel filtration.

1. Molecular weight markers (from Sigma)

The following protein MW standards were used:

. Cytochrome C, Horse Heart (MW ≈ 12,400).

. Carbonic Anhydrase, Bovine Erythrocytes (MW ≈ 29,000).

. Albumin, Bovine Serum (MW ≈ 66,000).

. Alcohol Dehydrogenase, Yeast (MW ≈ 150,000).

. Blue Dextran was used as a tracking dye (MW ≈ 2,000,000).

2. Gel preparation

McIlvaine buffer, pH 7.0 (Enzymes II^1 and II^2) and pH 5.0 (Enzyme I) containing 0.1M KCl was used as the equilibration and elution buffer. Sephacryl S-200 was prepared in the same buffer. The resin was suspended in about 2 volumes of buffer, the gel allowed to settle for

15-30 min, and the smallest particles decanted. The last step was repeated. The gel suspension was degassed before use.

3. Column packing and equilibration

A slurry of approximately equal volumes of buffer and gel was poured into a vertical column that was one-third filled with buffer. The slurry was allowed to settle for about 15 minutes without flow, then the excess buffer was drained through the gel bed. Gel slurry was added continuously until a bed height of 90 cm was obtained. The inner diameter of the column was 1.45 cm. The buffer reservoir was connected to the top of the column and 2-3 column volumes of buffer was allowed to pass through the column. The final height of the column was 87 cm. This column was built and run at 4° C. The column was continuously eluted with buffer when not in use (Sigma, 1986).

4. Void volume determination

Blue Dextran was dissolved in equilibration buffer. Two ml of the Blue Dextran (2.0 mg/ ml) were carefully applied to the column to determine V_0 and to check column packing. Fractions of 2.3 ml were collected. The flow rate was 23 ml /hr. V_e for Blue Dextran and the V_0 for the column were determined spectrophotometrically at 280 nm.

5. Elution volume determination for protein standards

Individual protein standards were dissolved in equilibration buffer at the following concentrations (Cooper, 1977; Andrews, 1964): Cytochrome C (2 mg/ml), Carbonic Anhydrase (3 mg/ml), Albumin (10 mg/ml) and Alcohol Dehydrogenase (5 mg/ml).

The following proteins were mixed (1:1) and run together (2.0 ml) on the column: Cytochrome C with Albumin and Carbonic Anhydrase with Alcohol Dehydrogenase. Two ml protein standards were applied to the column and fractions were collected. The V_e for the protein standards were determined spectrophotometrically (absorbance at 280 nm) by measuring the volume of effluent collected from the point of sample application to the center of effluent peak.

6.Standard curve

Molecular weights versus relative elution volume (REV) and distribution coefficient (K_{av}) for each protein standard were plotted on semilog paper. K_{av} defined as $V_e - V_0 / V_t - V_0$ (V_t is the total volume of the column) and REV is defined as V_e / V_0 (Andrews, 1964). MW determination of unknown proteins were made by comparing the ratio of V_e/V_0 for the protein in question to the V_e/V_0 of protein standards. The V_e of the enzymes was determined by measuring fractions at 405 nm after assaying for enzyme activity using PNPG as substrate.

MW Estimation Using SDS-PAGE

The following steps were followed to obtain the MW of the enzymes using the SDS-PAGE estimation method.

1. MW markers

The following protein MW standards were used.

. Albumin, Bovine ≈ MW 66,000

. Albumin, Egg (Ovalbumin) ≈ MW 45,000

. Glyceraldehyde-3-phosphate dehydrogenase, Rabbit muscle ≈ MW 36,000

. Carbonic anhydrase, Bovine erythrocytes ≈ MW 29,000

. Trypsinogen, Bovine pancreas, PMSF treated ≈ MW 24,000

. Trypsin inhibitor, Soybean ≈ MW 20,100

. α-Lactalbumin, Bovine milk ≈ MW 14,200

. Bromophenol Blue was used as a tracking dye

2. Resolving gel-11% acrylamide

The resolving gel was prepared according to the following formula:

- Tris-SDS-stock solution (30 ml) pH 8.8, 0.75M Tris and 0.2% SDS.
- Acrylamide stock (22 ml) which contained 30% w/v acrylamide and 0.8% w/v N,N -bis-methylene acrylamide.
- Water (8 ml).
- Tetramethylethylenediamine (TEMED) (25 ul)
- Freshly prepared 1% w/v ammonium persulphate (1.5 ml)

3. Stacking gel-4% acrylamide

The gel was prepared as follows:

- Tris-SDS-stock solution (10 ml) with pH 6.8, 0.2M Tris and 0.2% SDS.
- Acrylamide stock (2.7 ml) which contained 30% w/v acrylamide and 0.8% w/v N,N'-bis-methylene acrylamide.
- Water (7.3 ml).
- Freshly prepared 1% w/v ammonium persulphate (0.65 ml)
- TEMED (18 ul)

4. Sample preparation (Laemmli, 1970)

Samples were dialysed against buffer (pH 7.25) containing 0.2% SDS and 0.025 M Tris. Then, 0.9 ml of each sample was heated to 100° C with 0.1 ml 10% SDS. To each sample 1-2 drops of 2-mercaptoethanol was added. The samples , 5 to 50 ug protein, were subjected to electrophoresis. The electrode buffer (pH 8.3) contained 0.025 M Tris, 0.192 M glycine and 0.1% SDS. The power was set at 1mA/channel (15 mA/slab gel) until the dye entered the resolving gel then increased to 2 mA/channel.

Gels were stained with Coomassie brilliant blue-R 250 (0.4% w/v) in 40% acetic acid and 10% methanol for 4 hr at room temperature. The gel was destained with a 7.5% (v/v) acetic acid and 25% (v/v) methanol solution.

5. Standard curve

Molecular weights vs. relative mobility (R_f) for each protein standard were plotted on semilog paper

POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE)

Anionic PAGE (pH 8.3)

All steps on this procedure were the same as for SDS-PAGE, except that SDS and 2-mercabtoethanol were not included in the preparations. Acrylamide , 7.5%, was used in anionic PAGE instead of 11% in SDS-PAGE.

Cationic PAGE ,pH 3.5 (Reisfeld et al., 1962)

The following stock solutions were used to prepare the resolving and stacking gel:

A: (pH 4.3)

1. KOH (1N) 48 ml;
2. Acetic acid 17.2 ml;
3. TEMED 4 ml;
4. Water to make 100 ml.

B: (pH 6.8)

1. KOH (1N) 48 ml;
2. Acetic acid 2.87 ml;
3. TEMED 0.46 ml;
4. Water to make 100 ml.

C:

1. Acrylamide 30 gm;
2. Methylene bis-acrylamide 0.8 gm;
3. Water to make 100 ml.

D:

1. Acrylamide 10 gm;
2. Methylene bis-acylamide 2.5 gm;
3. Water to make 100 ml.

E:

1. Riboflavin 4.0 mg;
2. Water to make 100 ml.

Resolving gel, 7.5% acrylamide pH 4.3

A- 7 ml

C- 14 ml

Ammonium persulphate, fresh prepared (0.28 gm /100 ml) 28 ml.

Stacking gel, 4% acrylamide pH 6.8

B- 2.5 ml

D- 5 ml

E- 2.5 ml

A fluorescent lamp (15 W) was placed about 10 cm from the gel. The stacking gel became opalescent and polymerized within 15 min.

The sample buffer was prepared from stock A or B using a 10-fold dilution. Methylene green was used as a tracking dye (positively charged). The electrode buffer (pH 3.5) contained 31.2 gm β-alanine and enough acetic acid to adjust the pH to 3.5. The electrode buffer was diluted 1:1 with H_2O before use. The staining and destaining were the same as in SDS-PAGE.

Detection of enzyme activity on polyacrylamide gels (cationic)

Enzyme activity on the gels was detected using 0.3% 6-bromo-2-naphthyl-α-D-galactoside in dimethylformamide as a substrate (Hadacova and Benes, 1977). The medium consisted of 20 ml MacIlvain buffer pH 5.5, 1 ml stock substrate

solution and 20 mg Fast blue RR diazonium salt. The gels were incubated in this substrate solution for four hours at room temperature. Bands containing active enzyme were yellow.

ENZYME KINETICS

Initial Velocity

In an end point assay the initial velocity is the amount of product formed in a reaction divided by the elapsed time (Suelter, 1985; Allison and Purich, 1979). For the determination of K_m and V_{max}, initial velocities were used which corresponded to different reaction times for different substrates. Initial velocities were based on 3 minute reaction times when PNPG was the substrate. With raffinose as substrate, 45 min, 30 min and 90 min reaction periods were used to calculate initial velocities for Enzyme I, II^1 and II^2, respectively. With stachyose as substrate, the initial velocities were based on 45 min and 30 min reactions for Enzyme I and II^1, respectively. Enzyme II^2 was not tested on stachyose since the reaction rate was too slow and little enzyme was available.

K_m and V_{max} Values for Various Substrates

All the lines in the plots were drawn based upon the method of least squares polynomials (first degree equation, $y = bx + a$). The statistical manipulation of the

experimental data was achieved with the aid of a computer and the estimated values of the coefficients, a and b, along with the indexes of determinations, r, are indicated with the plots.

The K_m and V_{max} values were calculated from the following equation and formulas:

$$\frac{1}{v} = \frac{K_m}{V_{max}} \times \frac{1}{[S]} - \frac{1}{V_{max}} \qquad (1)$$

where v = initial rate of reaction

S = initial substrate concentration

V_{max} = maximum reaction rate

K_m = Michaelis constant

$$K_m = \frac{b}{a}, \qquad V_{max} = \frac{1}{a}$$

The following substrates were used to determine K_m and V_{max} using the Lineweaver-Burk equation:

1. p-Nitrophenyl-α-D-galactopyranoside (PNPG) as an artificial substrate.
2. Raffinose and stachyose as natural substrates.

PNPG

The K_m and V_{max} values for each enzyme, with PNPG as substrate, were determined using the Lineweaver-Burk double reciprocal plot. Five or six PNPG concentrations were used.

Raffinose

A series of solutions, in MacIlvain buffer (pH 5.5), with the following molarities of raffinose were prepared: 2, 3, 5, 8, 13, and 20 mM. Solutions of enzyme I (0.2 ml), enzyme II^1 (0.1 ml) and enzyme II^2 (0.15 ml) were added to 1 ml of raffinose solution. The mixtures were allowed to react at 30° C for 45, 30 and 90 min for enzymes I, II^1 and II^2, respectively (These times were based on preliminary experiments which determined the linearity of the enzymic reactions, after trying reaction periods varying from 15 to 120 min, at 30° C with a 10 mM raffinose substrate). At the end of the reaction time the mixtures were placed in boiling water for three minutes.

The following procedure was used to determine the amount of liberated galactose (Kurz and Wallenfels, 1981):

The absorbance (A'_{334}) of 3.0 ml 0.1 M Tris buffer, pH 8.6, containing 0.1 ml 16.5 mM nicotinamide-adenine dinucleotide (NAD) and 0.2 ml of the D-galactose-containing solution was measured.D-Galactose dehydrogenase, 0.02 ml, (25 U/ml) was added and the mixture incubated at 37° C for 20 min or until the absorbance became constant, A''_{334}. Delta A = A' - A" was used for the calculations.

Under the above conditions the reaction proceeded stoichiometrically and the following formulla was used to

determine the concentration of galactose (Bergmeyer et al., 1981):

$$C = \frac{\text{delta A} \times V}{\epsilon \times d \times v} \text{ [umole/ml]}$$

ϵ : extinction coefficient [cm^2 /umole]

delta A: extinction change

V: assay volume [ml]

v: volume of sample used in assay [ml]

d: light path [cm]

C: concentration [umole/ml], [ug/ml]

For a total assay volume of 3.32 ml and a sample volume of 0.20 ml the D-galactose concentration of the sample was obtained using the following equations:

C = delta A x 2.72 [umole/ml] or

C = delta A x 0.490 [mg/ml].

Stachyose

The K_m and V_{max} values for enzyme I and II[1] were determined using stachyose as substrate. The disappearance of stachyose was measured by HPLC: An aliquot of the solution was passed through a 0.22 um memberane filter and a Sep-pak C_{18} cartridge. A 20 ul sample was injected into an HPLC system (Waters Assoc.,Milford, MA), composed of an M-45 pump, a guard-pack precolumn, an u-

Bondapak/carbohydrate column and an RI-401 refractometric detector. A Kontes model-100 recorder was used. The solvent (acetonitrile/water, 65/35 v/v) flow rate was 2.0 ml/min.

Enzyme I

The following concentrations of stachyose in MacIlvain buffer, pH 5.5, were prepared: 5, 8, 13, 20 and 30 mM. Enzyme I solution, 0.1 ml, was added to 1.0 ml of each stachyose concentration. The reaction was carried out at 30° C for 45 min. After stopping the reaction by boiling for 3 min, each mixture was injected in to the HPLC. The area of the stachyose peak was measured and compared to the peak area of standard stachyose solutions subjected to the same HPLC separation. The linearity of the enzymic reactions (0.1 ml enzyme and 1.0 ml stachyose) was confirmed after trying reaction times of 0, 30, 45 and 60 min at 30° C with a 5 mM stachyose substrate.

Enzyme II[1]

A 0.1 ml of the enzyme was used with 1.0 ml of each of the following concentrations of stachyose in MacIlvain buffer (pH 5.5): 3, 5, 8, 13 and 20 mM. The reaction was carried out at 30° C for 30 min. The 30-min reaction was used as the initial velocity was linear by a mixture of 0.1 ml enzyme and 1.0 ml stachyose solution 3 mM for 30, 45 and 60 min. The calculation of the disappearing of stachyose was as with enzyme I above.

Effect of Substrate (Raffinose) Concentrations on Enzyme Activity

The following raffinose concentrations were used for enzymes I, II^1 and II^2: 10, 20, 40, 80 and 160 mM. At 30° C, enzymes I, II^1 and II^2 were incubated for 45, 30 and 90 minutes, respectively. Solutions of I, II^1 and II^2 0.1, 0.1 and 0.4 ml respectively were added to 1.0 ml raffinose. Enzyme quantities were chosen to give readable results. For galactose quantitation , the galactose dehydrogenase test was used (Kurz and Wallenfels, 1981).

STABILITY

The stability of enzymes I, II^1 and II^2 during 24 hrs storage at four different temperatures, 4, 22, 37 and 45° C, and at various pH values from 2.0 to 8.0 was studied. MacIlvain buffer and PNPG as substrate were used. Enzyme activity was assayed at time zero and after 24 hr. Activity at time zero was considered 100% in order to calculate the relative activity. The MacIlvain buffer (0.5 M citric acid and 1 M Na_2HPO_4) was used to keep the pH of the assay around 5.5.

OPTIMUM ACTIVITY pH AT 30° C

The effect of pH on the rate of PNPG hydrolysis at 30° C was studied with a series of MacIlvain buffers ranging between 2.0 and 8.0 pH. The pH of the reaction mixture was

measured before the initiation of the reaction.

OPTIMUM ACTIVITY TEMPERATURE AT OPTIMUM pH

The optimum temperature for activity was determined between 0° and 70° C at the optimum pH for each enzyme. PNPG, was used as substrate to assay the enzyme.

GERMINATION AND INCUBATION OF COWPEAS

1- Experimental Design

Experiments were designed to follow the activity of α-galactosidase and changes in oligosaccharides during the germination, incubation and germination plus incubation of cowpeas. All experiments were performed in duplicates. The results were statistically analyzed (ANOVA) using a computer program (Microstat). The statistical analysis was used to show the significant differences within and between the treatments.

2- Quantitation of Oligosaccharides

Samples from the germinated and incubated cowpeas, 1-3 g dry wt., were ground and extracted twice with 25 ml 80% ethanol in an 80° C bath for 4 hr with occasional shaking. The extract was centrifuged at 2500 x g for 15 min. The supernatants were saved and 1 ml 10% lead acetate was added twice. After 10 min at 60° C, the solutions were centrifuged at 2500 x g for 15 min. Five drops of 10% oxalic acid were added to the supernatants which were then

centrifuged at 2500 xg for 15 min. The purified extracts were concentrated by vacuum evaporation below 50° C to a final volume of 25 ml. An aliquot of the extract was passed through a 0.22 um membrane filter and a Sep-pak C_{18} cartridge. A 20 ul sample was injected into an HPLC system as previously described. The concentrations of the sucrose, raffinose and stachyose standard solutions ranged from 0.2-0.6%, 0.02-0.06% and 0.1-0.3%, respectively. Results were expressed as g sugar/ 100 g dry wt.

The A.O.A.C. procedure No. 14.003, was used for moisture determination.

3- α-Galactosidase Activity and Oligosaccharides Content of Germinating Cowpeas

Cowpea seeds, 2-3 g dry wt, were soaked in distilled water for 2 hr and germinated at 24° C for 24 hr for oligosaccharides measurment. The grmination time for α-galactosidase activity measurment was 30 hr at 24° and 30° C. The seeds were germinated in darkin Petri dishes lined with wet filter paper. At intervals of 6 hrs, samples were removed from the incubators and frozen at -20° C. The frozen seeds were ground and analyzed for oligosaccharide content and α-galactosidase activity.

4- Incubation

Flour was made from cowpeas by the dry method (Dovlo et al., 1976). The cowpea seeds were ground in a Wiley mill

and passed through a 20-mesh seive after blowing off the seed coats. A paste was made from cowpea flour by mixing 100 g flour with 140 ml distilled water. Samples, 2-3 gm dry wt., were incubated at 24° C and 34° C for 0, 3, 6, 12 and 24 hr. After each period of time, the samples were removed from the incubator and their pH was measured. Samples were kept in the freezer at -20° C until analyzed for oligosaccharide content using HPLC as previously described.

5- Germination and Incubation

Samples were soaked for 2 hr, germinated for 6 hr, ground, incubated for 0, 3, 6 and 12 hrs at 24 and 34° C and analyzed for oligosaccharides using HPLC. The pH of the suspension was also determined.

6- α-Galactosidase Activity During Sprouting

The seeds started to sprout after 24 hr of germination. The α-galactosidase activity was measured at 42 and 72 hr of germination at 30° C in the sprouts and the remainder of the seeds.

INVERTASE ACTIVITY

Samples from zero time, 12 hr incubation and 12 hr germination were analyzed for invertase activity. The analysis were performed on samples obtained by the following extraction method:

Extraction Method (Al-Baker and Whitaker, 1978)

Five gram samples were ground with 20 ml distilled water and centrifuged at 15,000 xg for 30 min. The pellets were extracted three times with 0.25 M potassium phosphate buffer (1:1,w/v), pH 7.5, in a Vortex shaker for 2 min. The three extracts were combined after centrifugation at 15,000 xg for 30 min. The pH of the supernatant was adjusted to 4.0 with 30% acetic acid. Ammonium sulfate was added to 30% saturation. The supernatant was 95% saturated with ammonium sulfate and the precipitate formed was removed by centrifugation at 20,000 xg for 25 min. The pellet was dissolved in 0.01 M sodium acetate buffer, pH 4.0, dialyzed against the same buffer for 40 hr with several changes in buffer and tested for invertase activity.

Invertase assay using HPLC

Enzyme solution, 0.3 ml, was added to 5 ml of 0.1 M sucrose-0.1 M sodium acetate buffer, pH 4.0, at 30° C for 10 min. The reaction was stopped by boiling for 2 min. The reaction solution was diluted four-fold with distilled water before being injected in to the HPLC. Sucrose solution (25 mM) was used as a standard. Substracting the sucrose peak height of the enzyme reaction mixture from the peak height of the standard represented the mM of sucrose disappeared.

One unit of invertase activity was that amount of invertase which hydrolyzed 1 umole of sucrose per min at pH 4.0, 30° C at an initial sucrose concentration of 100 mM.

RESULTS AND DISCUSSION

ENZYME ASSAY

The validity of the PNPG assay for α-galactosidase activity was established.

The stopped assay was linear at a reaction time of 15 min or less (Figures 6). The wavelength of maximum absorbance for p-nitrophenol was 405 nm (Figure 7). Figure 8 shows the standard curve for determining p-nitrophenol concentration.

ENZYME PURIFICATION

The purification of cowpea α-galactosidases is summarized in Table 1 and 2. The difference between method I (Table 1) and method II (Table 2) was in using different ion exchange columns. The elution time, using CM-cellulose column was longer than the elution time when Accl-CM column was used. Method I resulted in better yields.

In stage 1, the extraction pH which resulted in the maximum enzyme activity and the clearest extract was 5.5 (Figure 9). In stage 2, acid precipitation (citric acid) almost tripled the specific activity of the extract with very little loss of total activity. The addition of citric acid also increased the specific activity obtained after ammonium sulfate precipitation (Table 3). The optimum pH

for acid precipitation of the inactive protein was 3.5 (Figure 10).

Table 1. Purification of cowpea (Vigna unguiculata) α-galactosidases, method I.

Stage	Volume (ml)	Activity (nKat/ml)	Protein (mg/ml)	Sp. activity (nKat/mg)	Recovery[1] (%)
1. Crude Extract	1575	63	174	0.4	---
2. Citric Acid Precipitation	2060	42	37	1.1	87
3. $(NH_4)_2SO_4$ 25-55%	154	324	220	1.5	50
4. Sephadex G-100					
α-galactosidase I	152	180	4	45.0	28
α-galactosidase II	113	77	40	1.9	8.8
5. CM-cellulose chromatography					
I	18.5	395	0.1	3950	7.4
II[1]	15.5	368	10.0	37	5.8
II[2]	13.5	5.3	0.2	27	0.1

[1]% recovery was calculated relative to the first stage, i.e. crude extract. Seed powder wt. taken = 1 kg.

In stage 3, ammonium sulfate fractionation was used rather than acetone precipitation as suggested by Dey and Pridham (1969), because -10° C acetone inactivated the enzyme. The cut point in ammonium sulfate precipitation for maximizing the activity of the precipitation was found to

be 25% to 55% ammonium sulfate (Table 4). The specific activity after this stage increased considerably.

Table 2. Purification of cowpea (Vigna unguiculata) α-galactosidases, method II.

Stage	Volume (ml)	Activity (nKat/ml)	Protein (mg/ml)	Sp. activity (nKat/mg)	Recovery[1] (%)
1. Crude extract	500	77	210	0.4	---
2. Citric Acid Precipitation	870	44	45	1.0	99
3. $(NH_4)_2SO_4$ 25-55%	55	299	160	1.9	43
4. Sephadex G-100					
α-galactosidase I	126	119	3	40.0	39
α-galactosidase II	110	65	39	1.7	19
5. Sephadex G-100, recycle					
α-galactosidase I	44	159	3	53	18
α-galactosidase II	57	67	16	4	10
6. Accl-CM-cation					
I	10	250	0.14	1786	6.5
II[1]	3	45	0.7	64	0.4
II[2]	8	13	0.3	43	0.3

[1]% recovery was calculated relative to the first stage, i.e. crude extract. Seed powder wt. taken = 0.5 kg.

In stage 4, the enzyme solution was dialyzed against McIlvain buffer, pH 5.5, and then passed through Sephadex G-100 column. Two protein fractions with α-galactosidase activity were resolved. Figure 11 shows the elution

profiles of enzyme I and II from this column. Recycling the fractions I and II removed some inactive protein and raised the specific activity as shown in Figures 12 and 13 and Tables 1 and 2.

Table 3. Effect of acid precipitation on the specific activity of the precipitate (stage 2)

	Specific activity (nKat/mg)	25% $(NH_4)_2SO_4$ Sp.act.(nKat/mg) supernatant	60% $(NH_4)_2SO_4$ Sp.act.(nKat/mg) suprnt.	ppt.
1.Extract at pH 5.5	0.30	0.30	0.01	0.50
2.Precipitate at pH 3.5	0.40	0.40	0.03	0.80

Table 4. Effect of ammonium sulfate concentration on the specific activity of the precipitate (stage 3)

	Specific activity (nKat/mg) supernatant	Specific activity (nKat/mg) precipitate
1.Extract at pH 5.5	0.43	
2.Acid precipitation at pH 3.5	0.63	
3.$(NH_4)_2SO_4$ precipitation		
a. 25%	0.65	
b. 35%	0.26	
4.Supernatant (from a):		
a. 55% $(NH_4)_2SO_4$	0.0	1.5
b. 60% $(NH_4)_2SO_4$	0.0	1.4

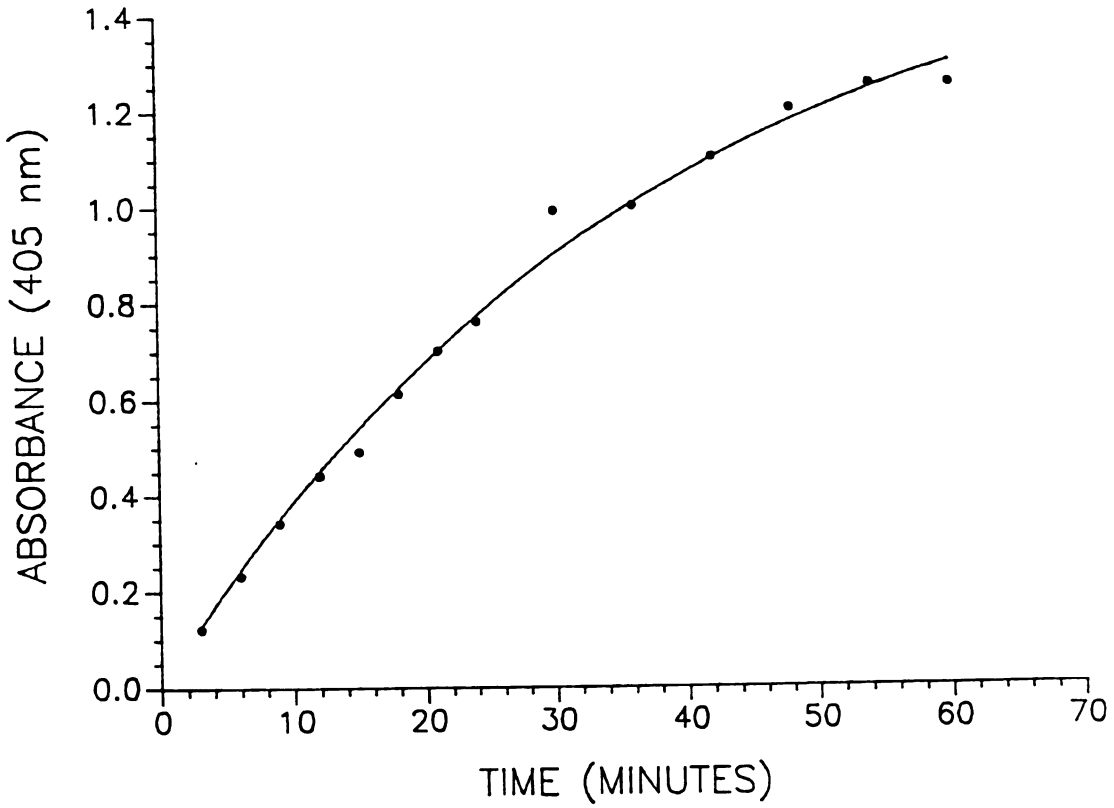


Figure 6. Time-dependence of PNPG hydrolysis by α-galactosidase

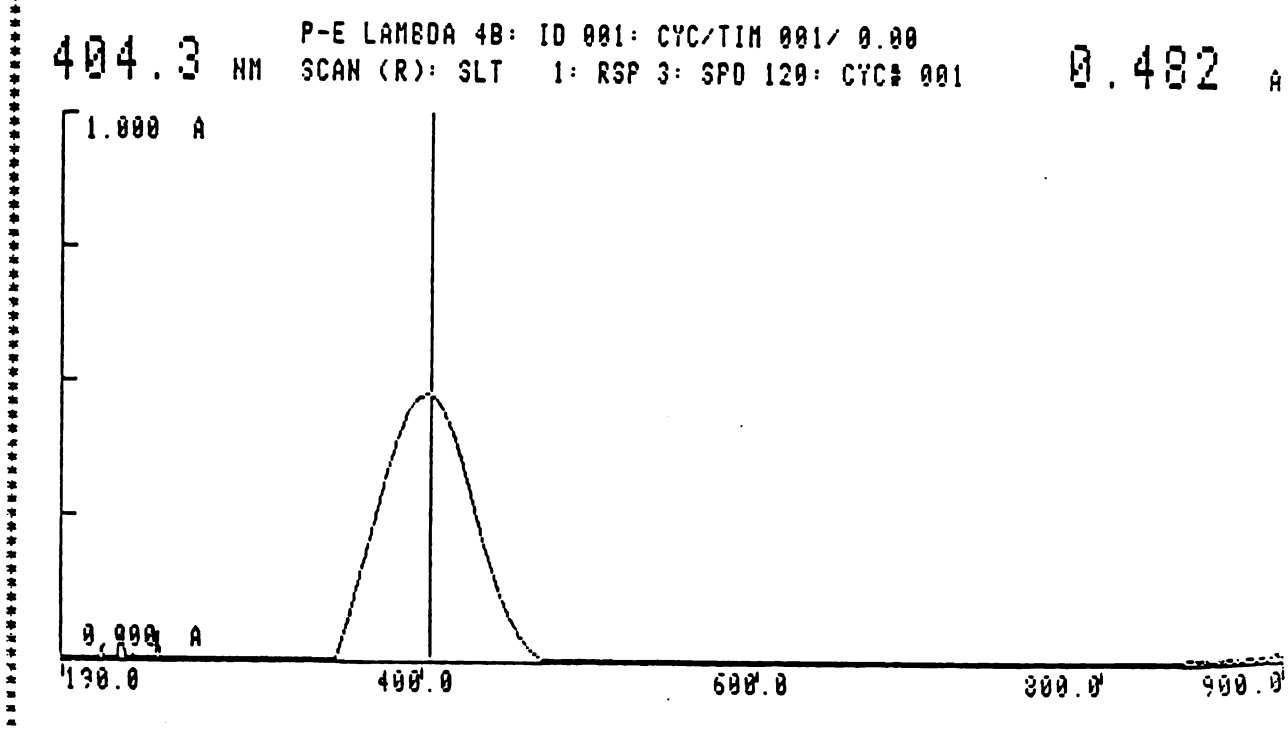


Figure 7. Absorption spectrum of p-nitrophenol freed from PNPG by α-galactosidase

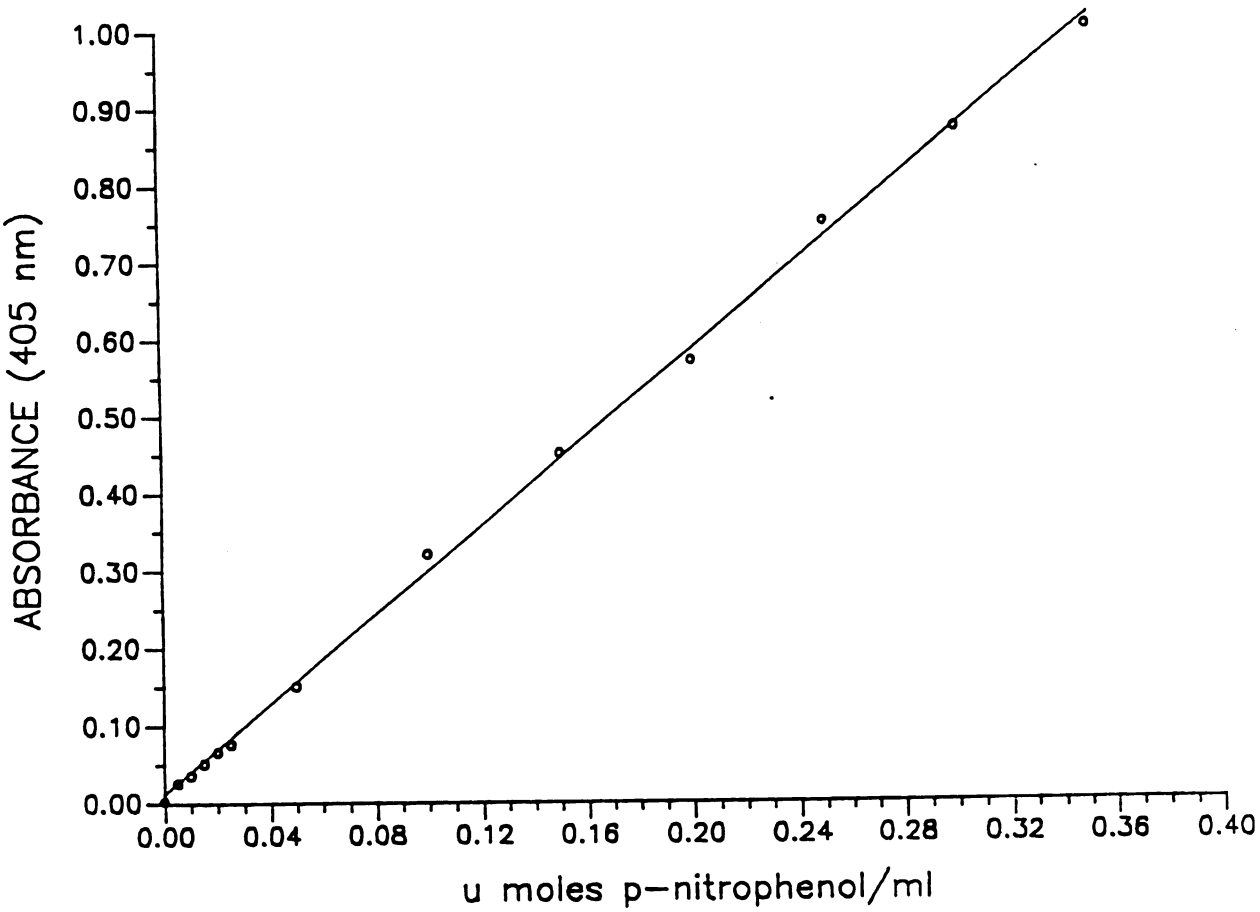


Figure 8. p-Nitrophenol standard curve measured at 405 nm

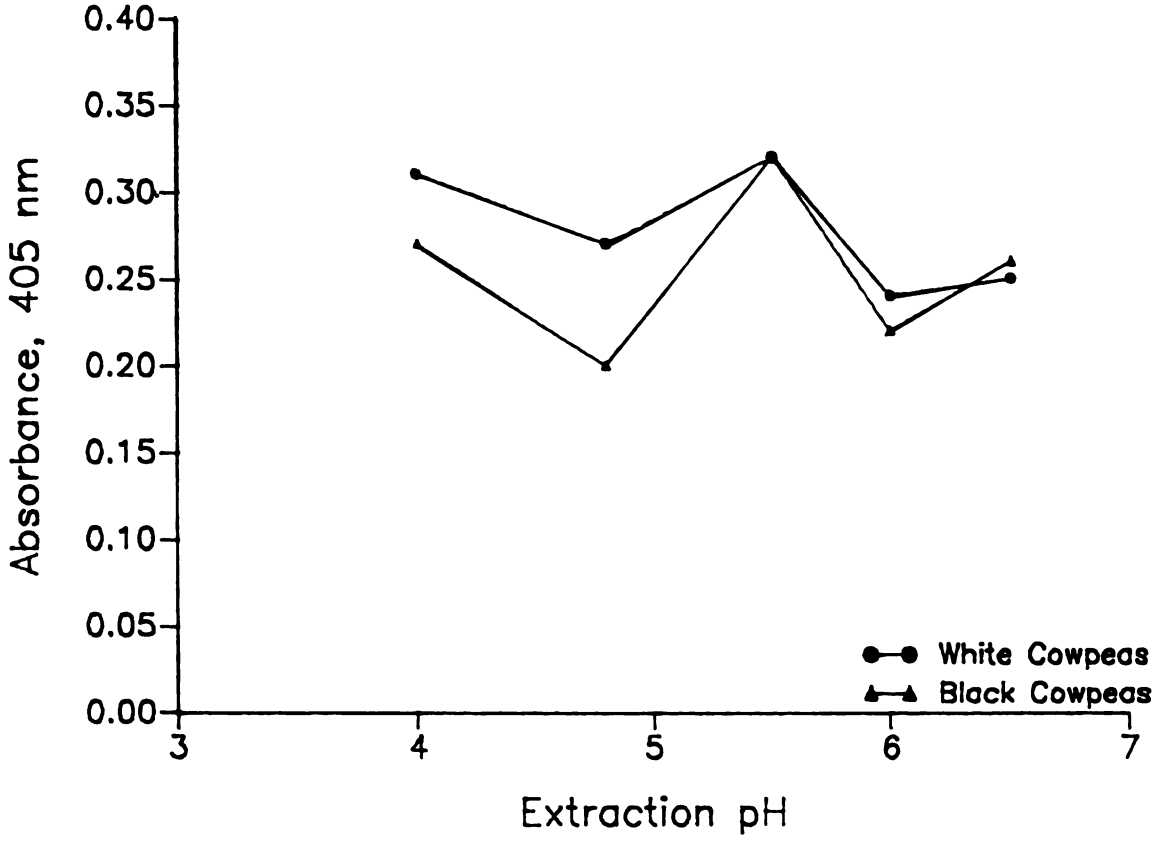


Figure 9. Effect of extraction pH on α-galactosidase activity measured at 405 nm

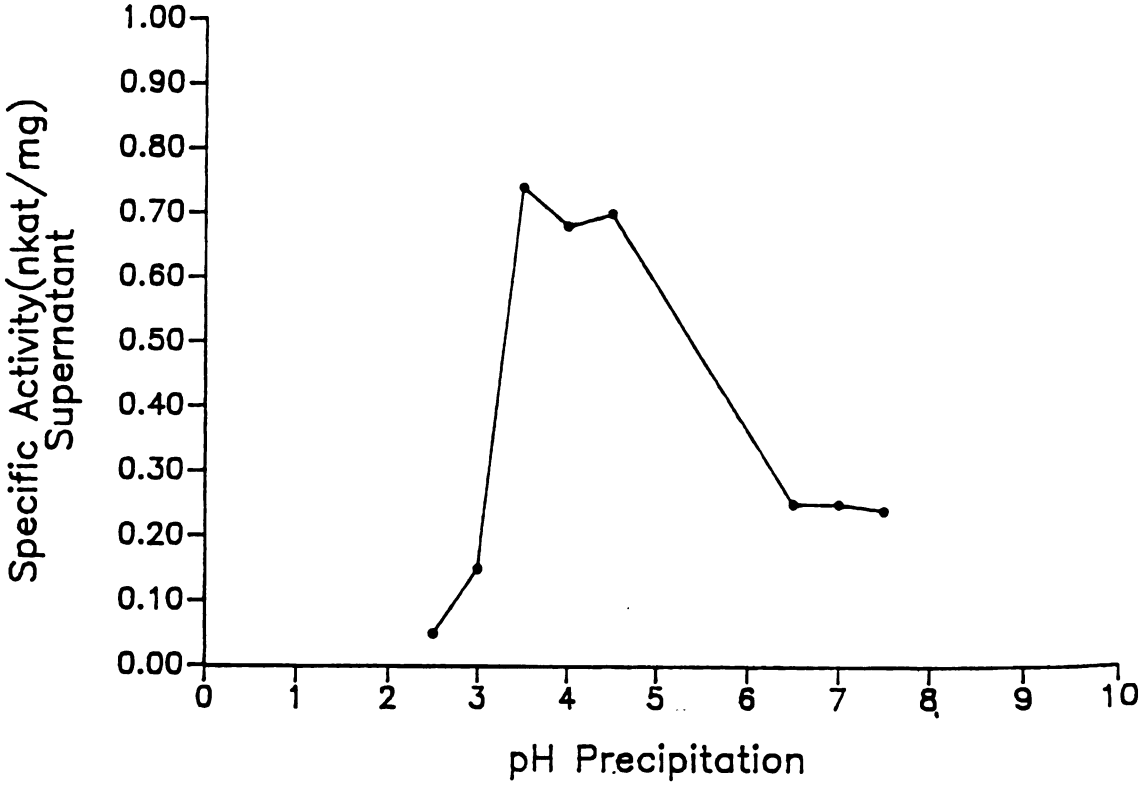


Figure 10. Specific activity of supernatant after precipitation of inactive protein at various pH, measured at 405 nm (stage # 2)

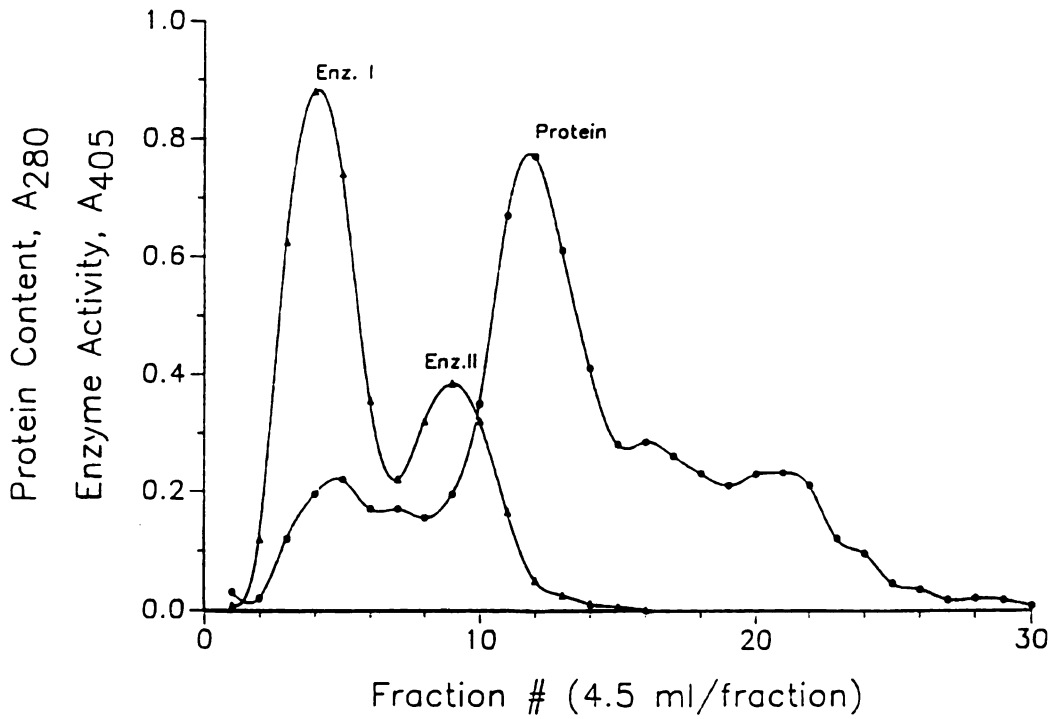


Figure 11. Elution profiles of α-galactosidases I and II from G-100 gel filtration column

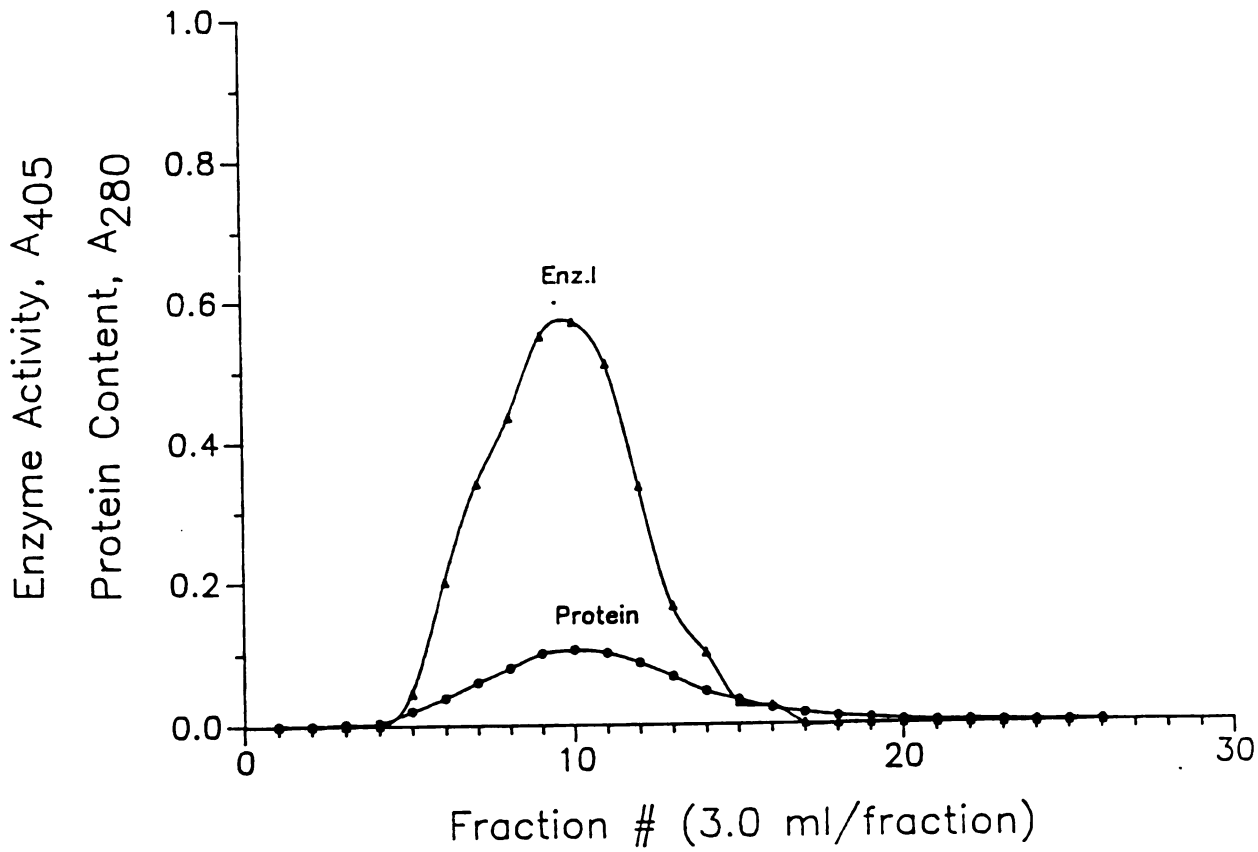


Figure 12. Elution profile of α-galactosidase I recycled through a G-100 gel filtration column

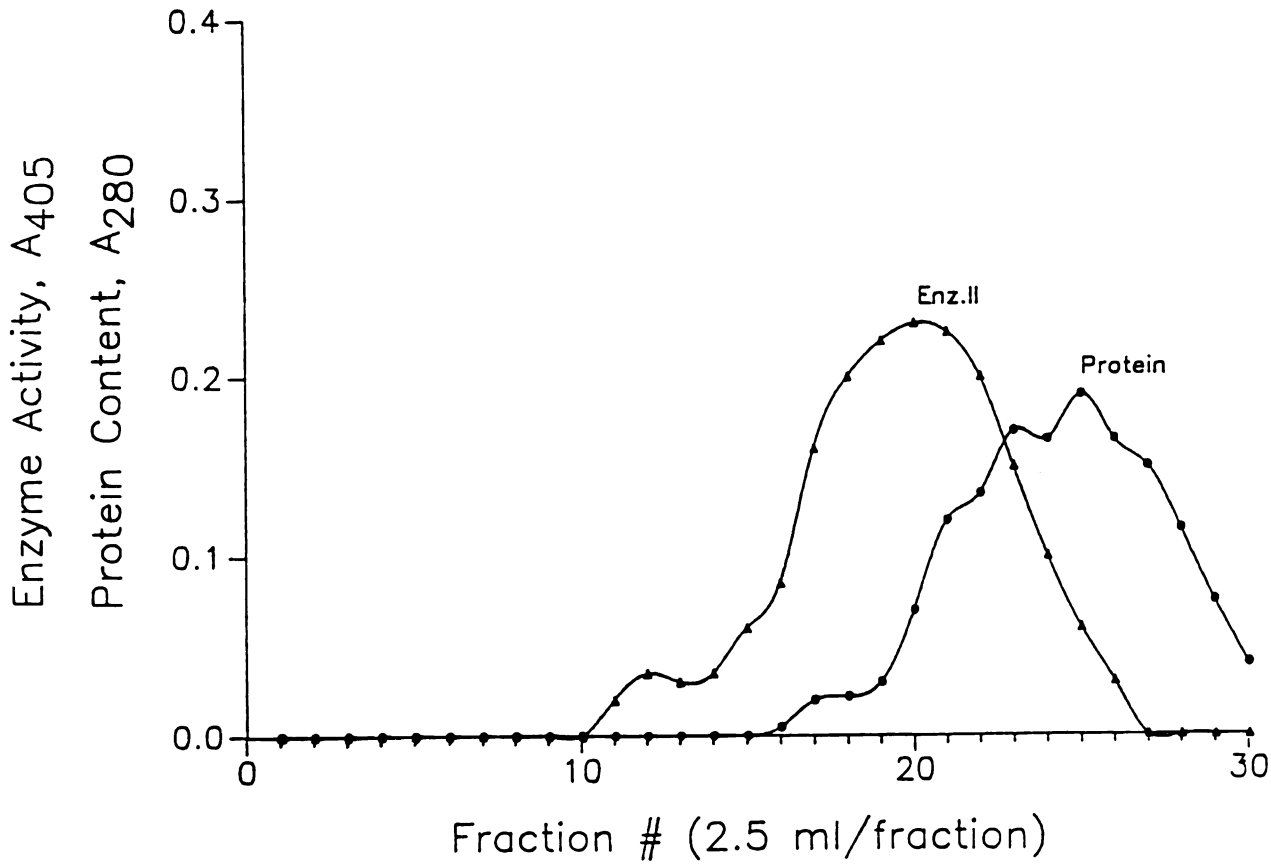


Figure 13. Elution profile of α-galactosidase II recycled through a G-100 gel filtration column

Similar results were observed on Sephadex G-200 but the elution was slower and the peaks were broader compared with Sephadex G-100. Figure 14 shows the elution profiles of enzyme I and II from Sephadex G-200.

Whatman CM-cellulose resin (Table 1) and a newly introduced cation Accell-CM by Waters Association (Table 2) were compared in stage 5. When enzyme I was passed through Accel-CM with the pH gradient of 4.0 to 7.0, one active peak was observed with higher specific activity (Figure 15). Two active fractions, enzymes II^1 and II^2 respectively, were obtained from enzyme II when passed through Accel-CM (Figure 16). Enzyme I and II were also fractionated on CM-cellulose using a NaCl gradient. Again, Enzyme I eluted as one peak, and enzyme II eluted as two peaks (Figures 17, 18 and Table 1).

The apparent specific activity of enzyme II^1 was higher than that of II^2 and the proportions of the two total activities varied with different batches of seeds. Enzymes I and II^2 were eluted from CM-cellulose and Accel-CM columns by similar sodium chloride concentrations and pH values, respectively, which indicated that they are charged in a similar way.

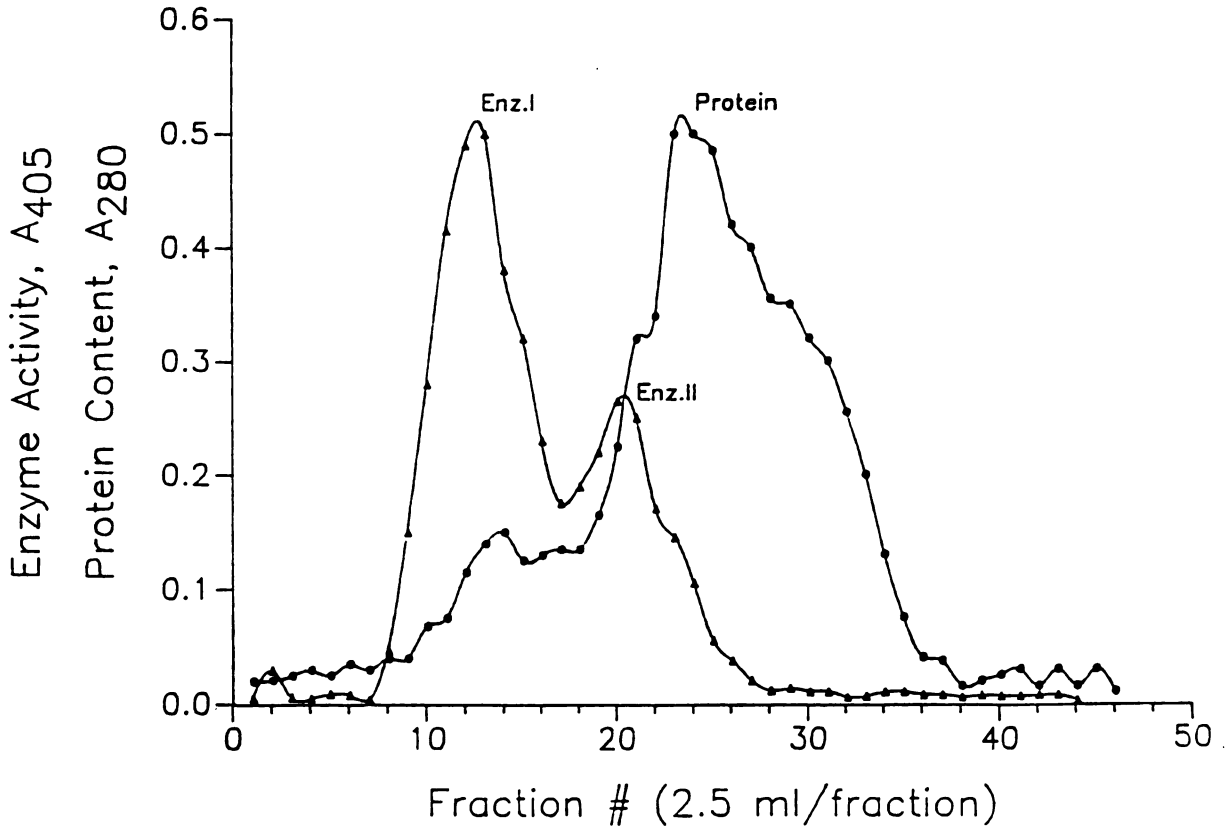


Figure 14. Elution profiles of α-galactosidases I and II on an G-200 gel filtration column

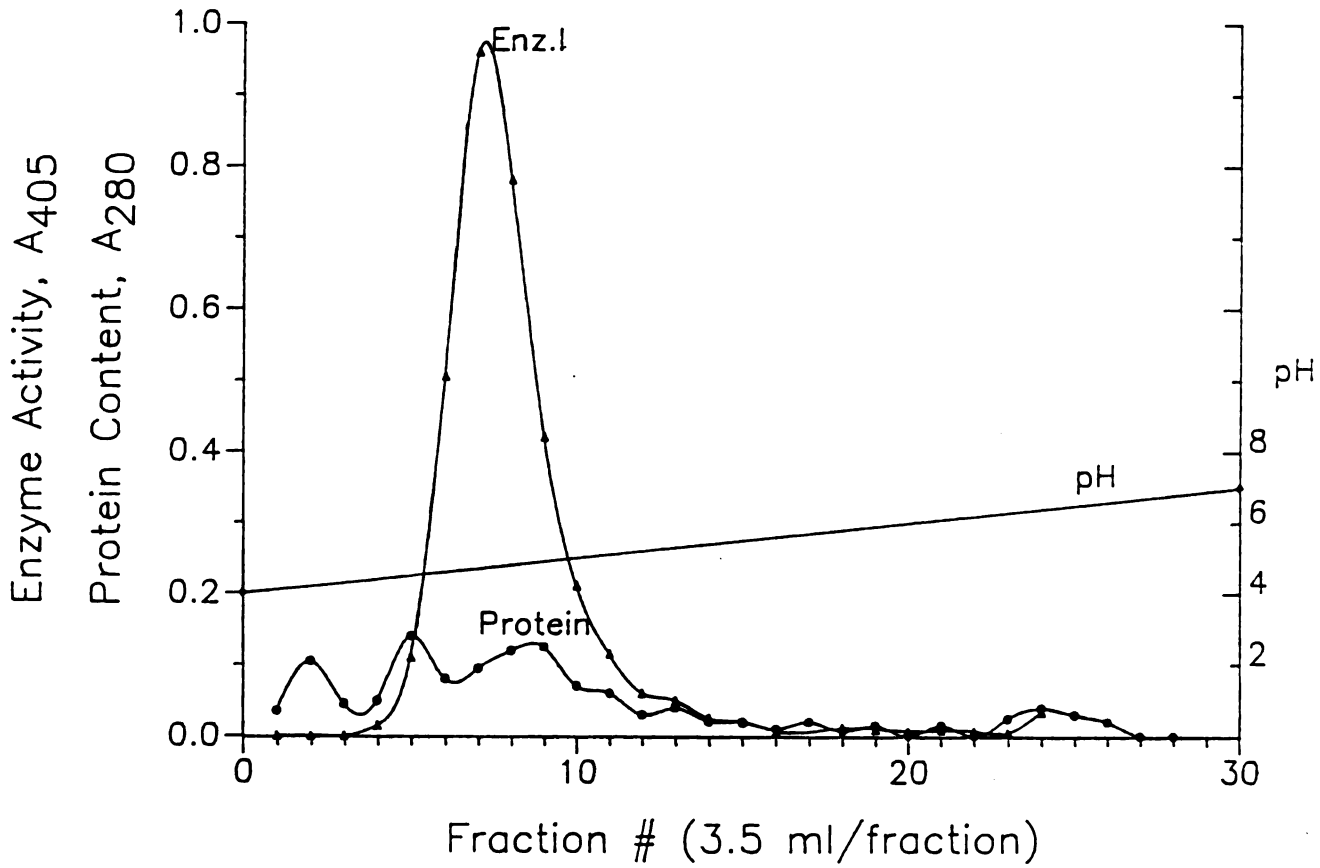


Figure 15. Elution profile of α-galactosidase I on an Accl-CM (Waters) column

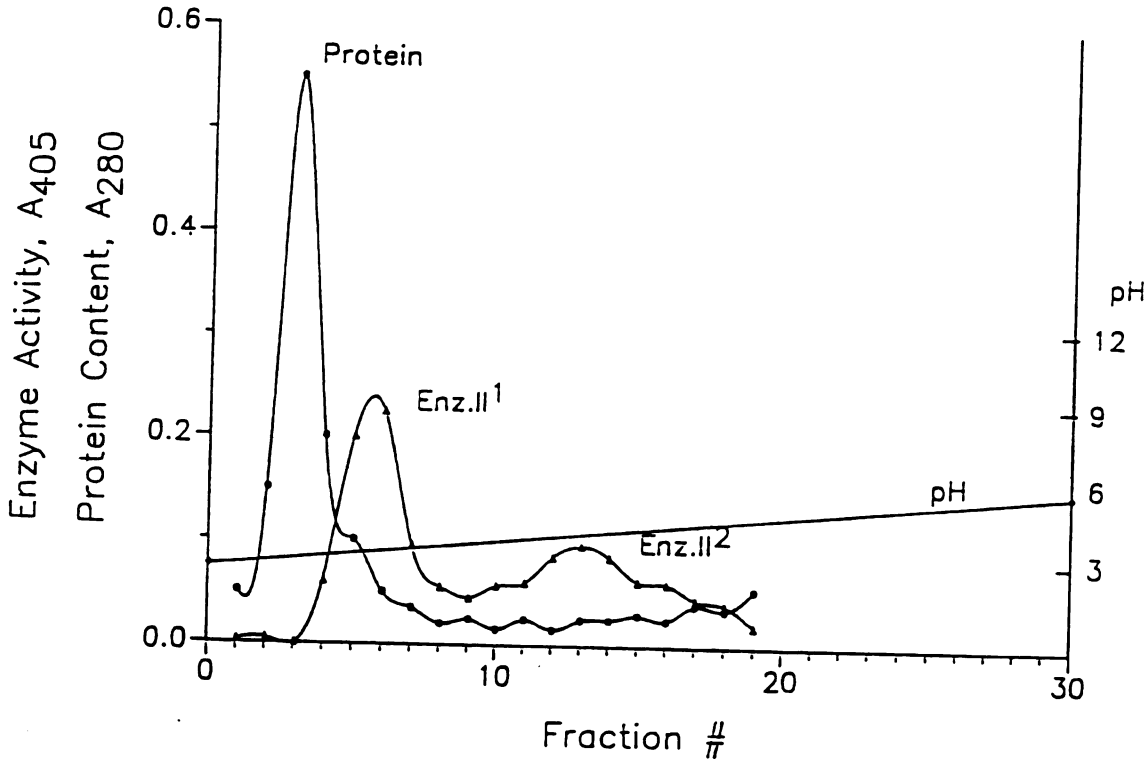


Figure 16. Elution profile of α-galactosidase II on Accl-CM (Waters) column

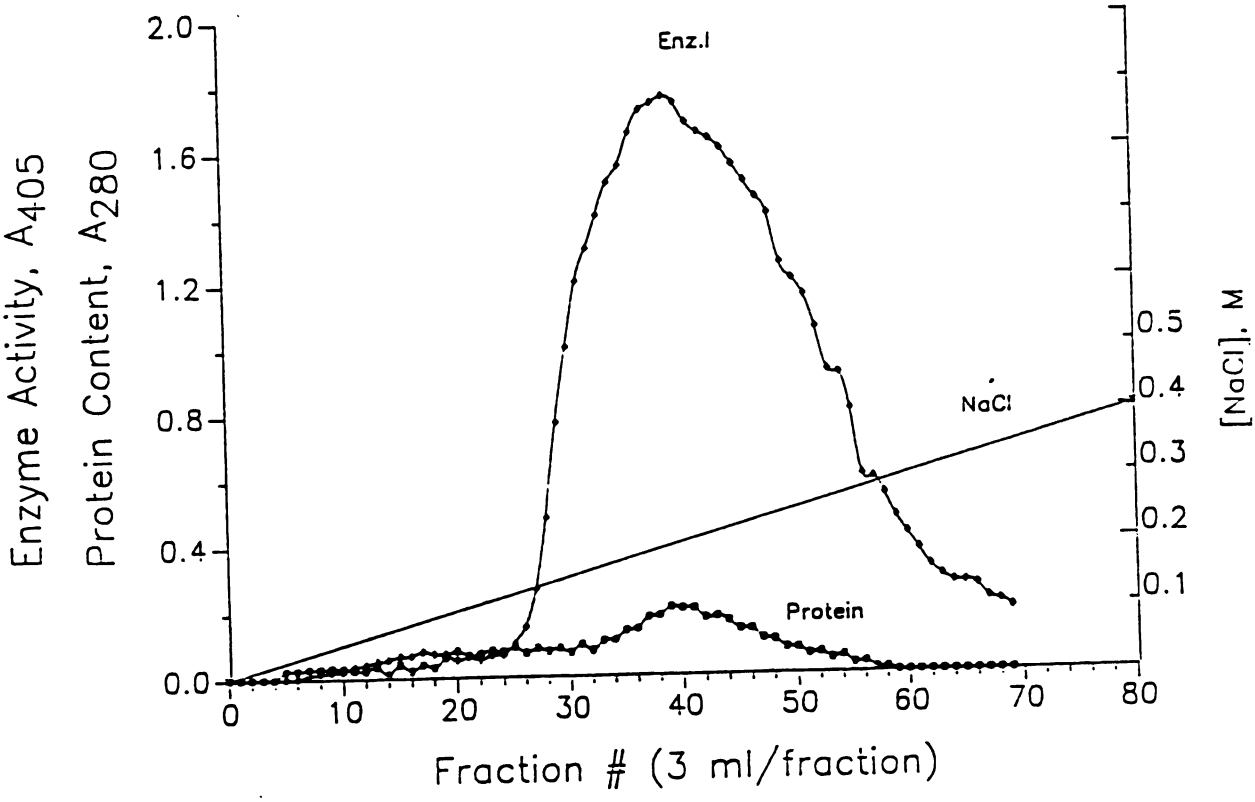


Figure 17. Profile of α-galactosidase I on an CM-cellulose column eluted with a sodium chloride gradient

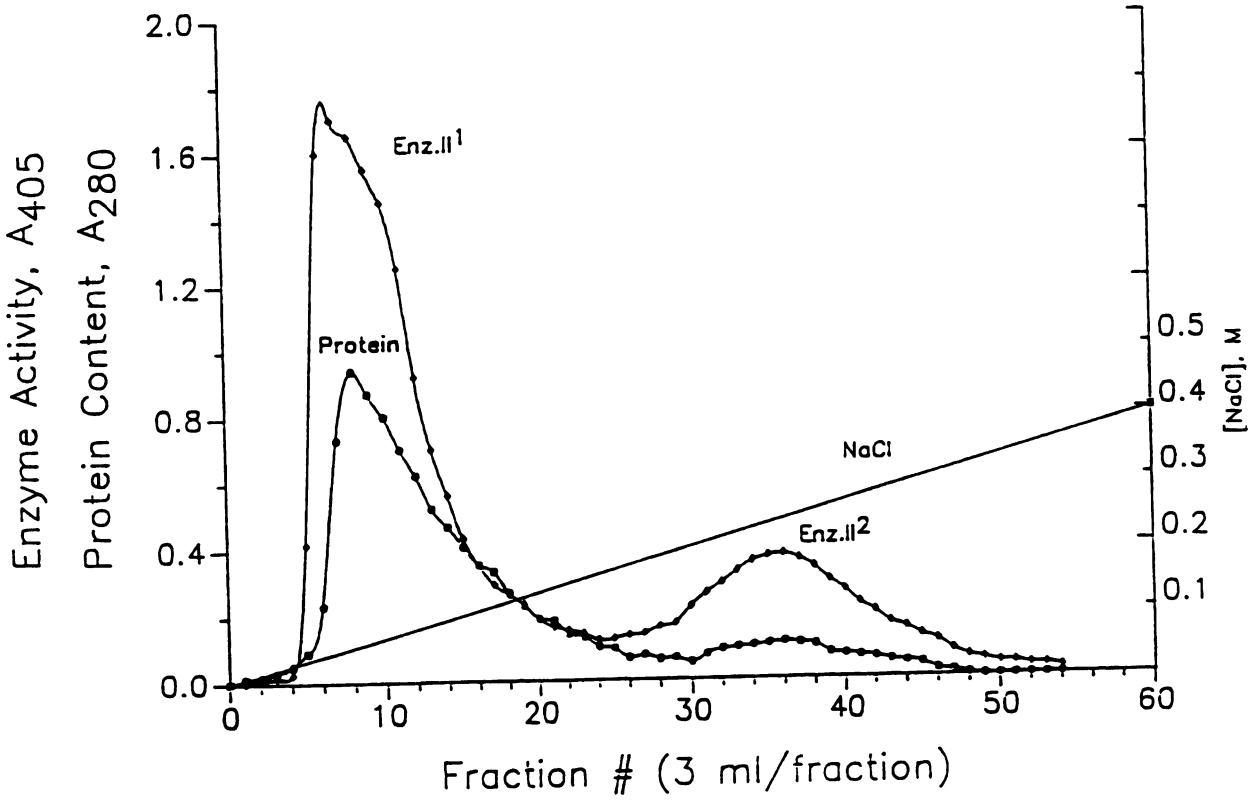


Figure 18. Profile of α-galactosidase II on an CM-cellulose column eluted with a sodium chloride gradient

POLYACRYLAMIDE GEL ELECTROPHORESIS

The purpose of this experiment was to test the purity of the enzymes and their mobility in different ionic environments. PAGE was carried out under cationic, pH 3.5, and anionic conditions, pH 8.3. A slight mobility for all three enzymes was observed on anionic PAGE. One protein band was observed for Enzyme I and II^1 while enzyme II^2 migrated in more than one band. Enzymes I,II^1 and the main band in enzyme II^2 migrated the same distance towards the anode (Figure 19). The R_f value for all enzymes was 0.26. At pH 3.5 the enzymes migrated towards the cathode as discrete bands (Figure 20) when detected with α-galactosidase-specific reagents and by Coomasie blue stain. The R_f value was 0.33.

Dey et al (1982) reported that the mobility of the three forms of the enzyme of Vicia faba on PAGE at pH 8.3 was zero. However, they all migrated at pH 3.5. The mobilities of I and II^1 appeared to be identical and these proteins were apparently less basic than II^1. In view of these results, they suggested that the order of elution of the enzymes from CM-cellulose columns by a NaCl gradient was not expected. They suggested that only a proportion of the charged regions of the enzymes interact with the ion exchange material and the elution is not a function of the total charges on the proteins. In our study the mobility of

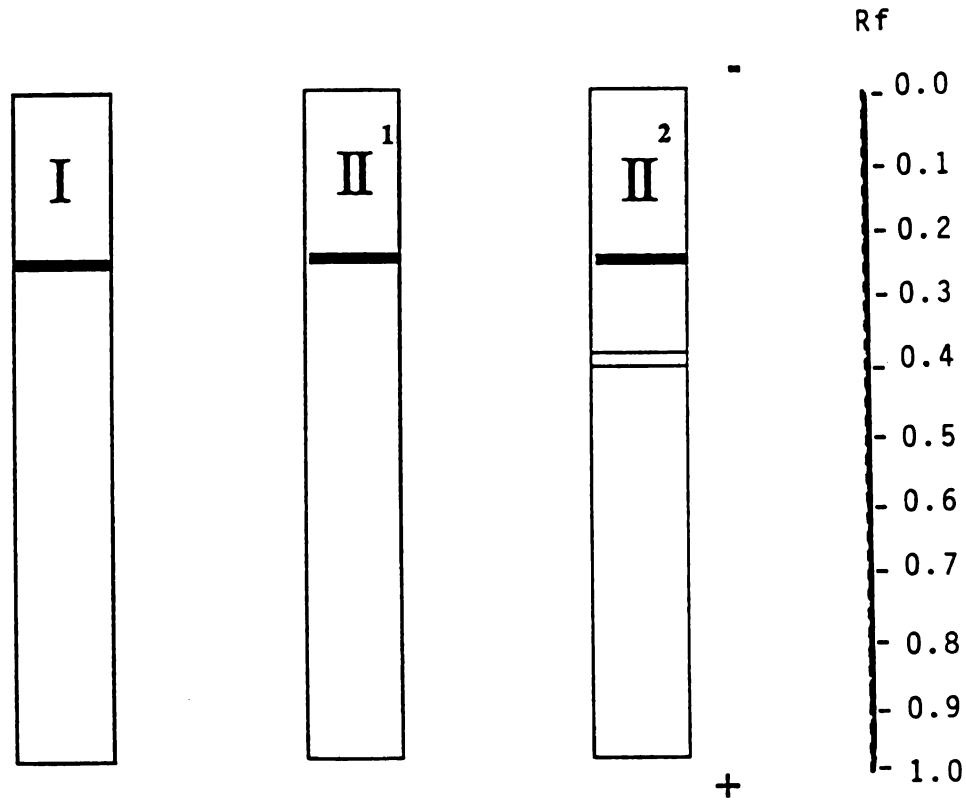


Figure 19. Anionic polyacrylamide disc gel electrophoresis of α-galactosidases I, II^1 and II^2 at pH 8.3 (Coomassie stain). R_f = 0.26

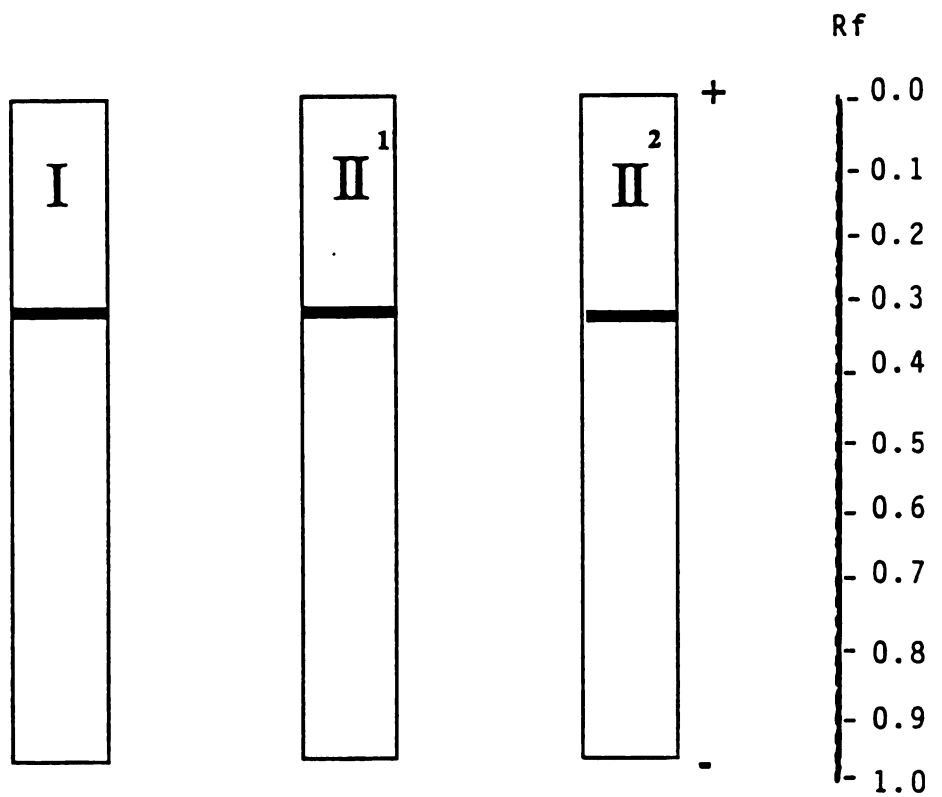


Figure 20. Polyacrylamide disc gel elecrophoresis of α-galactosidases I, II^1 and II^2 at pH 3.5. The same bands were visualized by Coomasie stain and by enzyme activity detection (Fast blue RR salt). R_f = 0.33

the Enzymes I, II^1 and II^2 appeared to be identical and these proteins may have the same charges.

MOLECULAR WEIGHT DETERMINATION

Two methods, gel filtration and SDS-PAGE chromatography, were used to determine the molecular weight of the enzymes.

Gel Filtration Chromatography Using Sephacryl S-200

Figures 21, 22 and 23 show the elution profiles of MW standards and Blue Dextran on the Sephacryl gel filtration column. Figures 24, 25 and 26 show the elution volume profiles of enzyme I,II^1 and II^2 respectively. The V_e (half maximum peak height of the leading edge of a protein peak after the extrapolation of the leading side to the base line) for all proteins, including the enzymes, were measured. This is more reliable than the peak crest because the position of that crest depends on the sample volume (Cooper, 1977).

Figures 27 and 28 show calibration curves for the MW determination of the enzymes by plotting REV and K_{av} vs. MW of the known proteins,respectively. Enzyme I, Enzyme II^1 and Enzyme II^2 have approximate MW of 110,720 ± 3780, 29,000 ± 355 and 30,000 ± 368, respectively. The most likely causes of error in protein MW estimated by gel-filtration are inaccurate estimation of elution volumes,

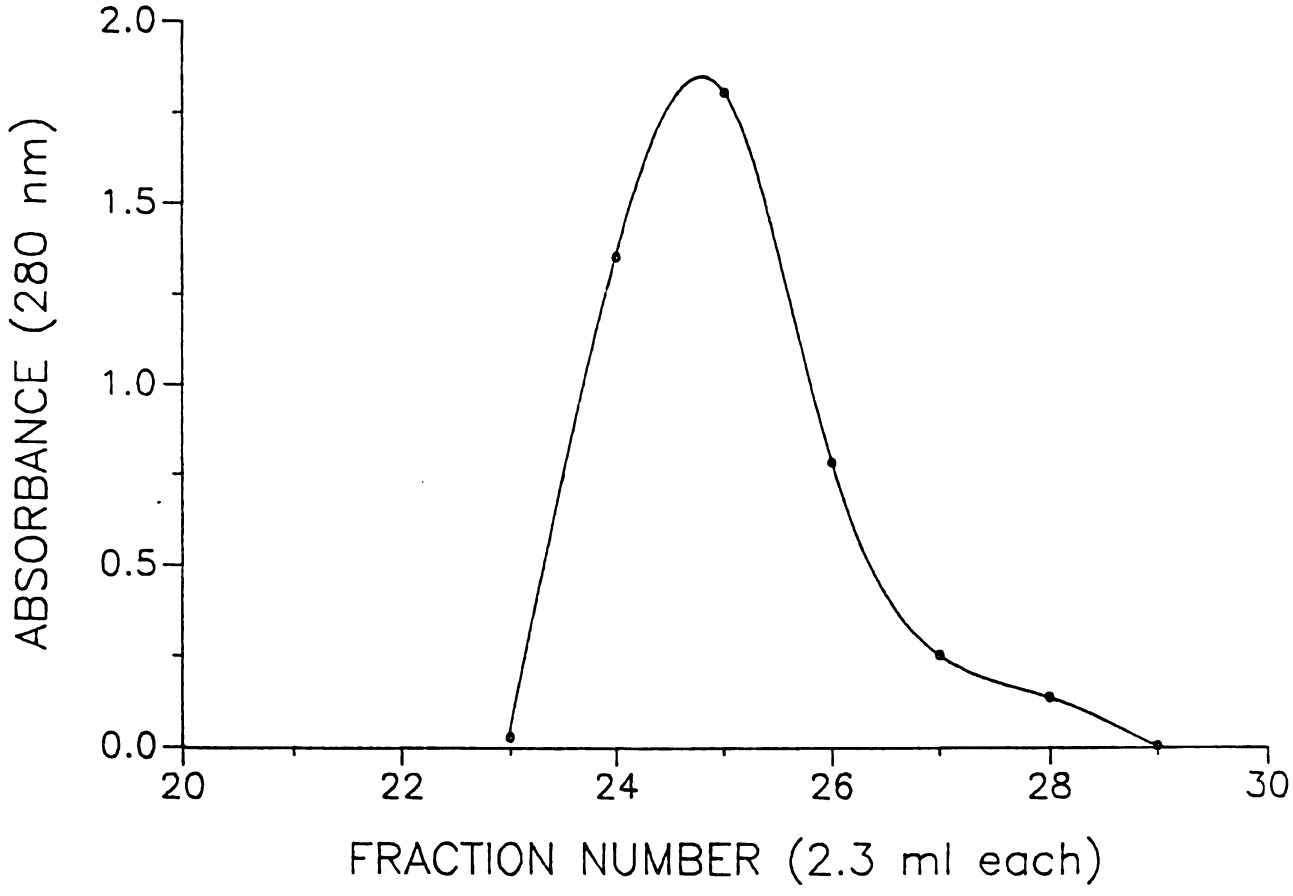


Figure 21. Elution profile of Blue Dextran (MW 2,000,000, V_0 = 54.5, V_T = 144 ml) from Sephacryl S-200 column

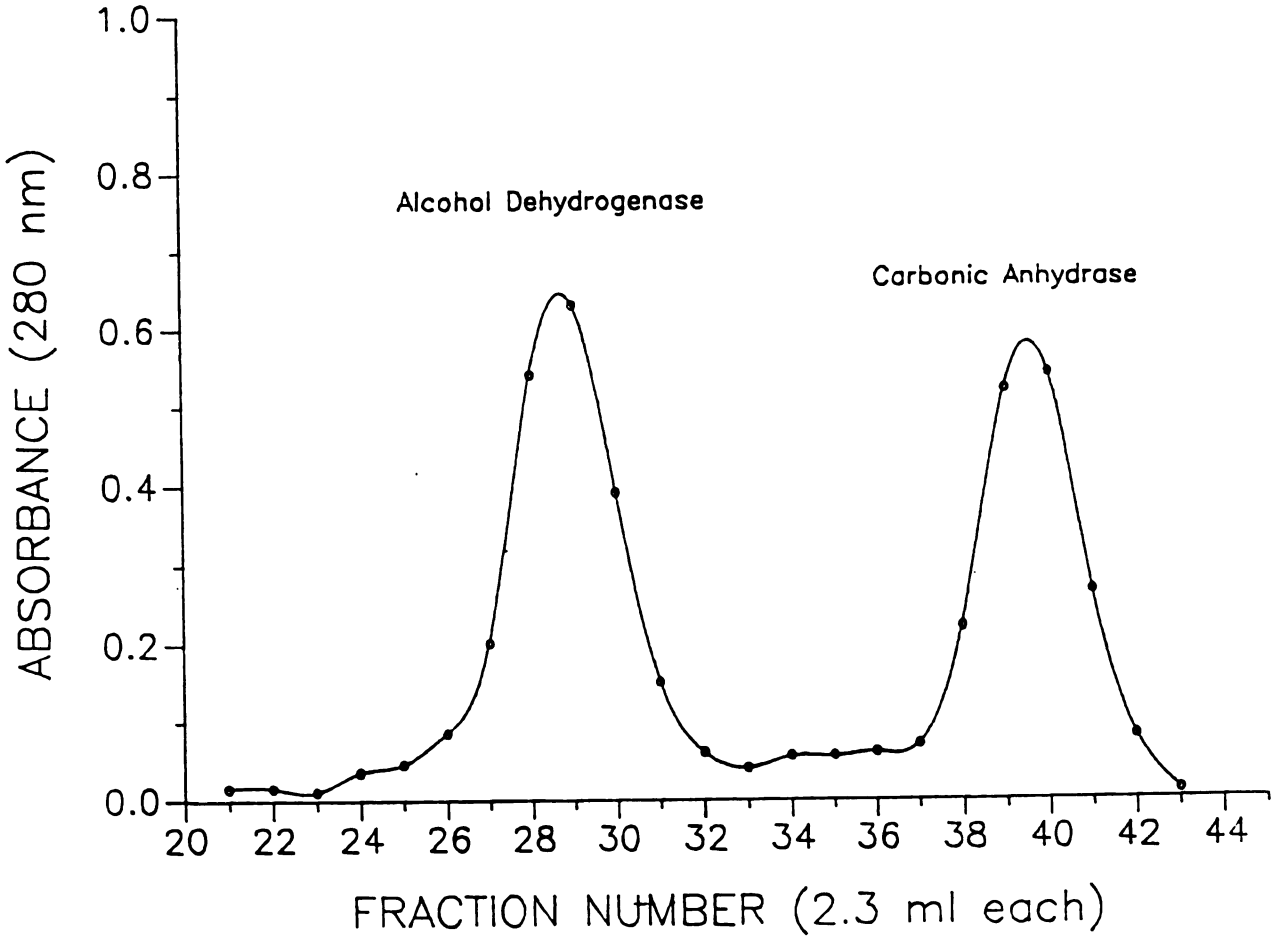


Figure 22. Elution profiles of alcohol dehydrogenase (MW 150,000, V_e = 62.0 ml) and carbonic anhydrase (MW 29,000, V_e = 87.6 ml) from Sephacryl S-200 column

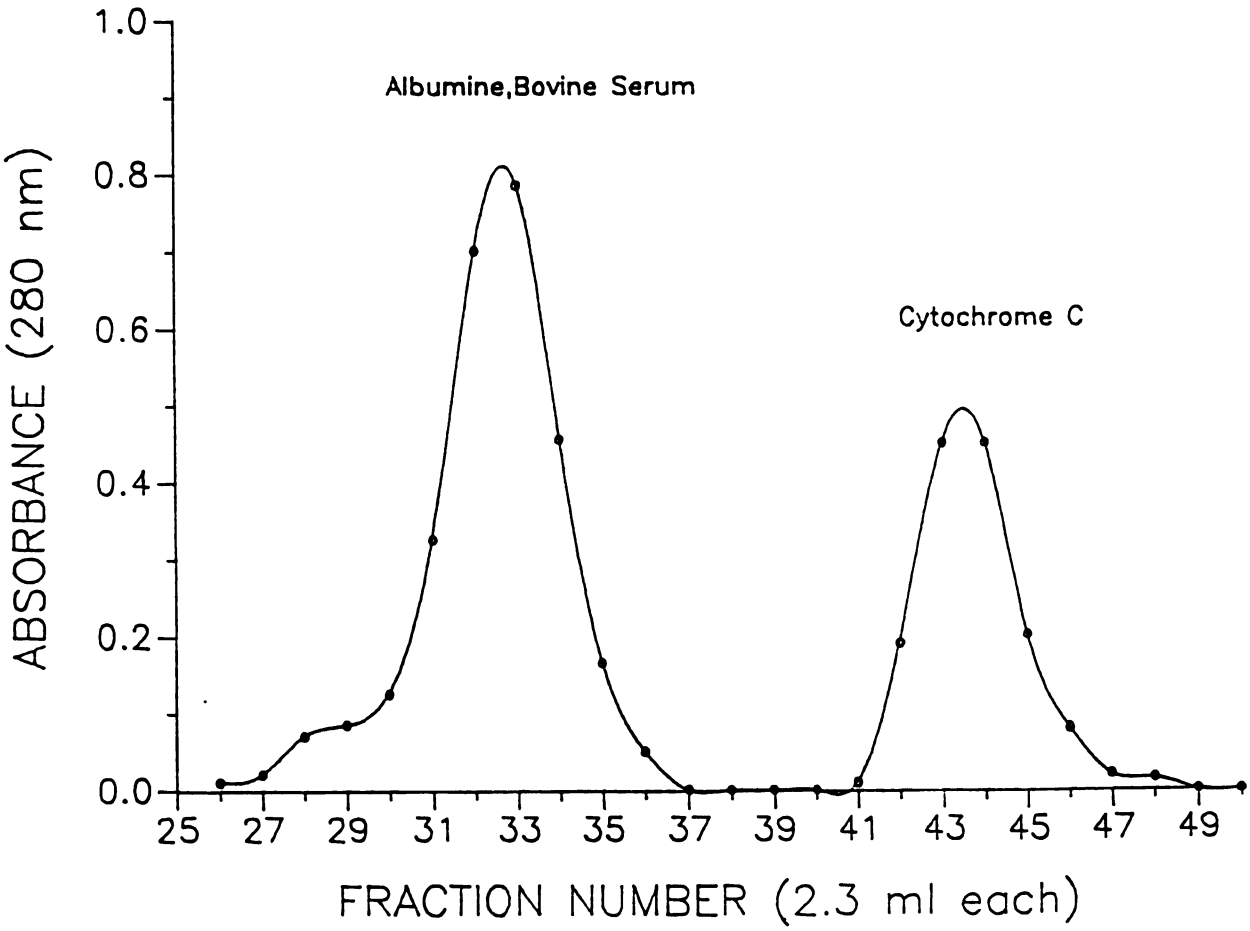


Figure 23. Elution profiles of bovine albumin (MW 66,000, V_e = 69.0 ml) and cytochrome C (MW 12,400, V_e = 97.0 ml) from Sephacryl S-200 column

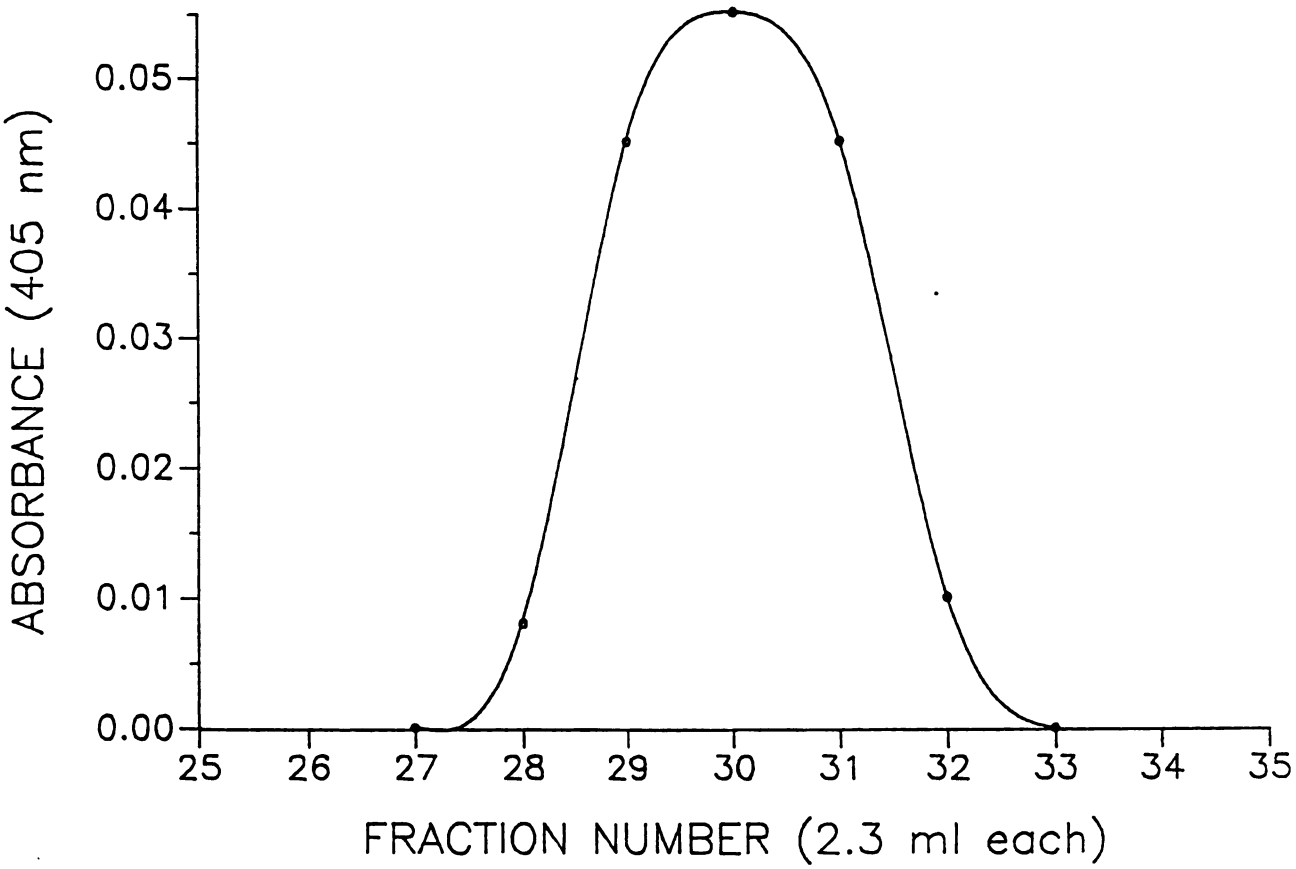


Figure 24. Elution profile of Enzyme I (at pH 5.0) from Sephacryl S-200 column. V_e = 65.5 ml

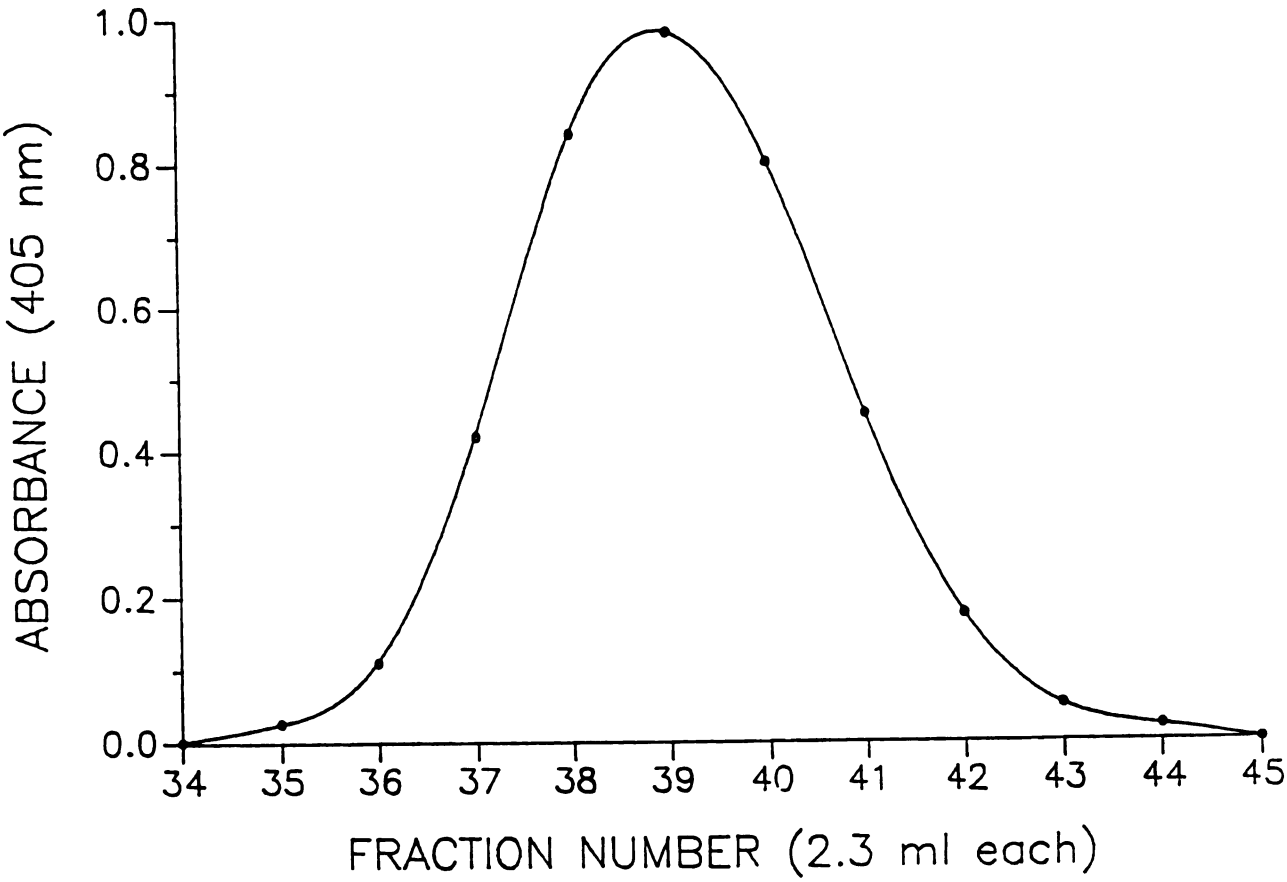


Figure 25. Elution profile of Enzyme II[1] (at pH 7.0) from Sephacryl S-200 column. V_e = 85.0 ml

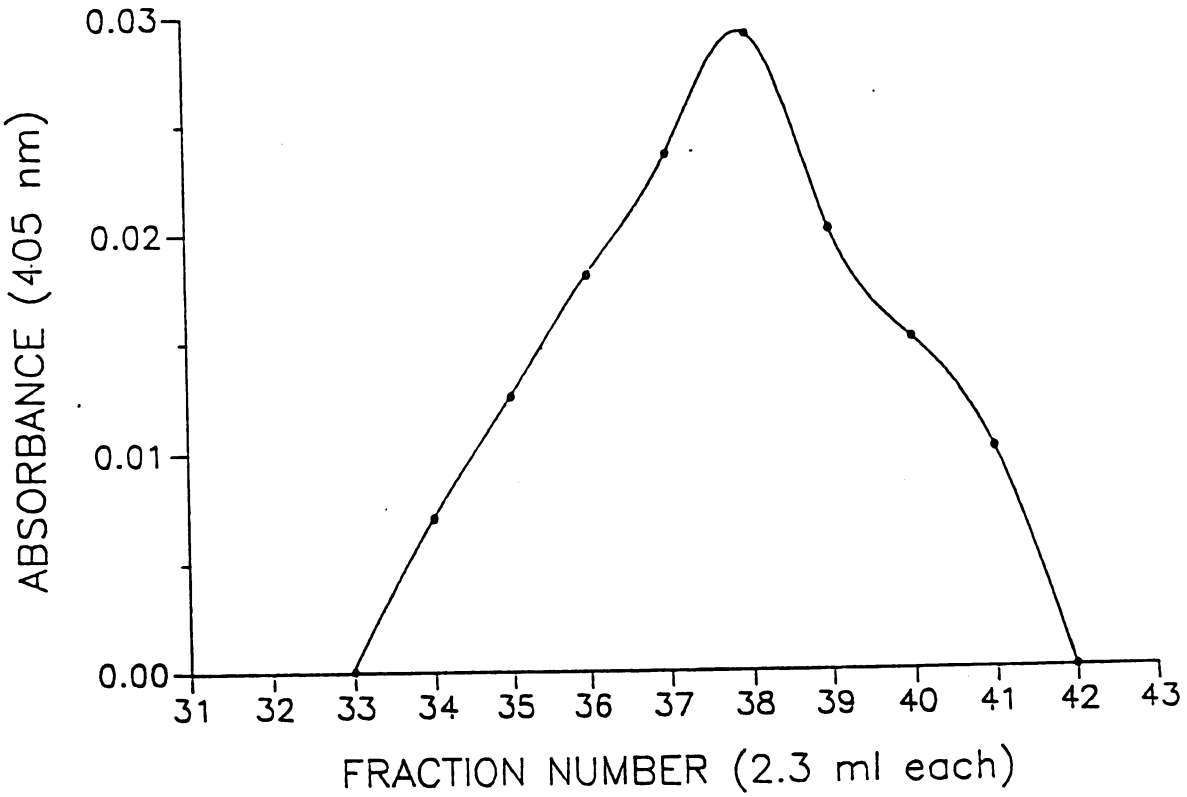


Figure 26. Elution profile of Enzyme II^2 (at pH 7.0) from Sephacryl S-200 column. V_e = 84.6 ml

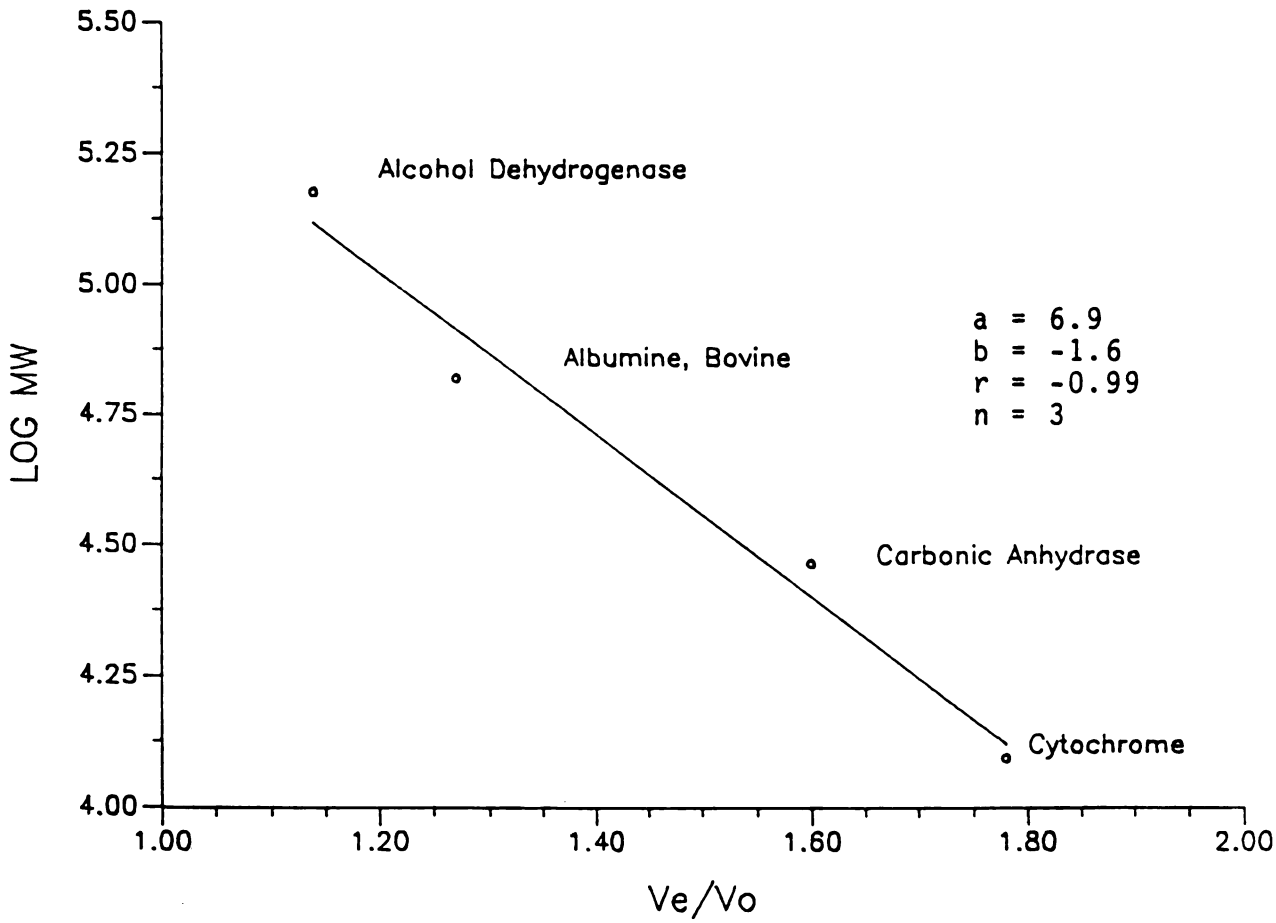


Figure 27. MW calibration curve using protein standards on Sephacryl S-200, based on V_e /V_0

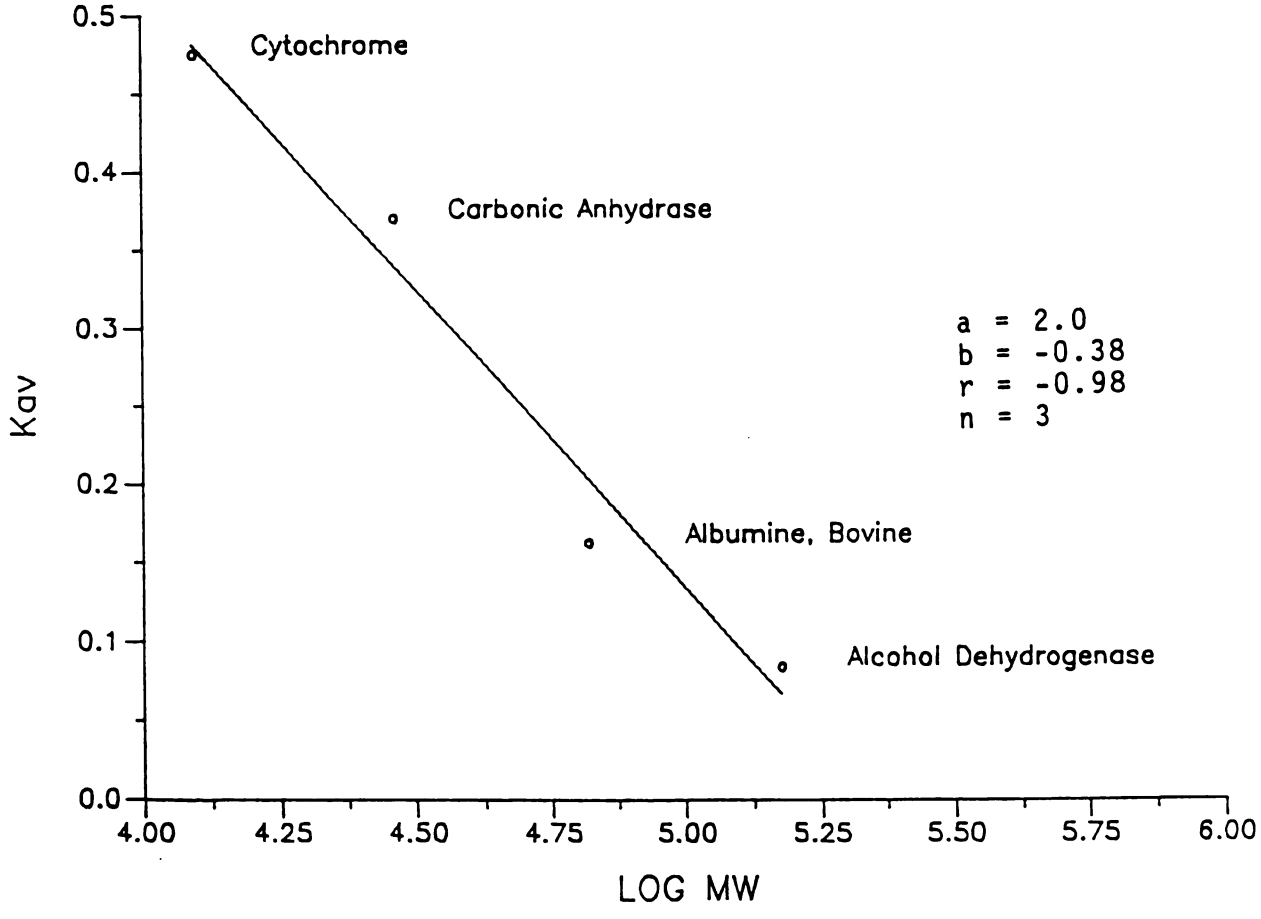


Figure 28. MW calibration curve using protein standards on Sephacryl S-200, based on K_{av}

differences in shape between one protein molecule and another, and density differences between solvated protein molecules, resulting in different ratios between sizes and MW (Andrews, 1964).

Dey and Pridham (1969) located two forms, I and II, of the enzyme in resting V. faba seeds. Using Sephadex gel filtration G-200, they found that the MW of I and II were 209,000 and 38,000, respectively. Later work (Dey et al., 1971) showed that form II was a mixture of two enzymes, II^1 and II^2 with MW (sedimentation equilibrium method) of 45,730 ± 3,037 and 43,390 ± 1,409, respectively. Examination of the MW of I by the sedimentation equilibrium gave a value of 160,400 ± 2,850 (Pridham and Dey, 1974).

MW Estimation Using SDS-PAGE chromatography

The mobility of a protein in acrylamide gels is a function of both its net charge and size. Hypothetically, two proteins of different MW may migrate toward the anode at the same rate if their size differences are balanced by compensating charge difference (Shapiro et al., 1967; Weber and Osborn, 1969). For this reason acrylamide gel electrophoresis using gels of only one pore size may not be used to gain information about the MW of a molecules. In an effort to surmount these problems Shapiro et al. (1967) attempted to separate a mixture of proteins in the presence of SDS.

Figure 29 shows the SDS-PAGE mobilities of α-galactosidases I, II^1 and II^2. These mobilities were sufficient to allow an estimation of their MW by plotting log MW vs. relative mobility (R_f) (Figure 30). Enzyme I, Enzyme II^1 and Enzyme II^2 have approximate MWs equal to 30,916 ± 1,042, 29,875 ± 413 and 30,350 ± 550 daltons with R_f values of 0.63, 0.65 and 0.64, respectively. The weak bands appearing with the main enzyme bands in the SDS-PAGE picture (Figure 29) may indicate the presence of minor polypeptides or contaminating proteins. Various MWs have been reported in the literature for α-galactosidases from different plant sources (Appendix 2).

The following observations suggest that Enzyme I is a tetramer of Enzyme II^2:

a) Gel filtration indicated that the MW of Enzyme I is 110,720 ± 3780 vs. 30,000 ± 368 for Enzyme II^2.

b) SDS-PAGE which depolymerized Enzyme I resulted in almost equal MW estimates of the two enzymes; 30,916 ± 1,042 vs. 30,350 ± 550 for Enzymes I and II^2, respectively.

c) The two enzymes are eluted at the same pH value (4.4-4.8) in Accel-CM chromatography and at the same NaCl concentration value (≈ 0.24M) in CM-cellulose chromatography. These methods separate compounds on the basis of charge only.

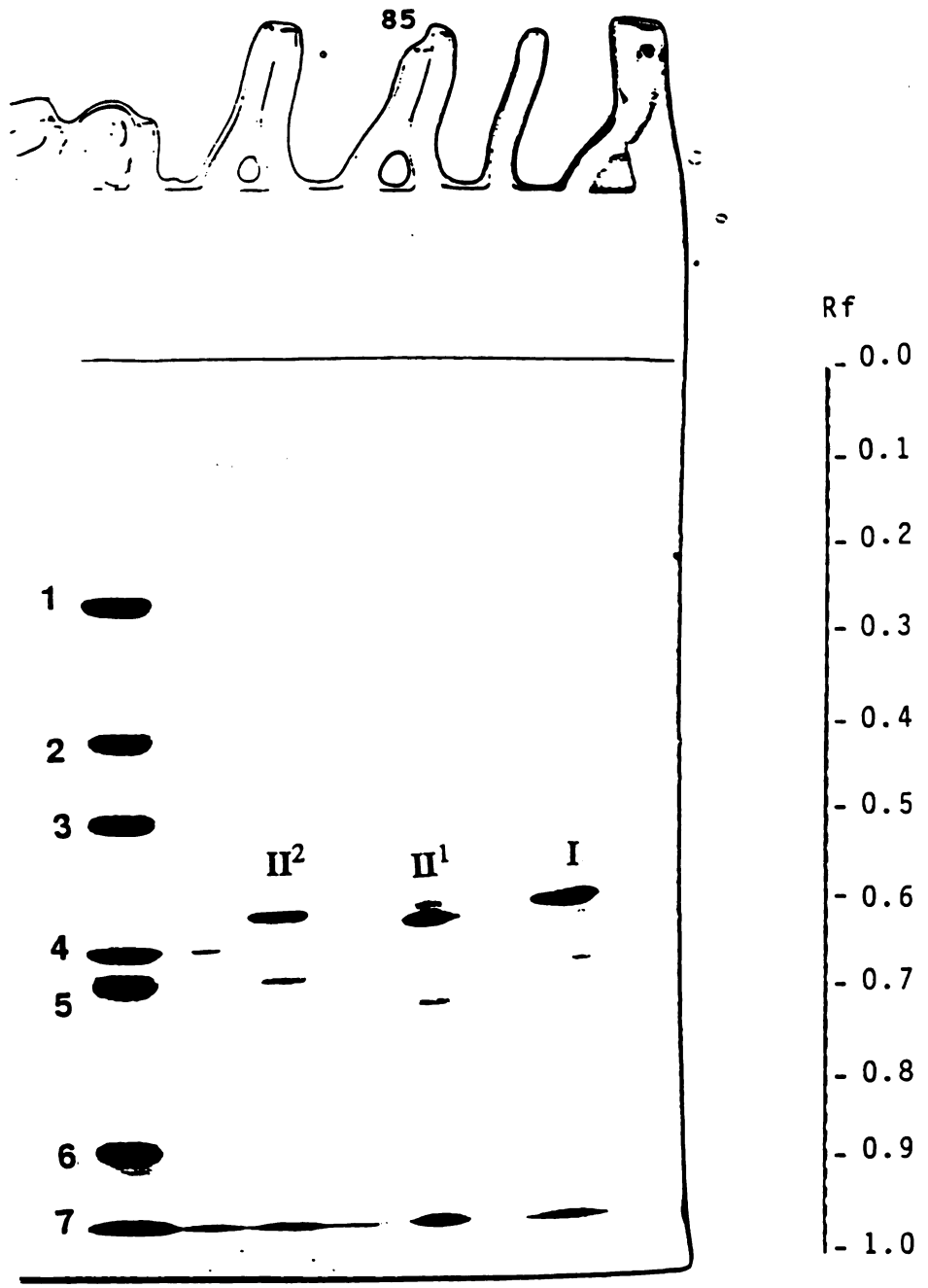


Figure 29. SDS-PAGE of cowpea α-galactosidases I, II^1 and II^2. MW of markers: 1, bovine albumin, 66,000; 2, egg albumin, 45,000; 3, glyceraldehyde-3-phosphate dehydrogenase, 36,000; 4, carbonic anhydrase, 29,000; 5, trypsinogen, 24,000; 6, trypsin inhibitor, soybean,20,000; 7, α-lactalbumin, 14,000.

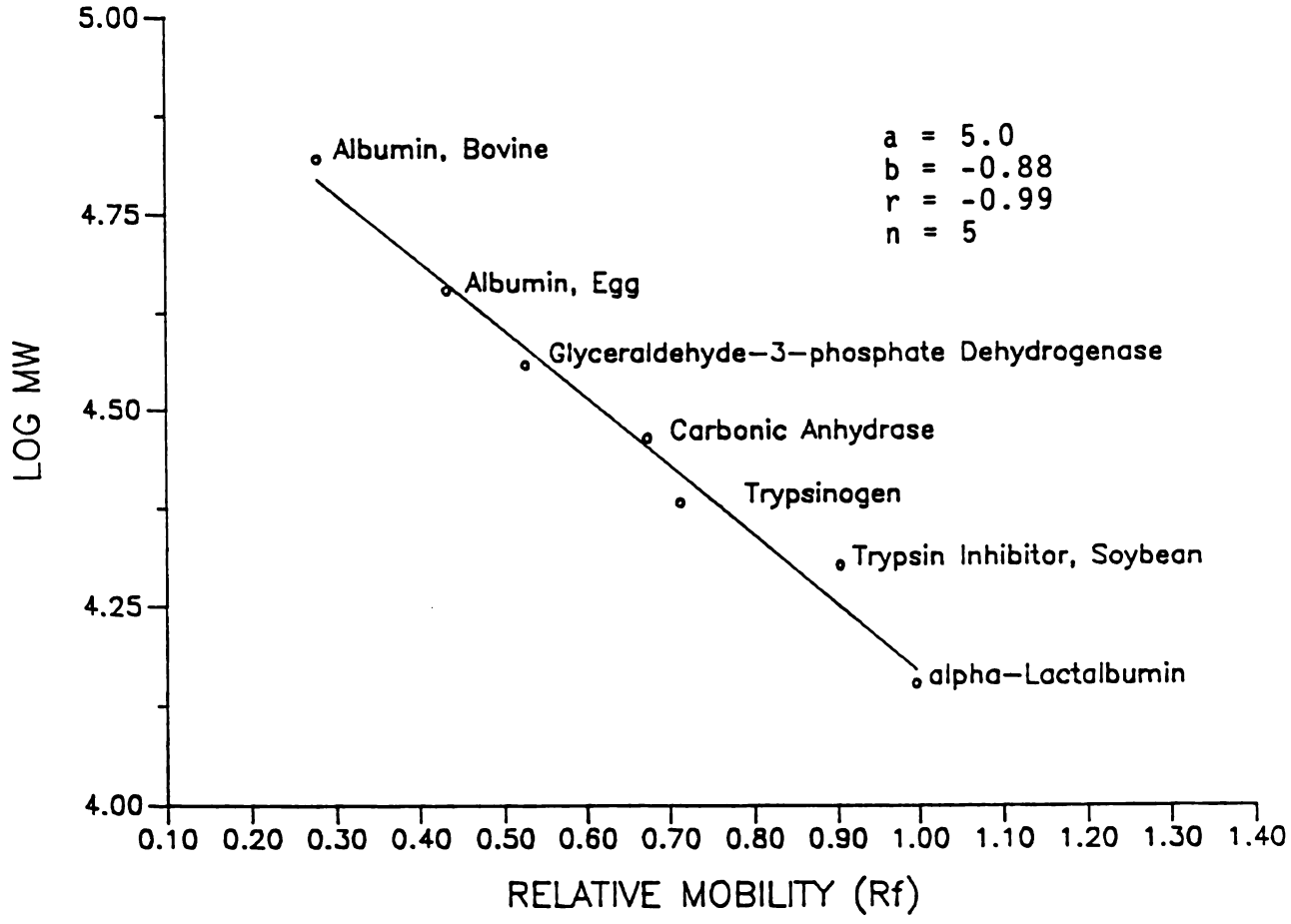


Figure 30. Molecular weight calibration curve using protein standards and SDS-polyacrylamide gel electrophoresis

ENZYME KINETICS

K_m and V_{max} for Enzyme I, II^1 and II^2 Using PNPG as Substrate

Lineweaver-Burk plots (equation 1) were used to calculate K_m and V_{max} values (Figures 31, 32, 33). Table 5 summarizes the K_m and V_{max} values for the enzymes. The K_m value of enzyme II^2 with PNPG (5.3 mM) was more than twice as high as the value obtained for Enzyme II^1 (2.2 mM) and approximately four times as high as the K_m of Enzyme I (1.5 mM). These results are not very different from other researchers (Appendix 2).

Table 5. K_m and V_{max} values of α-galactosidases using PNPG as substrate

Enzyme	I	II^1	II^2
K_m (mM)	1.5	2.2	5.3
V_{max} (umole/min/mg)	0.10	0.22	0.01

Determining K_m and V_{max} Values of Enzymes I, II^1 and II^2 Using Natural Substrates

Raffinose and stachyose as natural enzyme substrates were used to determine K_m and V_{max}. Stachyose is hydrolyzed more slowly, while raffinose seemed to be the substrate of preference because α-galactosidase affinity towards raffinose was higher than towards stachyose (Gavriel, 1985).

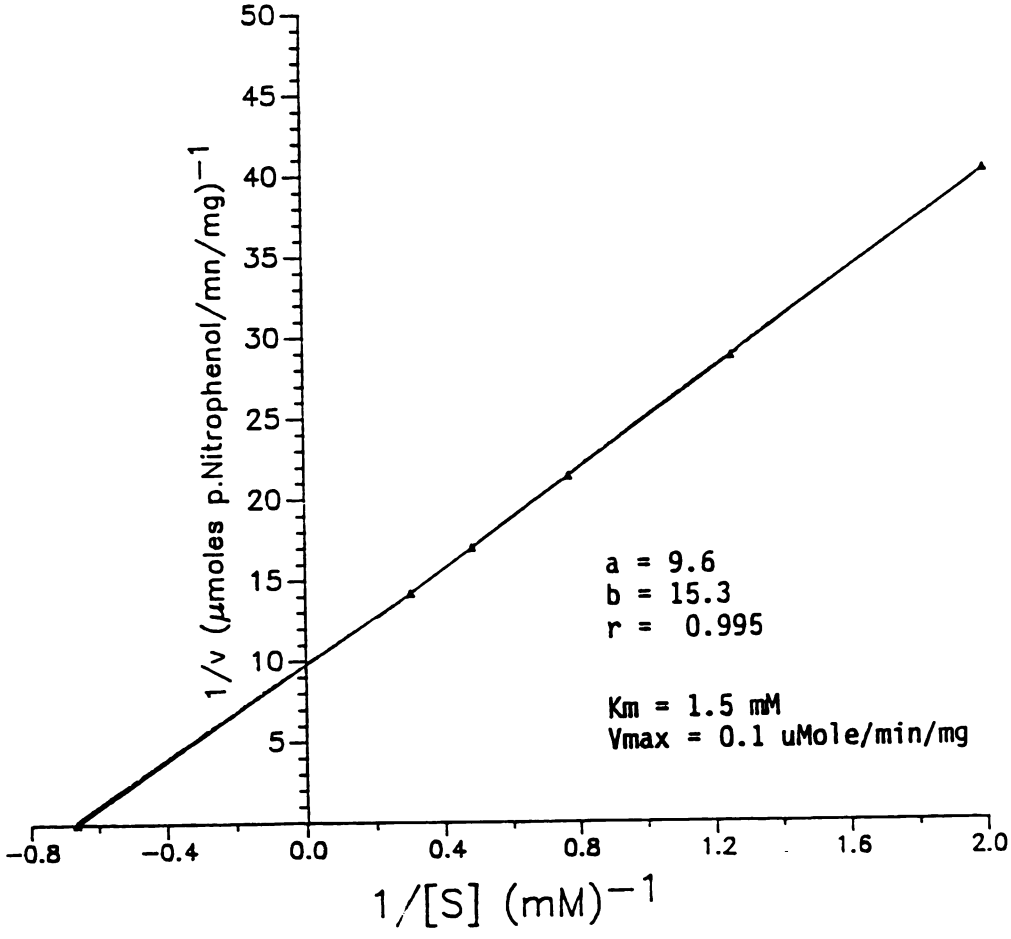


Figure 31. Lineweaver-Burk plot of the hydrolysis of PNPG by α-galactosidase I. n = 3

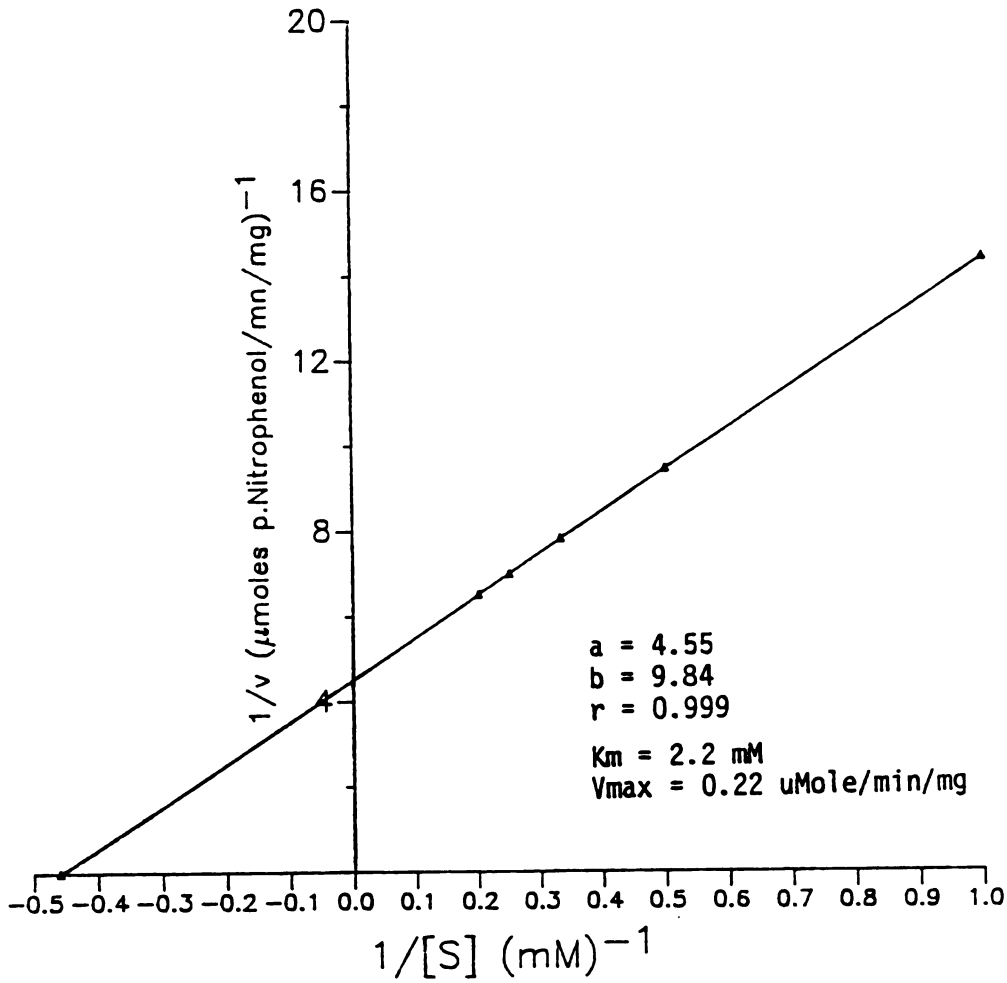


Figure 32. Lineweaver-Burk plot of the hydrolysis of PNPG by α-galactosidase II[1]. n = 3

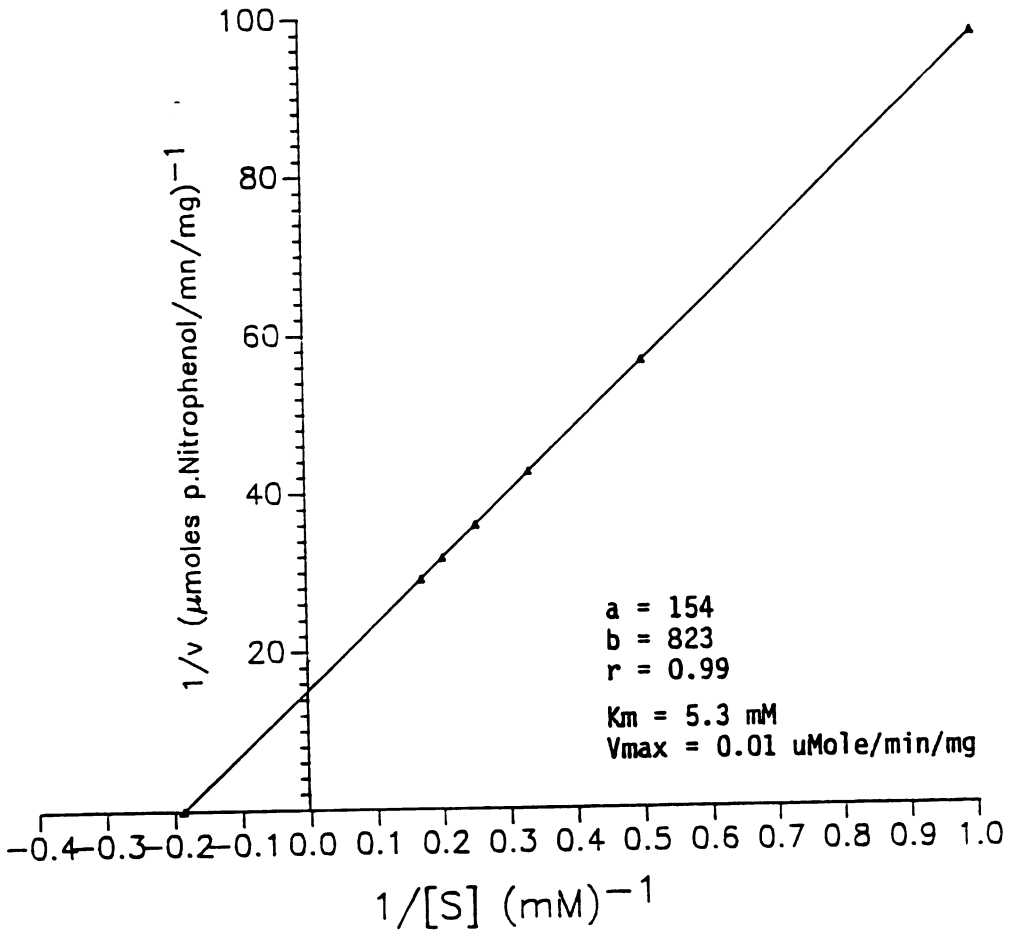


Figure 33. Lineweaver-Burk plot of the hydrolysis of PNPG by α-galactosidase II[2]. n = 3

Raffinose

When raffinose was the substrate, the enzymatic activity of Enzymes I, II^1 and II^2 was measured by the amount of liberated galactose. Originally, it was attempted to determine the freed galactose using HPLC but the method was not sensitive enough to measure the small quantities of galactose present. An enzymic method of determining galactose was used as microquantities of galactose had to be determined,

K_m and V_{max} for Enzymes I, II^1 and II^2 with raffinose as substrate were determined using Lineweaver-Burk plots (Figures 34, 35, 36). Table 6 summarizes the K_m and V_{max} values for the different enzymes. The K_m value of enzymes I (4.6 mM) and II^1 (5.0 mM) were close to each other, and were approximately three times as high as the K_m of Enzyme II^2 (1.6 mM). Thus, Enzyme II^2 has higher affinity ($1/K_m$) for raffinose than Enzyme I and II^1.

Table 6. K_m and V_{max} values of α-galactosidases using raffinose as substrate

Enzyme	I	II^1	II^2
K_m (mM)	4.6	5.0	1.6
V_{max} (umole/min/mg)	0.0034	0.043	0.001

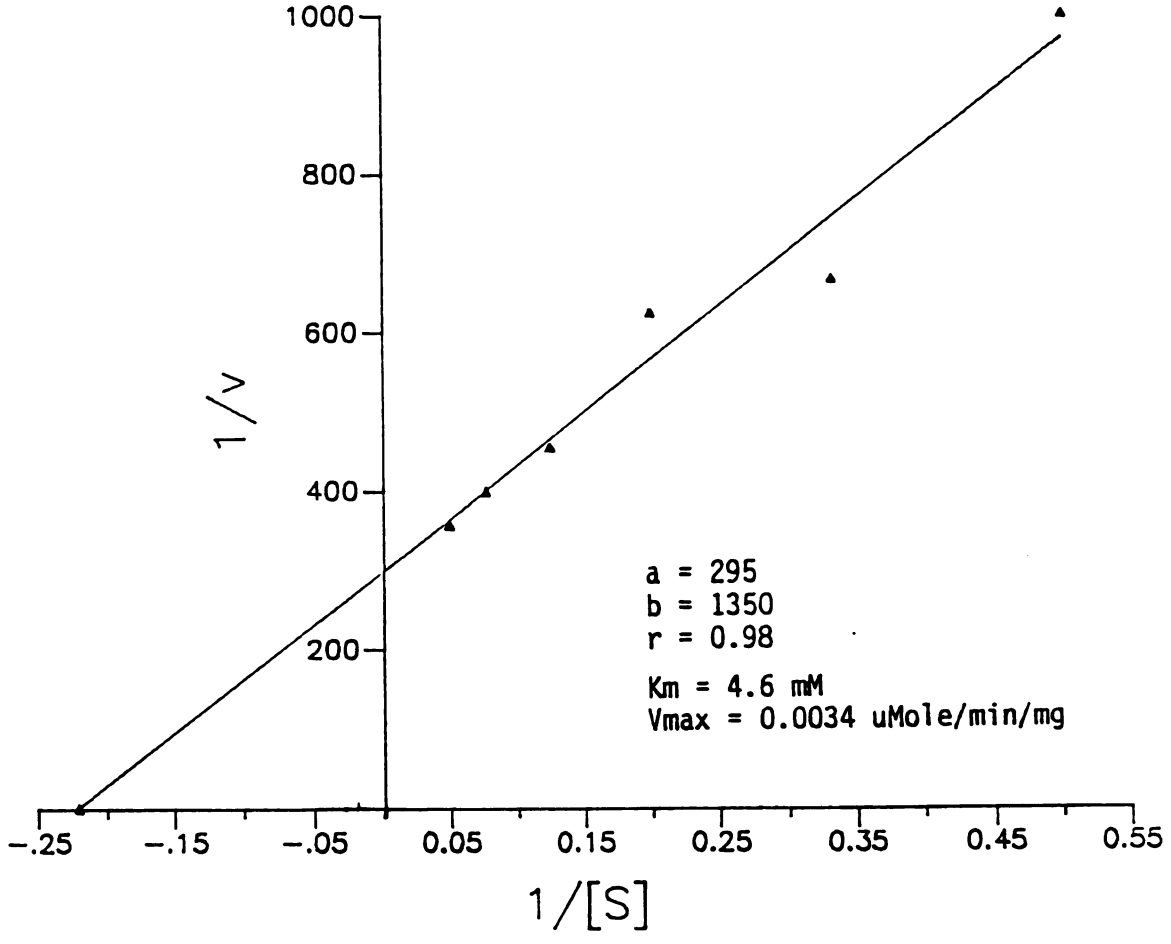


Figure 34. Lineweaver-Burk plot of the hydrolysis of raffinose by α-galactosidase I. n = 3

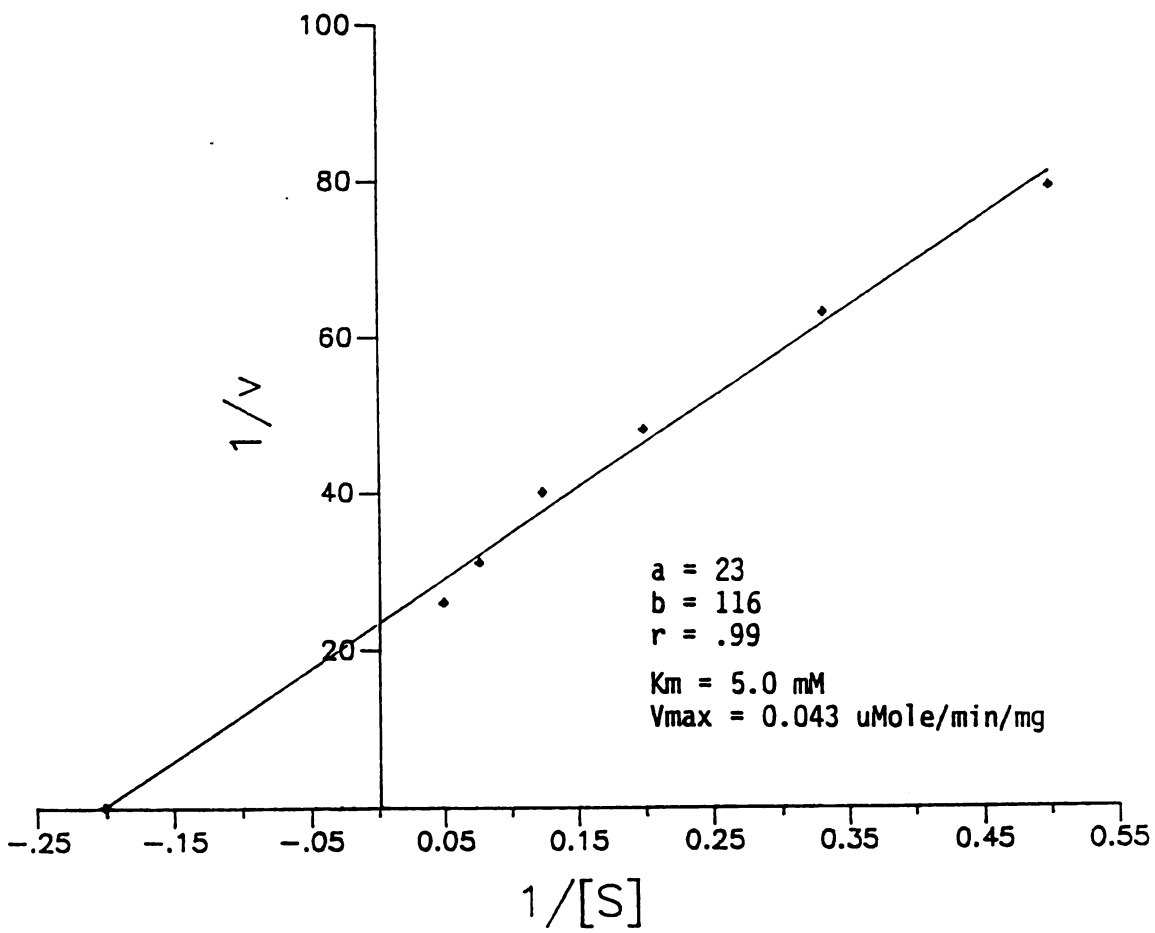


Figure 35. Lineweaver-Burk plot of the hydrolysis of raffinose by α-galactosidase II[1]. n = 3

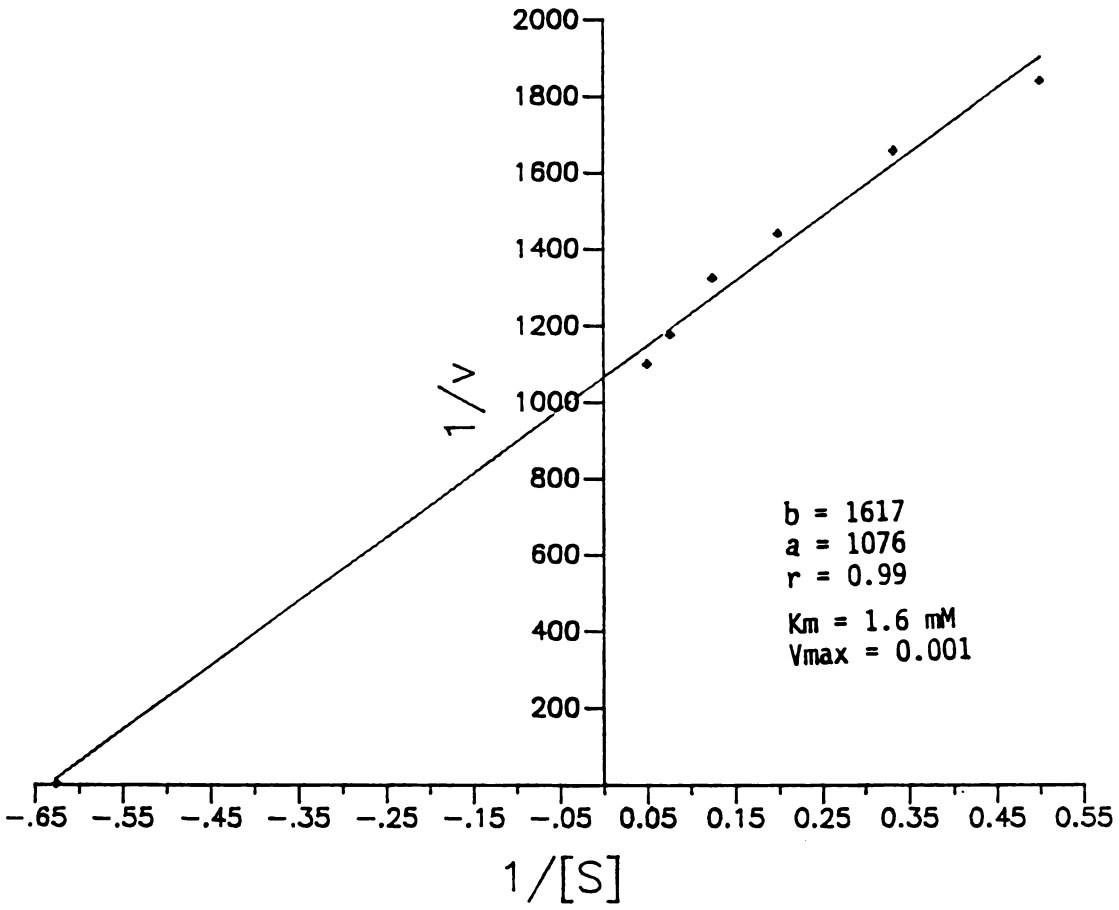


Figure 36. Lineweaver-Burk plot of the hydrolysis of raffinose by α-galactosidase II[2]. n = 3

Stachyose

It was difficult to determine the K_m and V_{max} for α-galactosidases using stachyose as substrate by measuring the amount of liberated galactose. Theoretically, two moles of galactose will be produced from hydrolysis of one mole of stachyose by α-galactosidases. While the reaction is in progress, it is impossible to assume a definite ratio of freed galactose to hydrolyzed stachyose. Therefore, K_m and V_{max} values were determined using HPLC to measure the disappearance of stachyose.

The Lineweaver-Burk plots of Enzymes I, and II^1 are shown in Figures 37, 38. Table 7 shows the K_m and V_{max} values of enzymes I and II^1. The K_m value for enzyme II^1 was higher than the value of Enzyme I, which means the affinity of Enzyme I towards stachyose was higher than the affinity of Enzyme II^1. The K_m values for Enzymes I and II^1 were 11.0 and 15.0 mM, respectively, using Lineweaver-Burk plot.

Table 7. K_m and V_{max} values of α-galactosidases using stachyose as substrate

Enzyme	I	II^1
K_m (mM)	11	15
V_{max} (umole/min/mg)	0.05	0.1

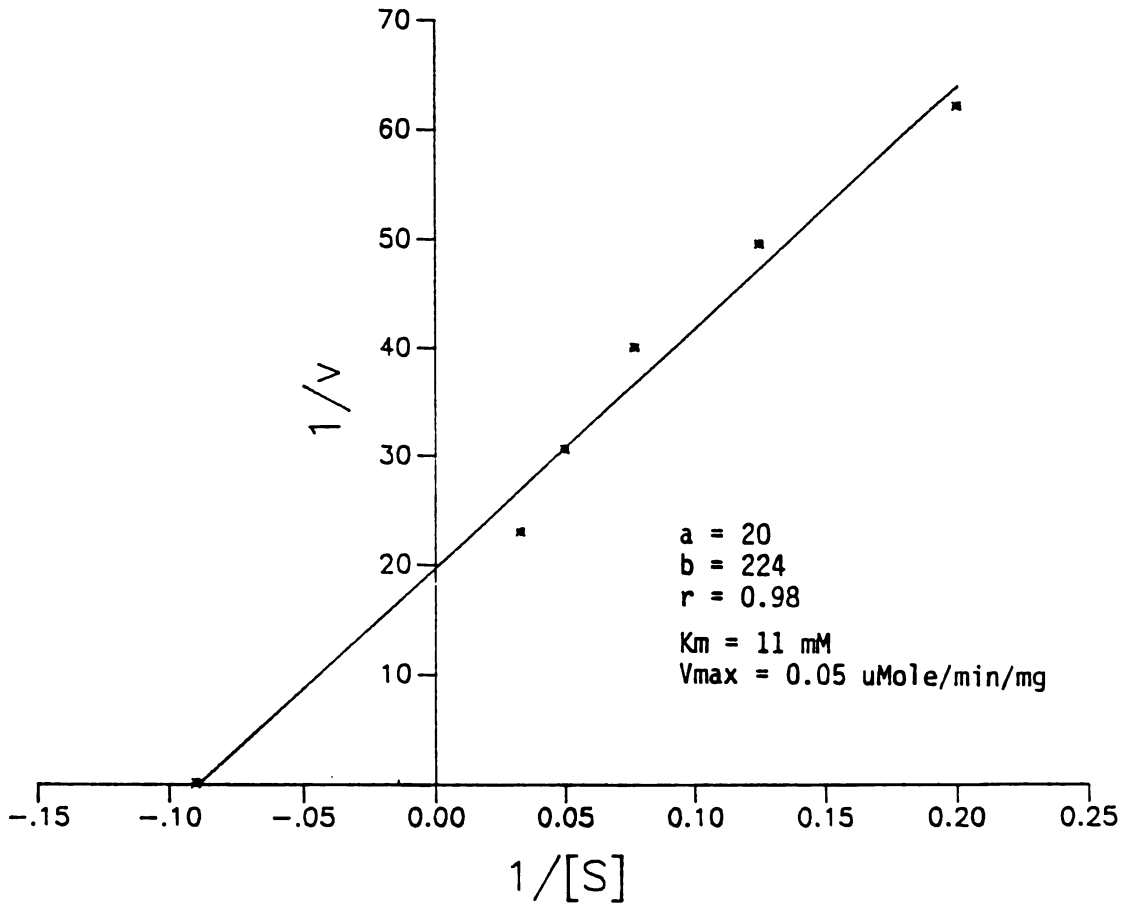


Figure 37. Lineweaver-Burk plot of the hydrolysis of stachyose by α-galactosidase I. n = 3

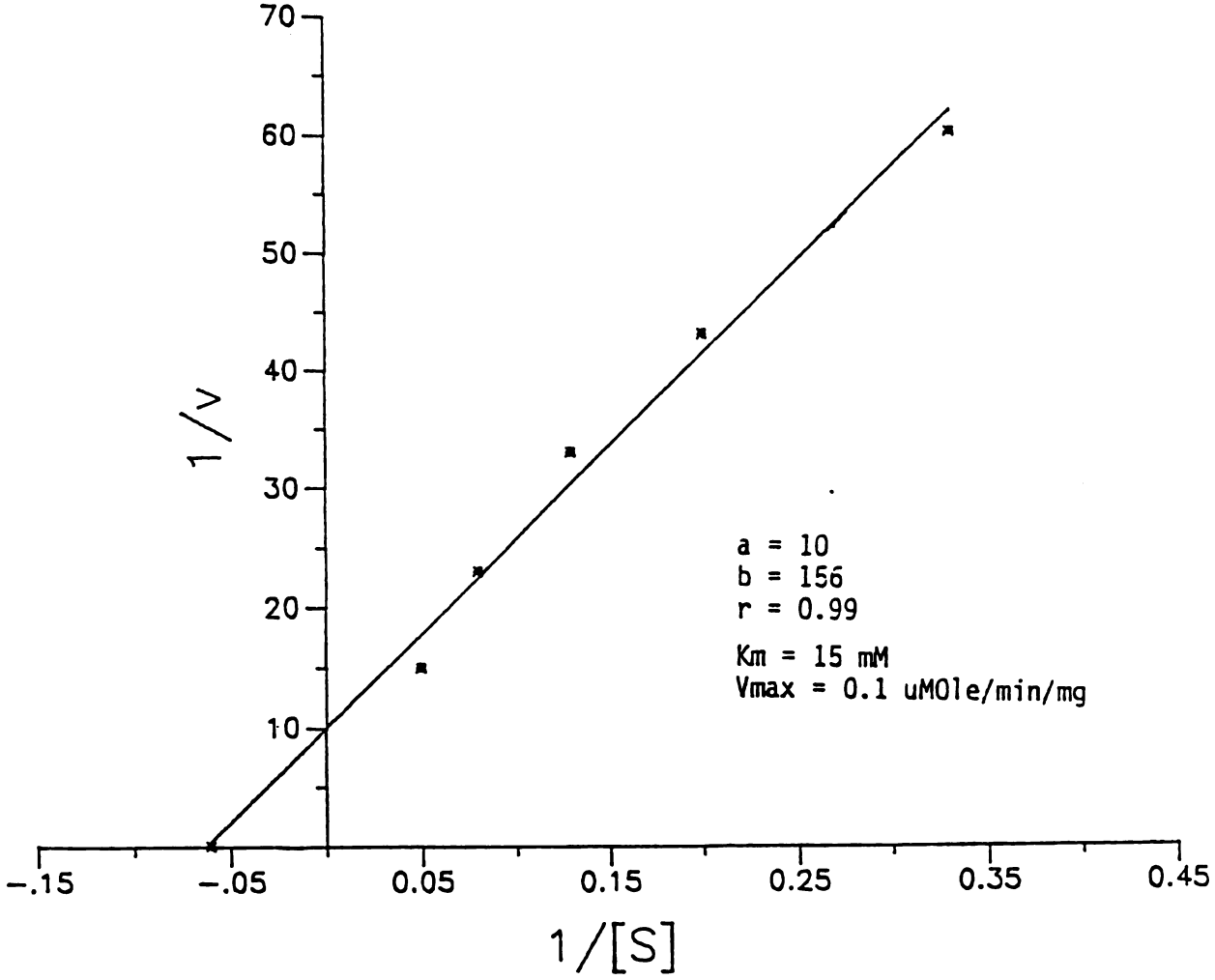


Figure 38. Lineweaver-Burk plot of the hydrolysis of stachyose by α-galactosidase II[1]. n = 3

Different values have been reported in the literature for the K_m of α-galactosidases of different sources using PNPG as substrate (Appendix 2). The K_m for α-galactosidases I and II from *V. faba*, using raffinose and stachyose as substrates were also reported (Dey and Pridham, 1972). The K_m values for Enzyme I were 4.0 and 7.5 mM and for Enzyme II were 5.0 and 5.26 mM using raffinose and stachyose, respectively. The method of measuring the K_m using stachyose as substrate was not mentioned.

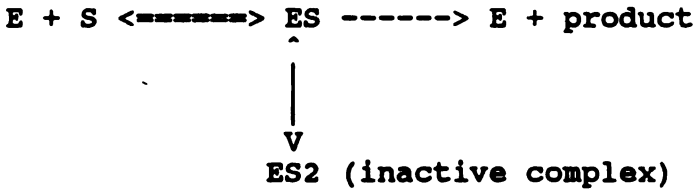
Effect of a Natural Substrate Concentration on Enzyme Activity

Table 8 shows the effect of increasing raffinose concentrations on Enzymes I, II^1 and II^2. The activity of Enzyme I started declining at concentrations higher than 40 mM of raffinose. The activity of Enzyme II^1 started diminishing at 160 mM raffinose and that of Enzyme II^2 showed a similar decline at 80 mM raffinose. With different K_m and enzyme amount, the comparison between the enzymes was not possible.

Table 8. Effect of substrate (raffinose) concentration on α-galactosidase activity

	Enzyme activity Kat/ml (at 30° C)		
Raffinose conc.(mM)	Enzyme I K_m 4.6 mM	Enzyme II[1] K_m 5.0 mM	Enzyme II[2] K_m 1.6 mM
10	217	552	98
20	233	665	108
40	227	756	133
80	207	816	118
160	200	718	93

The observations that, above a certain substrate concentration, for each enzyme, the reaction rate started decreasing indicates that substrate itself act as an inhibitor of the reaction, probably by forming inactive complexes with the enzyme as shown in the following scheme (Dey and Pridham, 1972).



STABILITY

The stability of Enzymes I, II^1 and II^2 during storage for 24 hr at four different temperatures and at various pH values was studied. Figure 39 shows the effect of pH and temperature on the stability of Enzyme I. The enzyme maintained its full activity in the pH range of 4.5 - 5.0 during 24 hr at 4° C. The enzyme lost almost 35% of its original activity in the pH range of 4.5 - 6.0 at 22° C. The enzyme was very unstable at 37° C, in general. The minimum loss of activity occurred at pH 5.8.

Figure 40 shows the effect of pH and temperature on the stability of Enzyme II^1. At 4° C and 22° C, Enzyme II^1 was more stable than Enzyme I and II^2; it maintained 100% activity in the pH range of 3.5 to 7.0 for 24 hrs. At 37° C almost 90% of original activity was preserved for 24 hr, at pH 4.5 - 5.5.

Figure 41 shows the effect of pH and temperature on the stability of Enzyme II^2. At 4° C, it maintained 75-80% of its original activity for 24 hrs in a large pH range (3.5 to 7.5). At 22° C the stability of the enzyme was somewhat lower at pH 4.0 but it did keep 75% of its original activity in the pH range of 5.0 to 6.5.

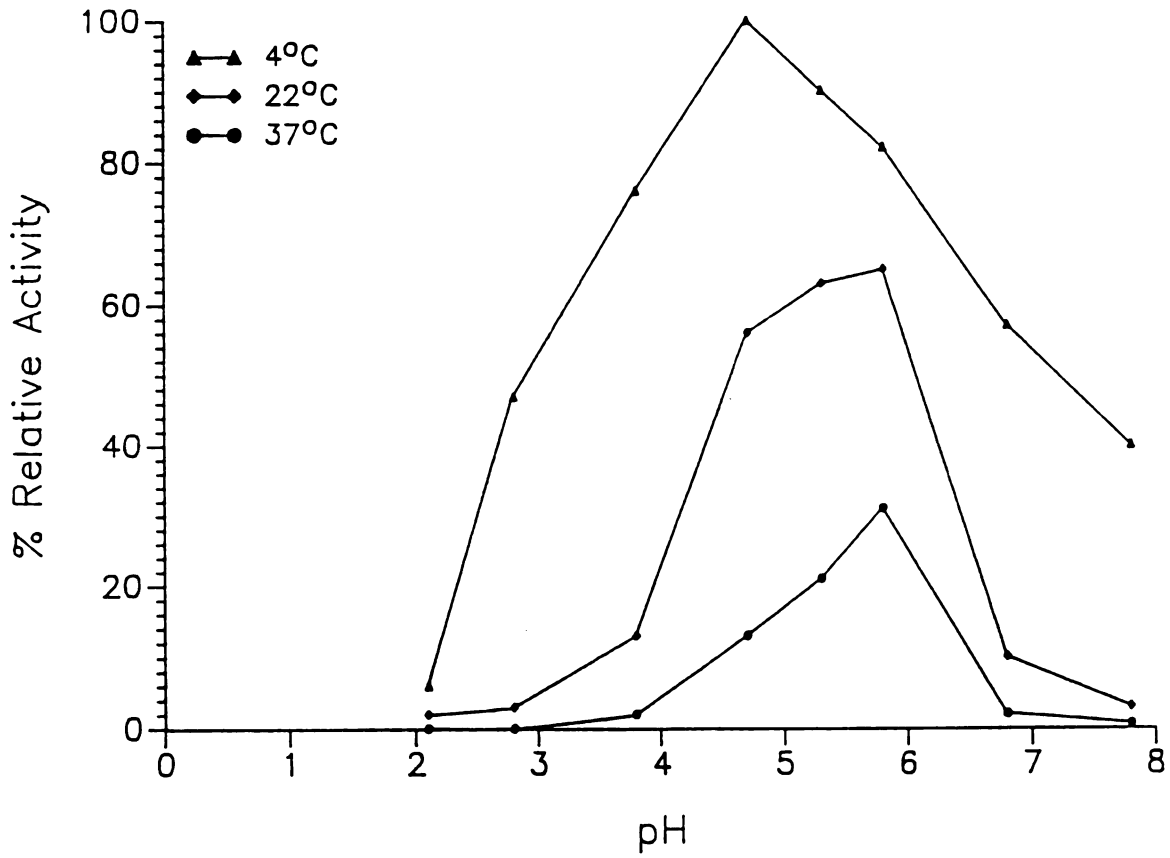


Figure 39. Effect of pH and temperature on the stability of Enzyme I after 24 hr of storage

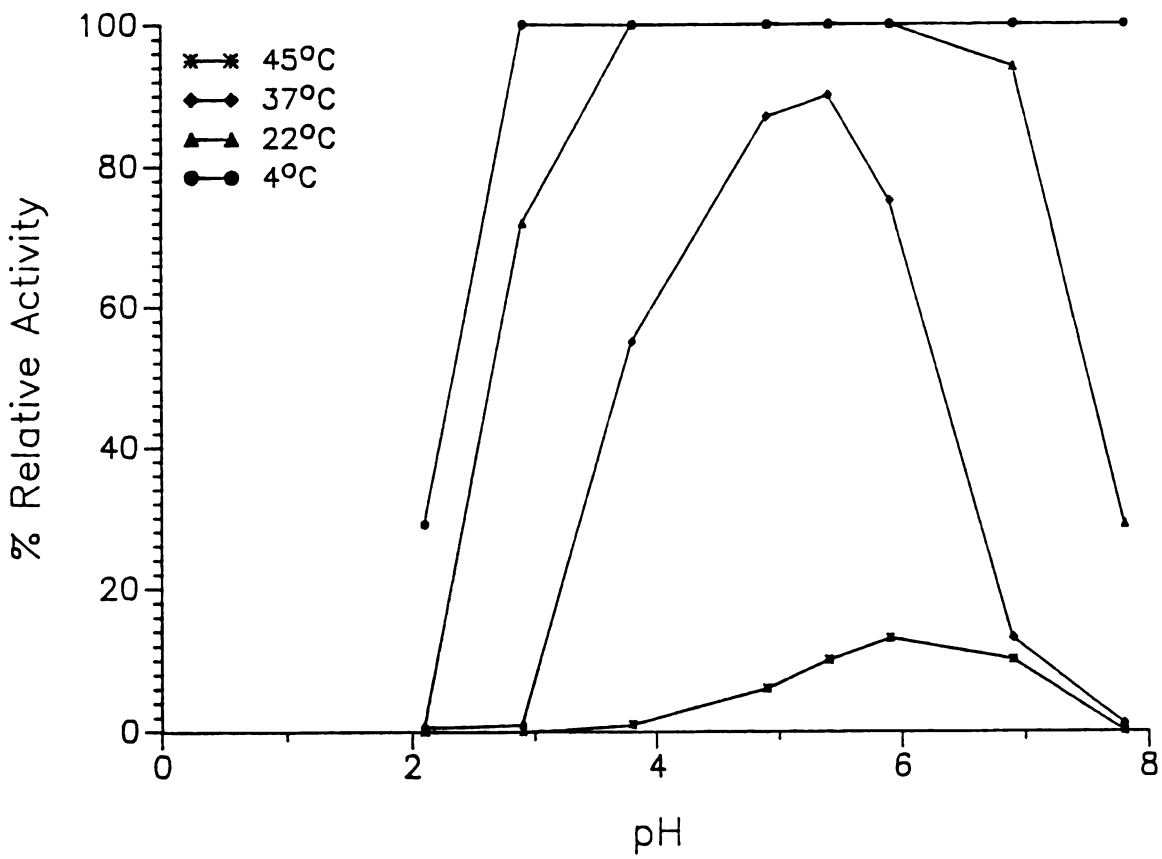


Figure 40. Effect of pH and temperature on the stability of Enzyme II[1] after 24 hr of storage

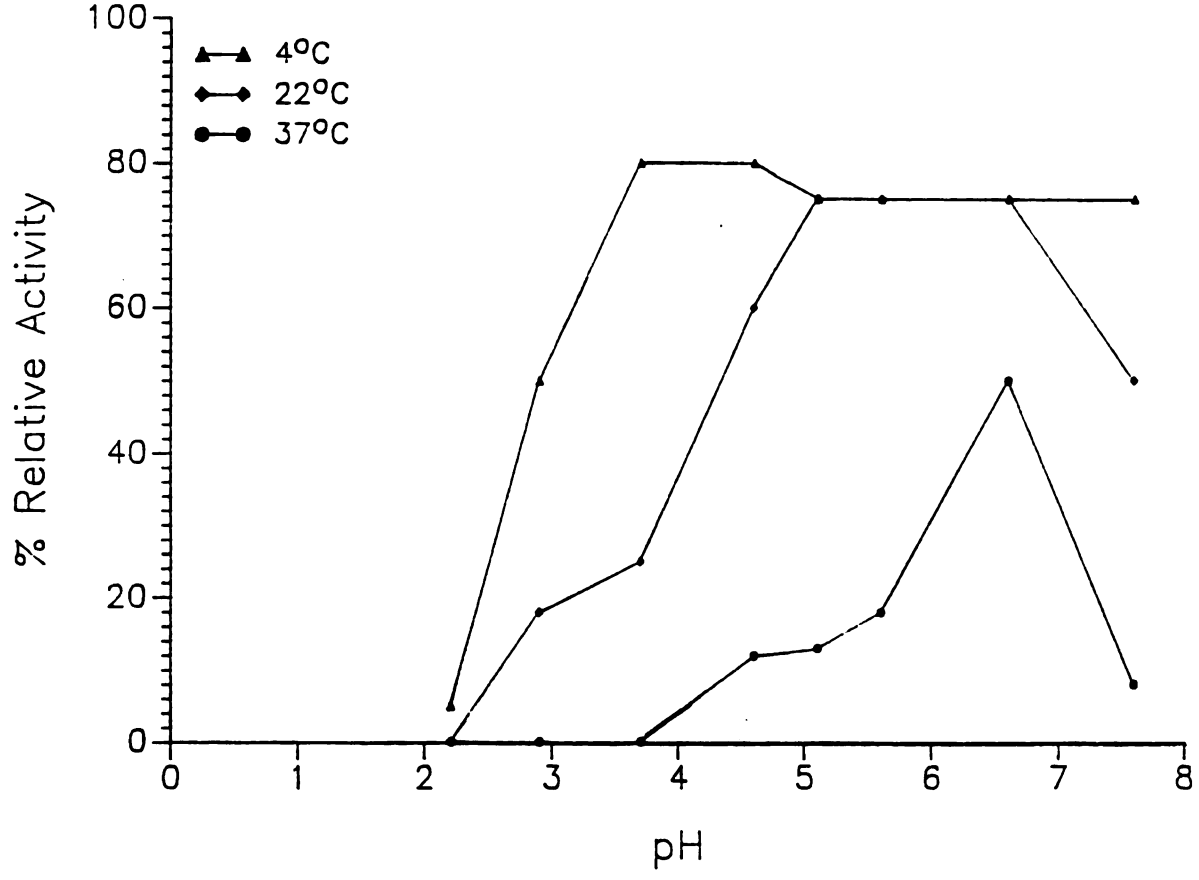


Figure 41. Effect of pH and temperature on the stability of Enzyme II[2] after 24 hr of storage

In general, all three enzymes showed their greatest stability at 4° C and at intermediate pH values (Table 9). Enzyme II^1 was more stable than Enzyme II^2 ; Enzyme I was the least stable under the conditions of this experiment. Enzyme II^1, the only one showed some activity at 45° C.

The thermal stability of other α-galactosidases, isolated from various species of the genus Aspergillus, such as A. satoi demonstrated an optimum temperature at 55° C (Sugimoto and Van, 1970).

OPTIMUM pH AND OPTIMUM TEMPERATURE FOR THE ENZYME ACTIVITY

The pH optimum for the α-galactosidases I, II^1 and II^2 were tested at 30° C and found to be 5.0, 5.9 and 5.3 respectively (Figures 42, 43, 44). The activity decreased rather rapidly on either side of the optimum pH. various figures have been reported in the literature as the optimum pH of α-galactosidase activity (Appendix 2).

The optimum temperature for the activity of each of the three enzymes was tested at the optimum pH. For all three enzymes the optimum activity temperature was found to be 50° C. The activity decreased rapidly at temperature higher than the optimum temperature indicating heat denaturation (Figures 45, 46, 47). Most α-galactosidases behave normally as rate of the enzyme-catalyzed reaction increases to an optimum value with increasing temperature

until inactivation of the enzyme occurs (Dey and Pridham, 1972). α-Galactosidase from Aspergillus awamori has an optimum temperature of 50° C (McGhee et al., 1978). Optimum temperature of α-galactosidase from A. oryzae for hydrolysis of PNPG was 50° C (Cruz and Park, 1982).

Table 9 summarized the results of the pH and temperature of optimum activity along with the pH of optimum stability at 22° C .

Table 9. Temperature and pH optima of α-galactosidases from cowpeas

Enzyme	I	II[1]	II[2]
Opt. activity pH (30° C)	5.0	5.9	5.3
Opt. activity temp.(at opt. pH)	50°C	50°C	50°C
Opt. stability pH (22°C)	4.5-6.0	3.5-7.0	4.5-6.5

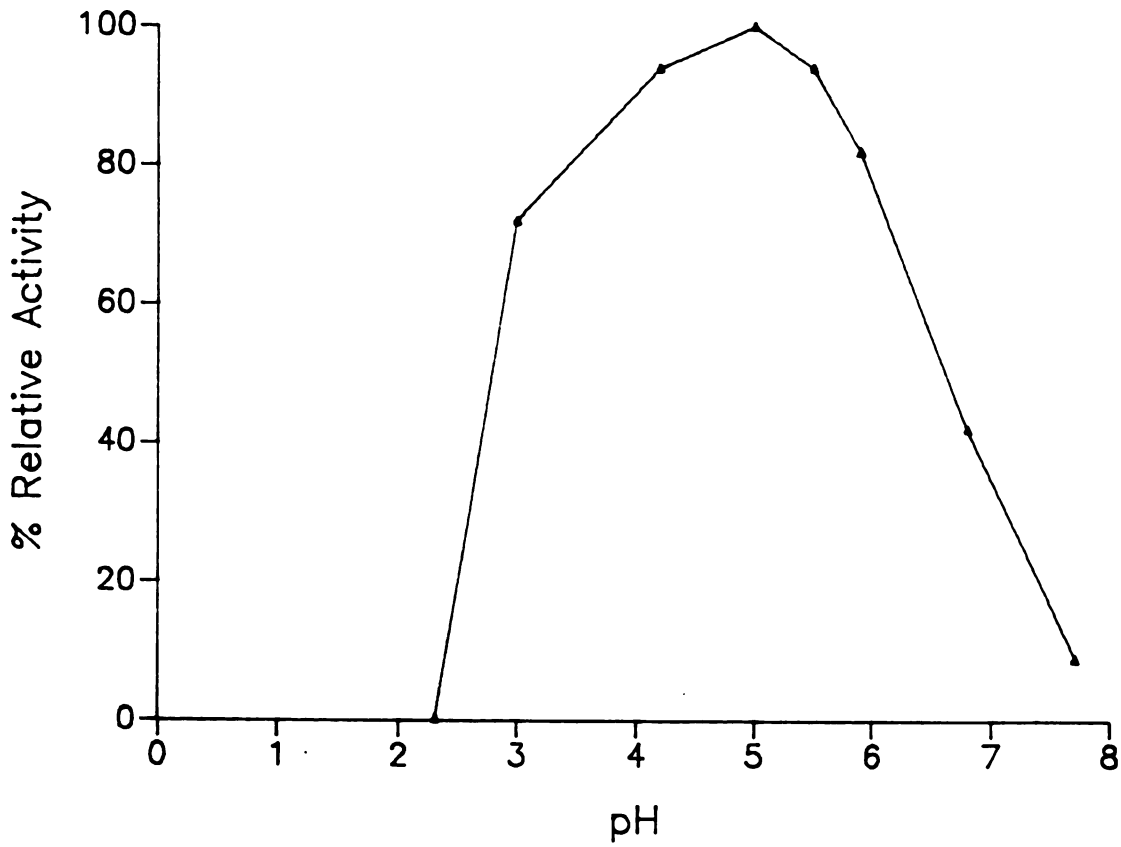


Figure 42. Effect of pH on the activity of Enzyme I

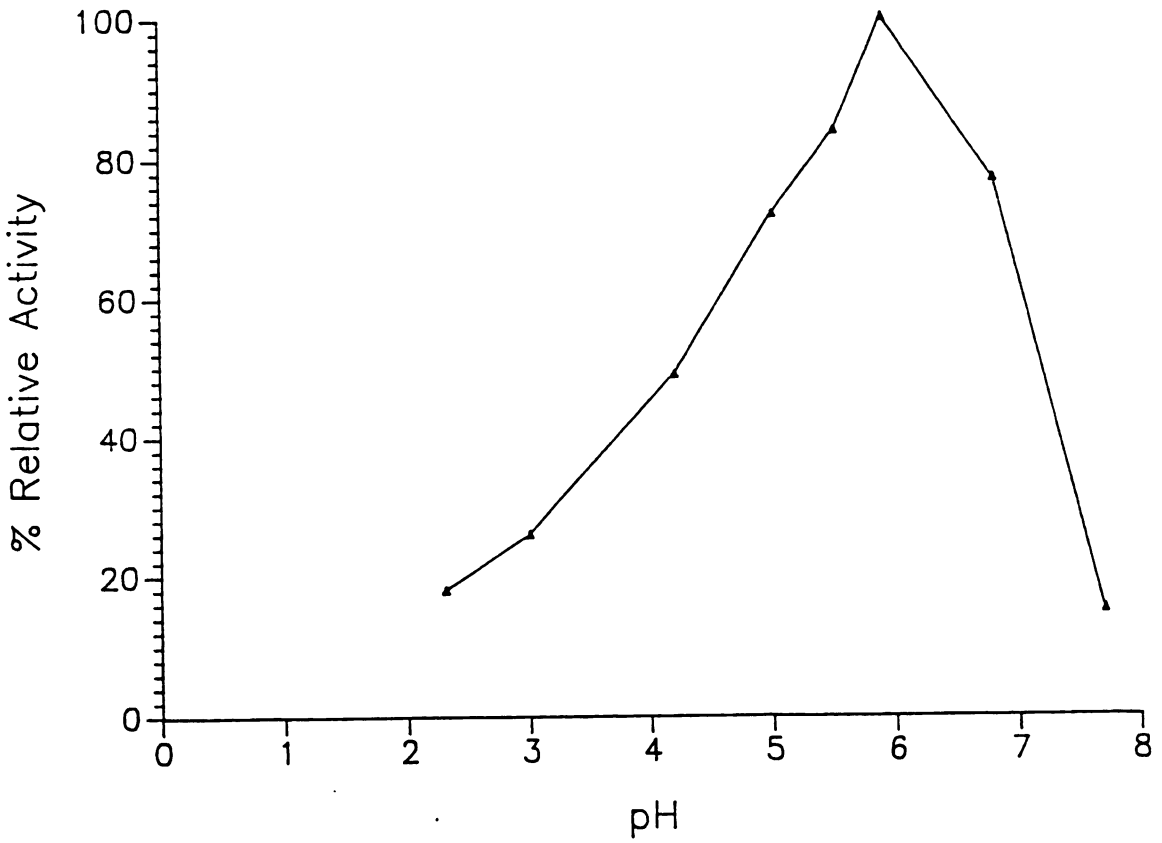


Figure 43. Effect of pH on the activity of Enzyme II[1]

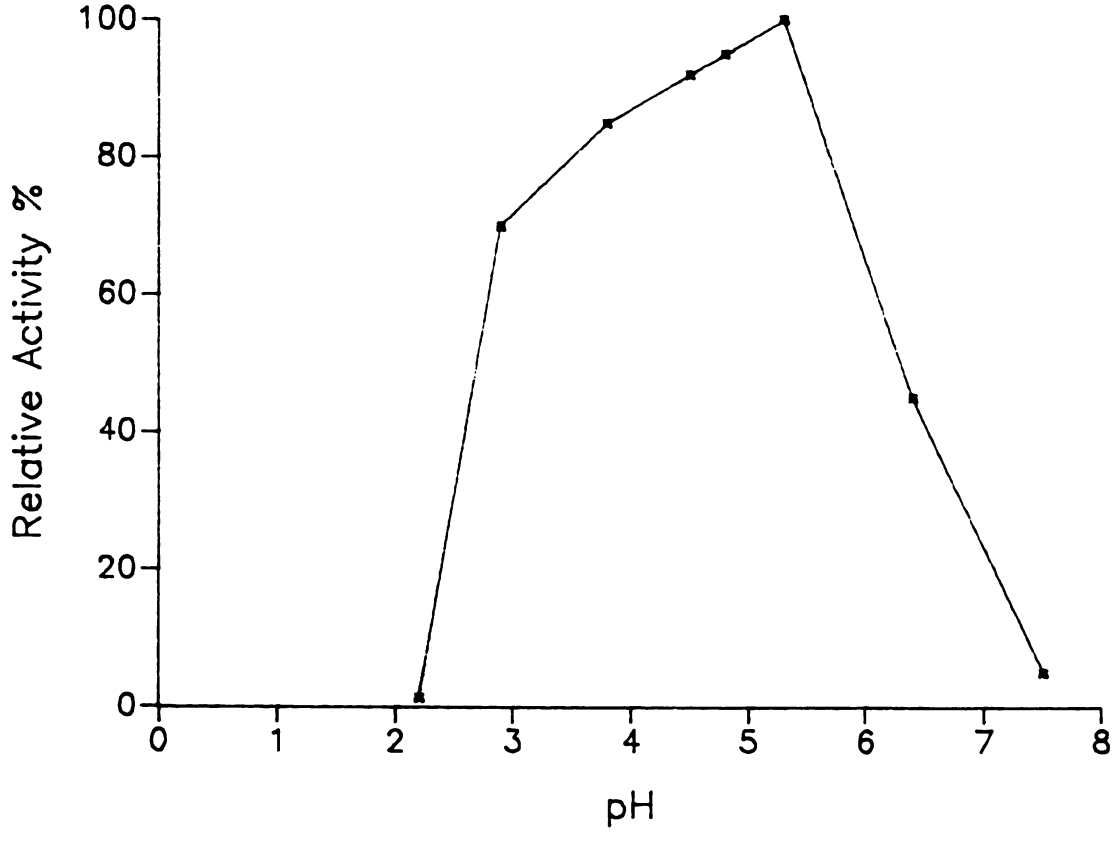


Figure 44. Effect of pH on the activity of Enzyme II^2

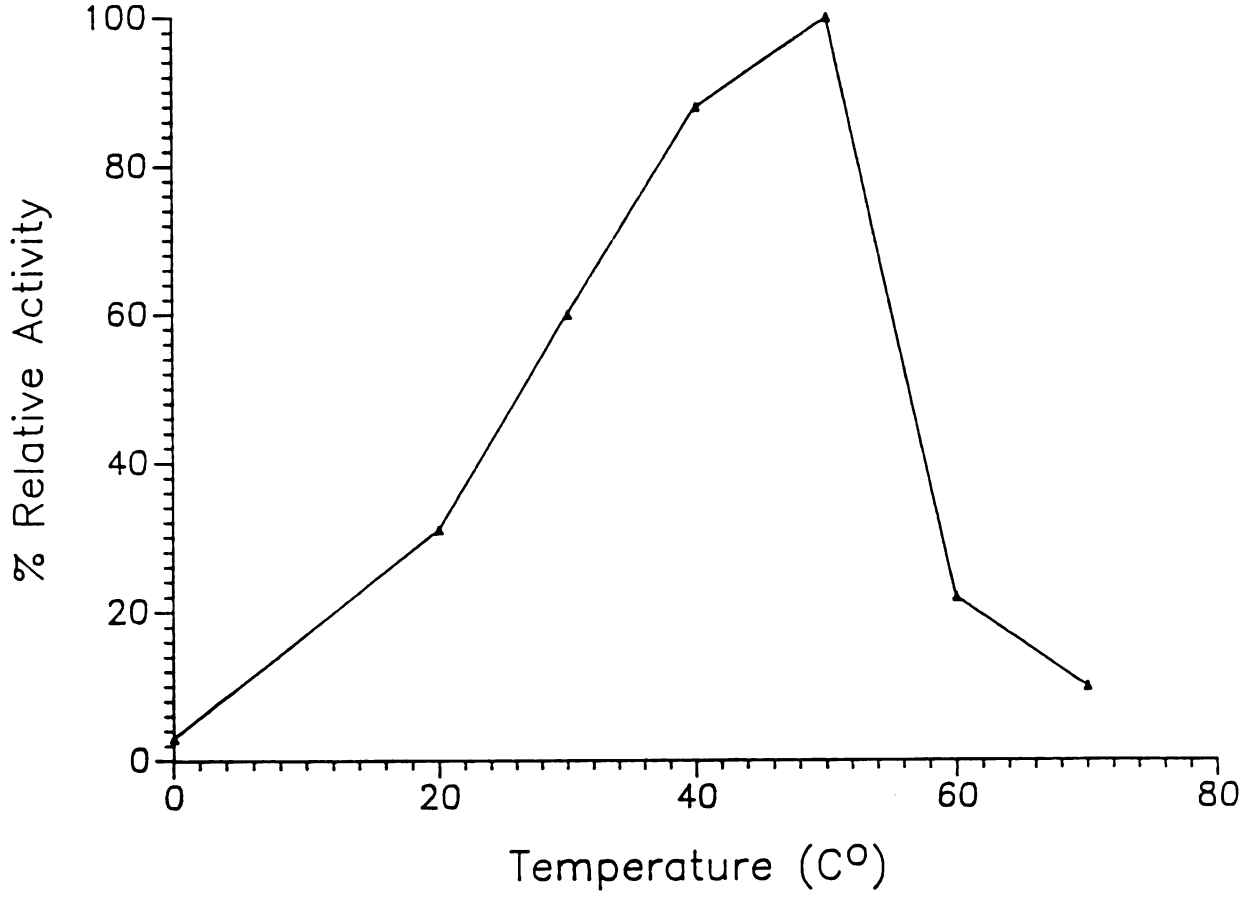


Figure 45. Effect of temperature on the activity of Enzyme I

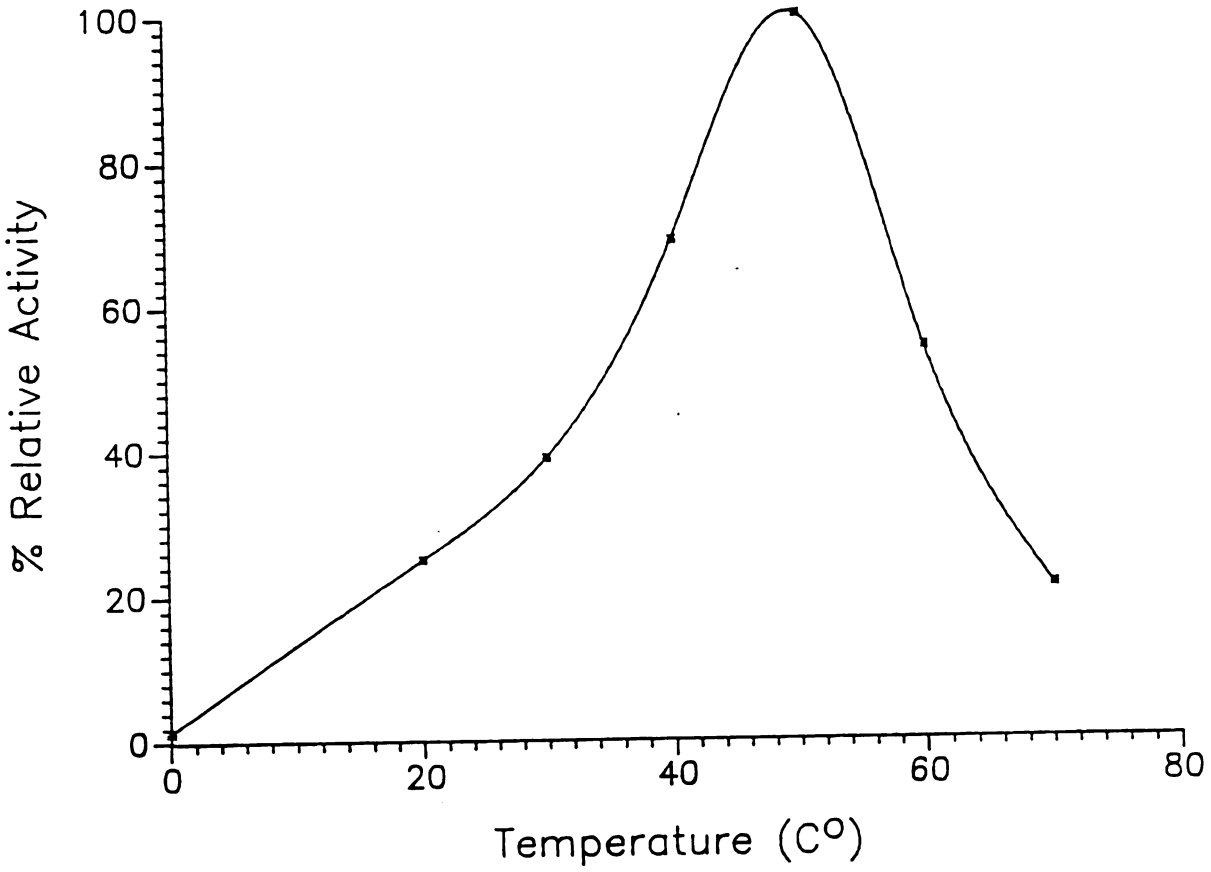


Figure 46. Effect of temperature on the activity of Enzyme II[1]

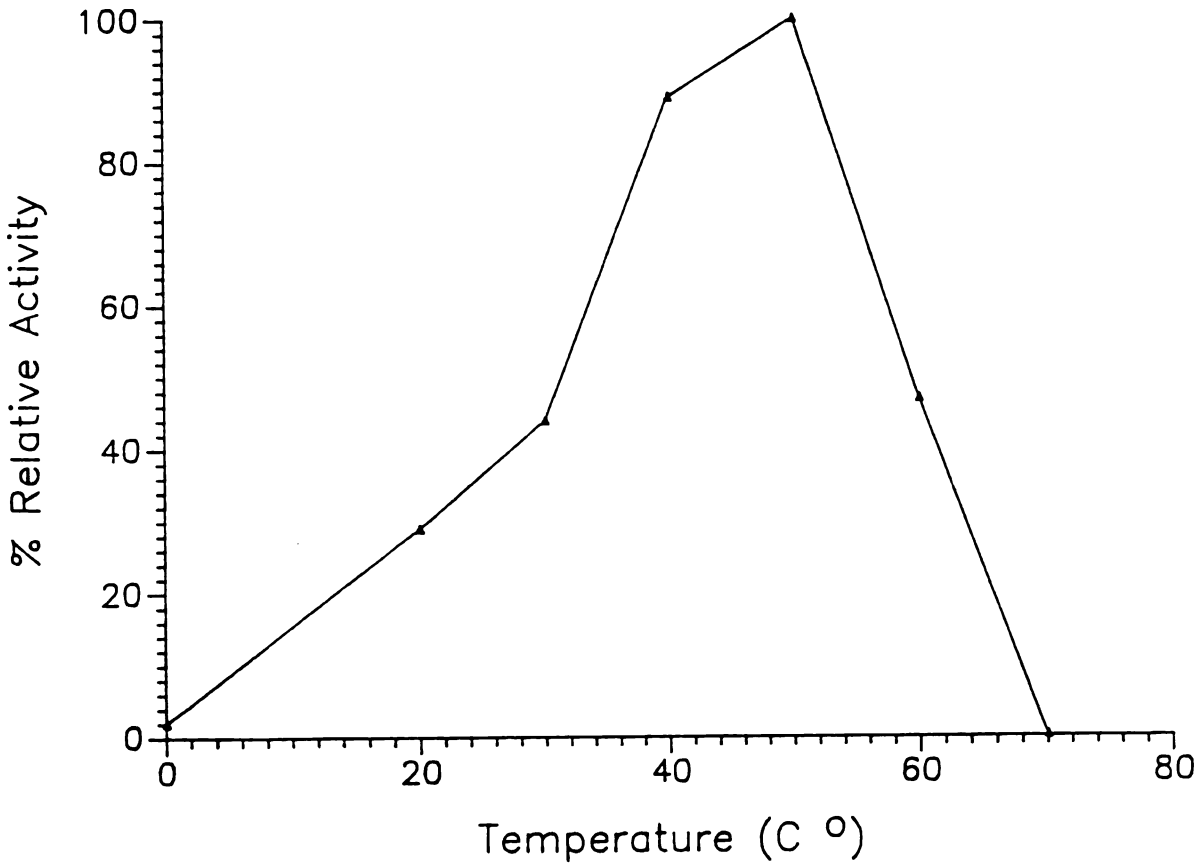


Figure 47. Effect of temperature on the activity of Enzyme II[2]

GERMINATION EFFECT ON COWPEA α-GALACTOSIDASE

The α-galactosidase activity increased rapidly during the first 6 hrs of germination at 24° C and leveled off during the subsequent 24 hrs (Figure 48). Germination at 30° C showed a similar trend in α-galactosidase activity.

In a second experiment, the α-galactosidase activity was assayed for up to 72 hrs of germination at 30° C in the sprouts and the remaining seeds. As shown in Table 10, this activity increased less rapidly in the sprouts during the first 42 hrs, than during the following 30 hrs, while the activity in the desprouted seeds did not change very much during the entire 72 hrs period.

Table 10. Total α-galactosidase activity in sprout and desprouted seeds of cowpeas at 30° C (pH 5.5, PNPG)

Time of sprouting (hr)	Part	Enzyme activity (A_{405} /g dry wt.)
0	whole seed	32
42	sprout	39
72	sprout	68
42	seeds w/o sprout	51
72	seeds w/o sprout	58

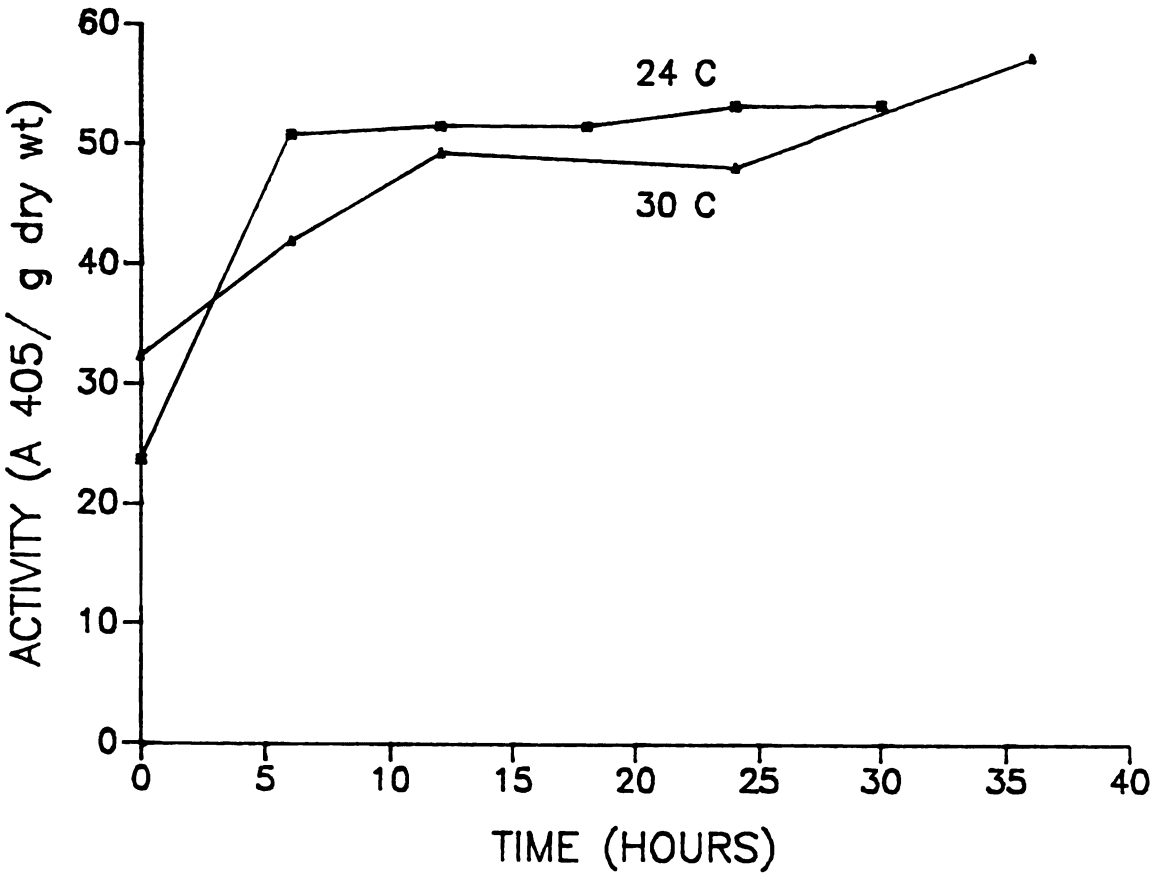


Figure 48. α-Galactosidase activity at different germination temperatures (2 hrs soaking prior to germination)

GERMINATION EFFECT ON COWPEA OLIGOSACCHARIDES

The oligosaccharide changes during germination for 24 hours at 24° C (room temp.) are shown in Figure 49. Sucrose, raffinose and stachyose concentration did not change significantly during the 24 hr germination period ($P < 0.05$). The correlation coefficients between concentrations of each sugar and time were around 0.8 (sucrose 0.78, raffinose -0.78 and stachyose -0.8). The statistical analysis of oligosaccharide changes during germination for 24 hours at 24° C shows in Table 11.

Table 11. The statistical analysis of oligosaccharide concentration changes during germination for 24 hrs at 24°

	Sucrose	Raffinose	Stachyose
Slope (arithmetic)*	0.029	-0.005	-0.042
Slope (logarithmic)	0.0065	-0.0182	-0.007
Intercept (arithmetic)	1.61	0.213	3.15
Intercept (logarithmic)	0.21	-0.64	0.49
r value (arithmetic)	0.78	-0.78	-0.80
r value (logarithmic)	0.74	-0.81	-0.77
probability (arith)	0.12	0.12	0.11
probability (logarith)	0.16	0.09	0.13
Standard error (arith)	0.013	0.0024	0.019
Standard error (log)	0.0034	0.0076	0.003

* arithmetic and logarithmic in the Table were for linearity. n = 2

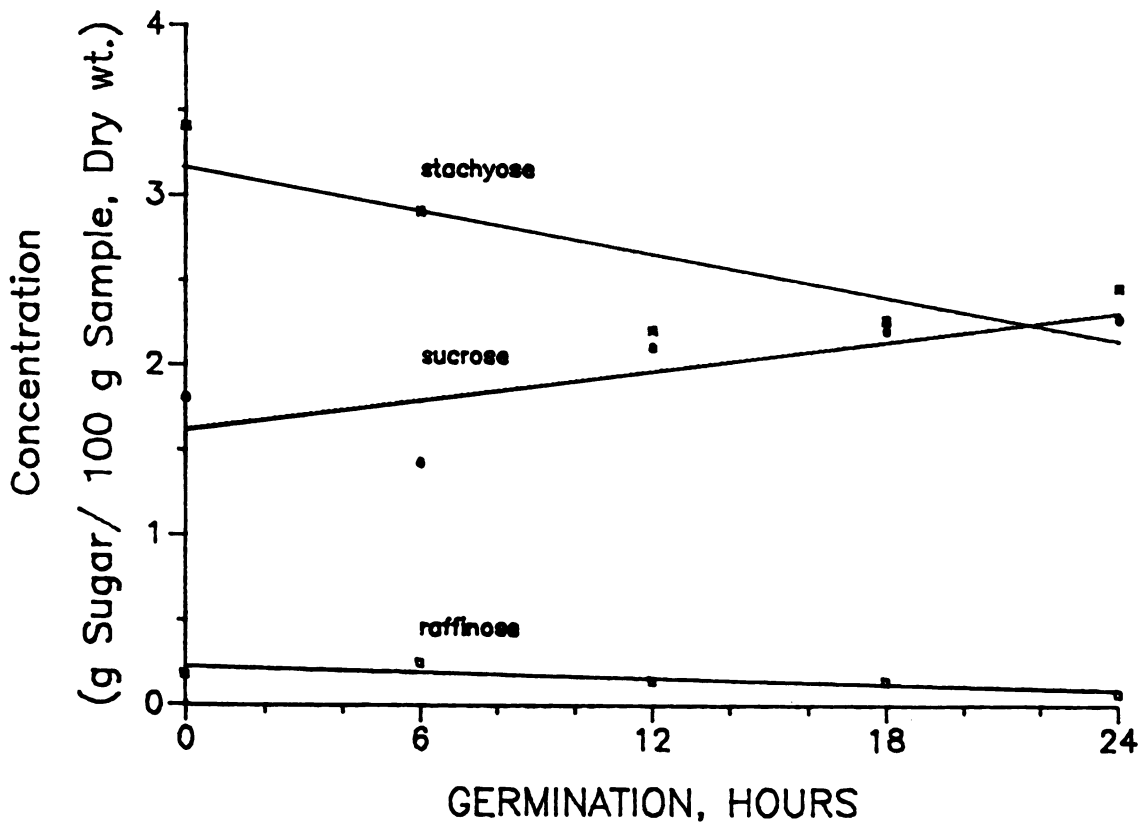


Figure 49. Oligosaccharide changes during germination for 24 hours at 24° C (2 hrs soaking prior to germination). n=3

SEED PASTE INCUBATION

Figure 50 shows the changes in oligosaccharide contents during 24 hrs of cowpea flour paste incubation at two different temperatures 24° C and 34° C. The 24 and 34° C were chosen to cover the temperature range of the places in which the cowpeas are consumed. Sucrose content significantly decreased during incubation at 24° C and 34° C ($P < 0.05$). The rates of sucrose decrease (slope) at 24° and 34° C were -0.057 and -0.061 % sugar/hr, respectively. Raffinose content did not change significantly at 24° C ($P < 0.05$) although a significant decrease was observed at 34° C ($P < 0.05$). Raffinose decreased twice as fast at 34° C than at 24° C (-0.0074% / hr, $P < 0.05$). The rate (slope) of stachyose depletion at 24° C and 34° C were -0.064 and -0.1 % sugar/ hr, respectively, and there was a significant difference between the two ($P < 0.05$). Stachyose and sucrose concentrations approached zero at 34° C after 24 hr incubation. The pH of the paste was 6.35 and did not change significantly ($P < 0.05$) at 24° C. At 34° C the pH changed significantly ($P < 0.05$) at a rate (slope) of -0.016. The pH decreased from 6.35 to 6.00. The rate of oligosaccharide content of the paste decreased in the following order during incubation: stachyose> sucrose> raffinose.

Since the arithmetic linear correlation is better than the logarithmic linear correlation for raffinose and

stachyose, the rate comparisons (slopes) between the two temperatures refer to the arithmetic linearities. The arithmetic linear correlations were easier to plot and to interpret although the logarithmic correlations were higher within the same temperature. Table 12 shows the statistical analysis of oligosaccharide and pH changes during flour paste incubation at different temperatures.

Table 12. The statistical analysis of oligosaccharide concentration and pH changes during flour paste incubation at different temperatures

	Sucrose		Raffinose		Stachyose		pH	
	24°C	34°C	24°C	34°C	24°C	34°C	24°C	34°C
slope[1]	-0.057	-0.061	-0.004	-0.0074	-0.064	-0.10	-0.021	-0.016
slope[2]	-0.034	-0.041	-0.008	0.018	-0.015	-0.05		
Incpt.[1]	1.54	1.46	0.27	0.28	2.66	2.50	6.46	6.34
Incpt.[2]	0.24	0.22	-0.57	0.53	0.43	0.50		
r value[1]	-0.94	-0.93	-0.75	-0.98	-0.90	-0.96	-0.85	-0.88
r value[2]	-0.98	-0.99	-0.78	-0.99	-0.94	-0.99		
Prob.[1]	0.017	0.023	0.14	0.004	0.036	0.008	0.07	0.047
Prob.[2]	0.004	0.001	0.123	0.002	0.02	0.001		
Std. E.[1]	0.012	0.014	0.002	0.001	0.018	0.016	0.01	0.005
Std. E.[2]	0.004	0.003	0.004	0.002	0.003	0.004		

1= arithmetic linearity, 2= logarithmic linearity, n= 3

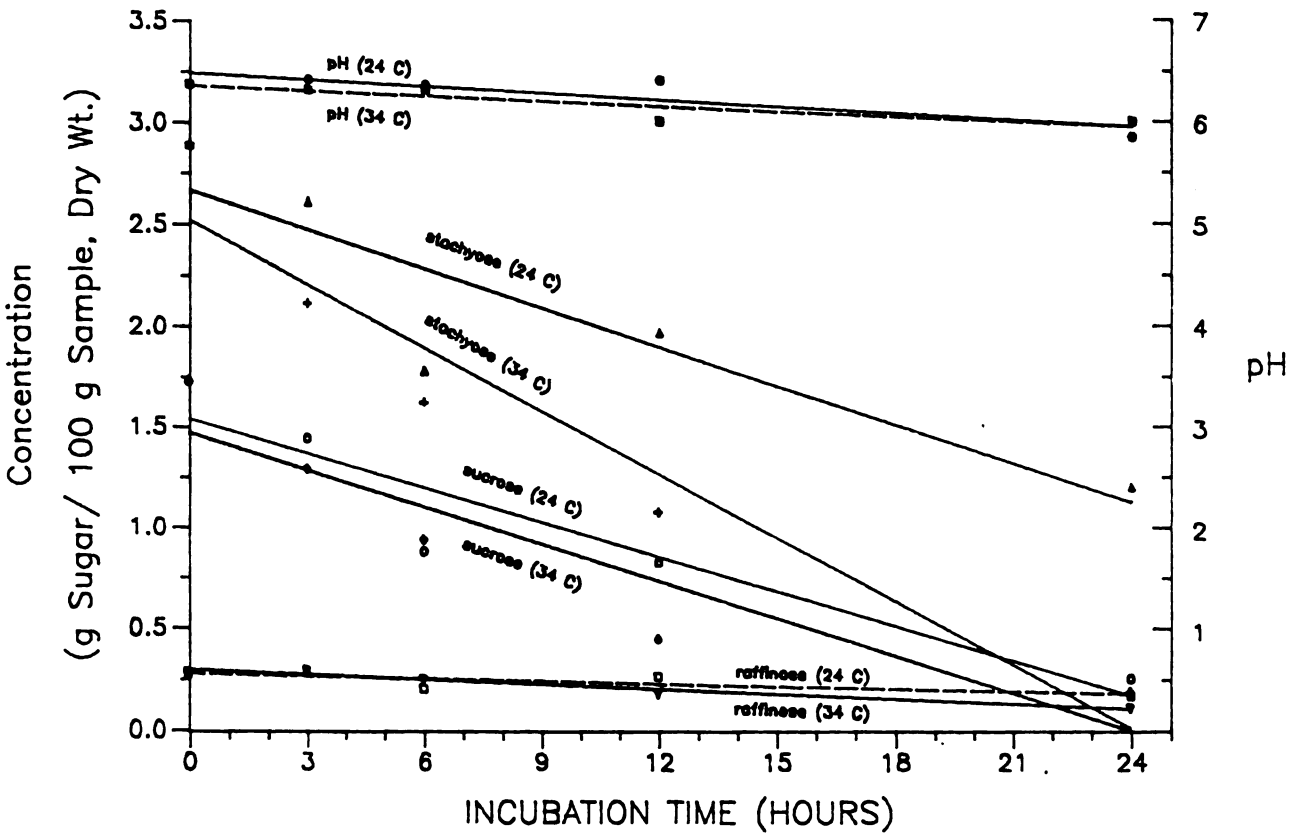


Figure 50. Oligosaccharide and pH changes during 24 hrs of cowpea flour paste incubation at different temperatures

GERMINATION PLUS INCUBATION OF THE SEED PASTE

Figure 51 contains plots of stachyose, raffinose and sucrose concentrations during two hours of soaking, six hours of germination, followed by 12 hours of incubation of a paste made from germinated seeds at two different temperatures, 24° C and 34° C. Two hours soaking was used prior to germination in order to speed up the germination process. The α-galactosidase activity in the germinating seeds peaked at about 6 hours (Figure 48). The incubation of the paste was stopped at 12 hrs because considerable oligosaccharide breakdown was achieved by then without apparent microbial spoilage.

Stachyose content significantly decreased ($P < 0.01$) at both 24° C and 34° C. Raffinose was significantly decreased at both 24° C and 34° C ($P < 0.05$). The rates of decrease (slope) at both temperatures were rather low (-0.004 % /hr at 24° C; -0.003 % /hr at 34° C). Sucrose increased significantly at 24° C (slope = 0.02) at $P < 0.01$, but it did not change significantly at 34° C (slope = -0.011). The pH increased significantly from 6.35 to 6.6, ($P < 0.01$) at 24° C (slope = 0.014) but did not change significantly (pH 6.35) at 34° C ($P < 0.05$). Raffinose content was not significantly altered with increasing temperature ($P < 0.05$). Stachyose, sucrose and pH changed significantly with increasing temperature ($P < 0.05$). Stachyose decrease was

significantly greater at 34° C than at 24° C. The rate comparisons (slopes) between the two temperatures ($P < 0.05$) refer to the arithmetic linearities which is better than logarithmic linear correlation for sucrose, stachyose and pH. Table 13 illustrates the statistical analysis of oligosaccharide and pH changes during the situation described in Figure 51.

Table 13. The statistical analysis of oligosaccharide concentration and pH changes during 2 hrs of soaking, 6 hrs of germination, followed by 12 hrs of incubation of a paste made from germinated seeds at two different temperatures, 24 and 34°C

	Sucrose		Raffinose		Stachyose		pH	
	24°C	34°C	24°C	34°C	24°C	34°C	24°C	34°C
slope[1]	0.02	-0.011	-0.004	-0.003	-0.072	-0.12	0.014	0.00
slope[2]	0.004	-0.003	-0.008	-0.006	-0.015	-0.04		
Incpt.[1]	1.68	1.78	0.28	0.27	2.92	2.98	6.30	6.37
Incpt.[2]	0.23	0.25	-0.54	-0.57	0.48	0.56		
r value[1]	0.96	-0.83	-0.95	-0.90	-0.99	-0.99	0.94	0.01
r value[2]	0.94	-0.89	-0.91	-0.88	-0.98	-0.94		
Prob.[1]	0.01	0.084	0.014	0.04	0.0001	0.001	0.005	0.99
Prob.[2]	0.018	0.044	0.03	0.046	0.002	0.02		
Std. E.[1]	0.003	0.005	0.001	0.001	0.003	0.007	0.002	0.01
Std. E.[2]	0.001	0.001	0.002	0.002	0.002	0.008		

1= arithmetic linearity, 2= logarithmic linearity, n= 3

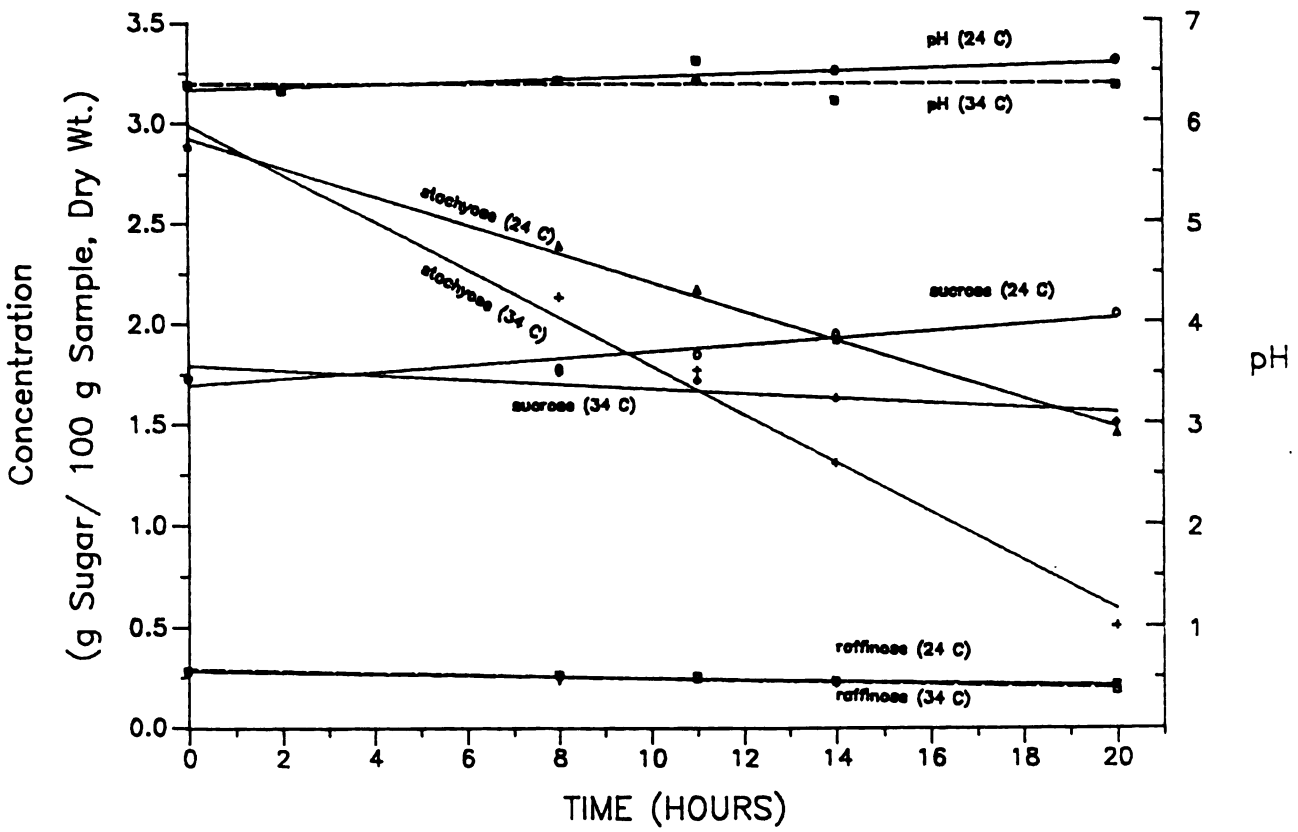


Figure 51. Oligosaccharide concentration and pH changes during 2 hrs of soaking, 6 hrs of germination, followed by 12 hrs of incubation of a paste made from germinated seeds at two different temperatures, 24° and 34° C

These three experiments show that it is possible to decrease the flatus-related sugar content of cowpeas, especially stachyose which is present in much larger concentration than raffinose. The most efficient way appears to be forming a paste with cowpea flour and let it stand in a warm place (around 30° C) overnight.

Safety Concerns

The relatively high pH of the paste makes it susceptible to spoilage by microorganisms, including Clostridium botulinum. Although microbiological studies were not conducted, the paste did not show signs of spoilage (smell, gas). The possibility of an outgrowth of C. botulinum may be eliminated, if Dovlo's et al. (1976) procedure is followed which includes aerating of the paste and subsequent frying.

INVERTASE ACTIVITY

The invertase activity of dormant seeds was 5.8 nkat/mg sample, that of seeds germinated for 12 hours at 24° C was 10.3 nkat/mg sample, and that of seed paste incubation for 12 hours at 24° C was 20.0 nkat/mg sample. The high invertase activity in the paste explains the quick disappearance of sucrose in that material. Invertase activity was lower in dormant and germinated seeds than in the paste made from cowpea flour. Invertase activity

increased in the paste probably as a result of the decompartmentation of the enzyme and its substrate.

Schwimmer et al. (1961) suggested that the low invertase activity in potato tubers may be due to the presence of an inhibitor. Enzyme action can be accelerated by decompartmentation of enzyme and substrate via cell and organella breakage (Schwimmer, 1981).

CONCLUSIONS AND DIRECTIONS FOR FUTURE RESEARCH

The objectives of this research focused on investigating the properties of cowpea α-galactosidases, activity, in dormant and germinating cowpeas, and determining the oligosaccharide changes during the germination of seeds and the incubation of a paste from these seeds. In the following sections, the main research contributions of this research are summarized and avenues for future research development are described.

Two forms of α-galactosidases, I and II, were isolated using gel filtration on Sephadex G-100 and G-200. Form II of α-galactosidase was further resolved into two fractions, II^1 and II^2, using Accl-CM and CM-cellulose cation exchange chromatography. The three enzymes exhibited different K_m , V_{max} and optimum activity pH. Table 14 shows the K_m values for enzymes I, II^1 and II^2 with different substrates. The values of the optimum activity pH obtained for enzymes I, II^1 and II^2 were 5.0, 5.9 and 5.3, respectively. All three enzymes displayed maximum stability at 4° C and at intermediate pH levels (4.5-6.5). The optimum activity temperature was the same for all three enzymes, 50° C.

The effect of increasing raffinose concentration on Enzyme I, II^1 and II^2 was studied. Above a certain level of substrate concentration, the reaction rate started

decreasing indicating that the substrate itself started acting as an inhibitor of the reaction.

Table 14. K_m values of Enzymes I, II^1 and II^2 with different substrates

	substrate		
Enzyme	PNPG	Raffinose	Stachyose
I	1.5	4.6	11.0
II^1	2.2	5.0	15.0
II^2	5.3	1.6	---

The enzymes were purified to homogeneity as determined by disc gel electrophoresis. Enzymes I, II^1 and II^2 moved slightly in alkaline pH (8.3) and acidic pH (3.5) PAGE. The protein bands on the gel at pH 3.5 were confirmed as the α-galactosidases I, II^1 and II^2.

Using gel filtration, the MW of Enzymes I, II^1 and II^2 were 110,720 ± 3,780, 29,000 ± 355 and 30,000 ± 368 daltons. Using SDS-PAGE polyacrylamide gel electrophoresis, the MW values obtained were 30,916 ± 1,042, 29,875 ± 413 and 30,350 ± 550 for the three enzymes, respectively. Based on the SDS-PAGE and two types of ion exchange chromatography, Enzyme I appears to be a tetramer of Enzyme II^2 rather than Enzyme II^2.

Stachyose decreased at a rapid rate while raffinose declined slowly during incubation and germination plus incubation of cowpeas for 24 hrs at 24° and 34° C. The sucrose concentration slightly increased during germination plus incubation of cowpeas for 24 hrs at 24° C. The activity of α-galactosidase increased during germination for 24 hrs, especially during the first 6 hrs. The change in oligosaccharide content during 24 hr of cowpea flour paste incubation was higher at 34° C than at 24° C. The rate of stachyose decrease was higher than sucrose which in turn was higher than raffinose. Germination did not have significant effect on the oligosaccharide changes during 24 hr. Incubation of a paste made from germinated seeds or flour of the seeds resulted in considerable reduction of the flatus-related oligosaccharide of the cowpea. As a result of these investigations, it appears that the most efficient way to decrease flatus-related sugar of cowpeas especially stachyose, is to form a cowpea flour paste and let it stand overnight in a warm place (30° C). Microbiological safety concerns of this procedure need to be evaluated.

Future research can be continued at the enzyme level and the nutritional level.

At the enzyme level, the following aspects of α-galactosidases may be studied.

- Purification of the cowpea α-galactosidases using affinity chromatography.

- Amino acid composition and sequence of cowpea α-galactosidases

- The effect of using invertase with α-galactosidase on the oligosaccharides in cowpeas.

- The influence of germination on the ratio of Enzyme I and II in cowpeas.

From a nutritional point of view, it has been shown that incubation of a cowpea paste resulted in oligosaccharide decrease. It will be of interest to study the effect of such pastes in reducing flatulence. Another issue of interest which requires further investigations, concerns the use of immobilized α-galactosidase to remove the oligosaccharides from cowpea slurries.

APPENDICES

APPENDICES

Appendix 1

Table. Nutritional value of cowpeas and certain other foods (Dovlo et al., 1976)

	Cal/ 100g	Protein %	Fat %	Carbo-hydrate %	Vit-amin A IU/ 100g	Thi-amin mg/ 100g	Ribo-flavin mg/ 100g	Nia-cin mg/ 100g	Ascorb acid mg/ 100g
Cowpea	340	22	1.5	60	20	0.9	0.15	2	0
Soybean	382	35	18	20	0	1.1	0.3	2	0
Rice, slightly milled	354	8	1.5	77	0	0.25	0.05	2	0
Sorghum flour	353	10	2.5	73	0	0.4	0.1	3	0
Fish, sea, lean, fillet	73	17	0.5	0	0	0.05	0.1	2.5	0
Beef, lean	202	19	14	0	0	0.1	0.2	5	0
Eggs, hen	158	13	12	0.5	1000	0.12	0.35	0.1	0

Cereal proteins are relatively deficient in the amino acid lysine, which is prsent in a relatively high amount in legumes.Legumes are a poor source of the sulfur-containing amino acids, methionine and cystine, which are adequate in cereals.

Appendix 2

Table. Molecular forms and properties of some plant seed α-galactosidases (Dey and Pridham, 1972)

Source	Form	Method of separation	MW	pH Optimum	K_m (mM) PNPG substrate
Cajanus indicus	I	Gel filtration	High	5.0	6.3
	II		Low	4.5	1.2
Ceratonia siliqua	I	DEAE-Cellulose	37,000	≈ 5.0	0.42
	II		37,000	≈ 5.0	0.42
	III		23,000	≈ 5.0	0.45
Glycine max	I	Gel filtration	160,000	6.8	0.15
	II		40,000	5.6	0.39
Medicago sativa	I	DEAE-Cellulose	34,000	≈ 5.0	0.49
	II		23,000	≈ 5.0	2.36
Vicia faba	I	Gel filtration	160,000	2.0 & 5.5	0.44
	II[1]	SDS-PAGE	43,000	2.0 & 5.2	0.97
	II[2]		41,000	2.0 & 5.5	0.33
Vigna radiata	I	Gel filtration	160,000	5.6 & 7.2	0.2
	II		40,000	5.6	0.1
Vigna unguiculata	I	Gel filtration	110,000	5.0	1.2
	II[1]	SDS-PAGE	30,000	5.9	2.2
	II[2]		29,000	5.3	5.3

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