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FROM ASPERGILLUS NIGER, COFFEE BEANS
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Penelope Gavriel

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ENZYMATIC CHARACTERISTICS OF α-GALACTOSIDASES FROM *ASPERGILLUS NIGER*, COFFEE BEANS AND *ESCHERICHIA COLI*

By

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ABSTRACT

ENZYMATIC CHARACTERISTICS OF α-GALACTOSIDASES FROM *ASPERGILLUS NIGER*, COFFEE BEANS AND *ESCHERICHIA COLI*

By

Penelope Gavriel

A crude laboratory preparation of α-galactosidase from *A. niger* and two commercial preparations of the enzyme, one from *E. coli* and another from coffee beans, were compared as follows: their optimum reaction pH and temperature and their storage stability were determined with p-nitrophenyl-α-D-galactoside (PNPG) as substrate. Their Km and Vmax values were assessed with PNPG, melibiose, raffinose and stachyose as substrates. The *A. niger* and coffee bean enzymes had similar pH and temperature optima, 5.0 and about 52°C, respectively. The *E. coli* enzyme had a pH optimum near 38°C. The latter enzyme was much more unstable in storage than the other two. The *E. coli* enzyme hydrolyzed melibiose faster and stachyose more slowly than the other two enzymes. Ag^{+} and Hg^{2+} inhibited all three enzymes, but while KI lifted the inhibition of the *A. niger* and coffee bean enzymes, it did not affect the inhibition of the *E. coli* enzyme. There were considerable differences among the Km and Vmax values of the three enzymes.

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INTRODUCTION

In 1895, Bau, Fisher and Lindner (21) isolated certain enzymes from bottom yeast, which hydrolyzed the disaccharide melibiose, and they named them melibiases. This name was later changed to α-galactosidases by Weidenhagen, who studied the specificity of action of the enzyme using a variety of carbohydrates possessing non-reducing, terminal α-D-galactosyl residues.

α-Galactosidases or α-D-galactoside galactohydrolases (E.C. 3.2.1.22) catalyze the following reaction (Figure 1).

The enzyme may hydrolyze a variety of simple α-D-galactosides as well as more complex molecules, such as oligo- and polysaccharides. The ease of hydrolysis of the α-galactopyranosyl residues decreases progressively with increasing size of the substrate molecule.

In addition to their hydrolytic ability α-galactosidases from certain sources (bacteria, plants) can catalyze trans-α-D-galactosylation reactions from aryl α-D-galactopyranosides to alcohols, mono- and oligosaccharides (21).

Interest has centered around the mode of action and physiological significance of these enzymes and their use as tools for structural studies of biological molecules as well as practical applications (40).

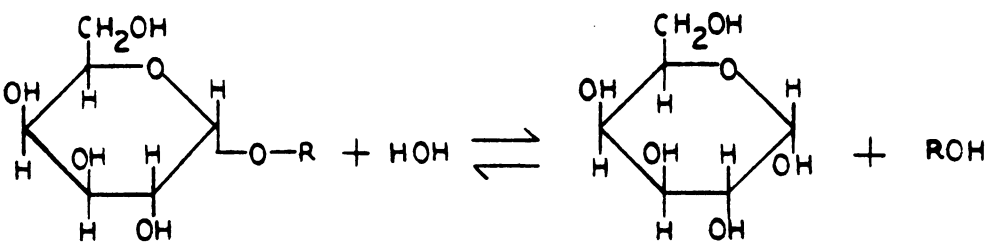


Figure 1. α-Galactosidase hydrolysis.

R: Aromatic or aliphatic residue.

Occurrence

α-Galactosidases have been reported to occur widely in microorganisms, plants and animals. In most cases they are found intracellularly in various organelles (mitochondria, chloroplasts, microsomes). In E. coli (8, 26, 51, 52, 53) and Aerobacter aerogenes α-galactosidase is not constitutive but can be induced by the introduction of several α-D-galactosides in the culture media (6).

Detection and Methods of Assay

Melibiose and raffinose, presumed to be natural substrates for plant α-galactosidase, are commonly used to determine enzyme activity (14, 42, 44, 58, 60). Following incubation the extent of hydrolysis is measured in terms of liberated molecules of galactose (product) or non-hydrolyzed molecules of substrate. The galactose determination can be achieved by various methods, such as colorimetry (23, 45), measurement of the increased reducing power of the hydrolyzate, chromatography (33) or enzymatically (22, 30), while the determination of the remaining substrate can be estimated with sufficient accuracy by GLC or HPLC (13, 14, 31). Often substituted phenyl-α-D-galactosides can be used as convenient assay substrates (35). In this case the enzymatic activity can be estimated spectrophotometrically, by the appearance of the phenyl compound, which is usually a chromogen.

Often cofactors such as NAD^+ or metal ions such as Mn^{++}, as in the case of cell free enzymic extracts of E. coli (8), or activators, such as K^+ ions for the α-galactosidase isolated from V. faba seeds (17), may be necessary in the assay medium.

Galactosyl transferase activity, as opposed to hydrolysis, is normally assayed by carrying out the enzymic reaction in the presence of a galactose donor, such as PNPG and an acceptor, such as raffinose (Figure 2). The resulting mixture is fractionated and the products are determined usually by chromatography (16).

Isolation

α-Galactosidases from various sources can be isolated by conventional methods of extraction. Often NaCl solutions are used to extract the enzyme from the cells of the microorganisms that produce it. At times, suspensions of cultures are marketed as sources of α-galactosidase (8). Commonly α-galactosidases occur in the cells in association with other glycosidases and often have been difficult to fractionate these activities.

The techniques of isolation include ammonium sulfate or cold acetone, fractionation, ion exchange, molecular exclusion and isoelectric focusing (8, 23, 28, 40, 57, 59). All or some of these techniques are employed, according to the desired purity of the enzymic preparation or the

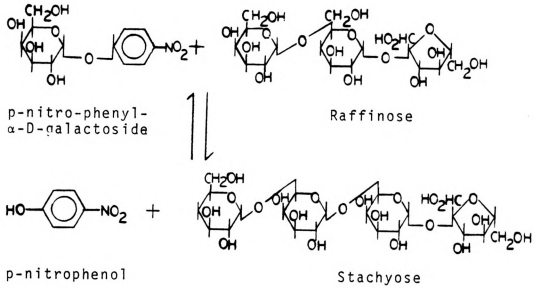


Figure 2. Transferase activity of α-galactosidase.

difficulty during fractionation. Highly purified and apparently homogeneous α-galactosidases have only been isolated in a few cases (19, 40).

Physical Properties

The existence of multi-molecular forms of α-galactosidases was first reported by Petek and his collaborators (48). They isolated two separate forms of this enzyme from coffee beans by chromatography on alumina columns. Dey and Pridham (20) later showed that dormant seeds of V. faba possessed two α-galactosidases, which had different molecular weights. The separation was achieved by Sephadex gel filtration. Multimolecular forms of α-galactosidases may have very similar properties and may be difficult to resolve. For example, the isolated enzyme from A. niger was homogeneous, as judged by gel filtration but, when passed through an ion exchange column, it was resolved into three active forms (23, 40). The α-galactosidases isolated from various sources have different molecular weights. Most of these were estimated by gel filtration and ranged between 25,000-200,000.

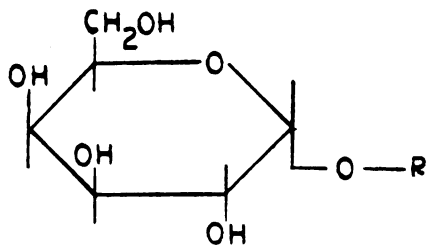
Hydrolase Activity

In general, change of configuration of hydrogen and/or hydroxyl groups or any single carbon atom of a galactoside substrate is sufficient to reduce or completely inhibit the

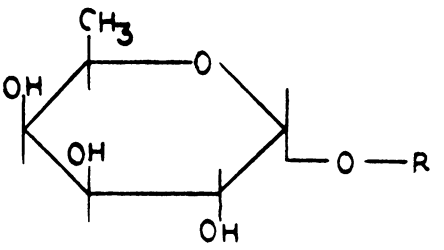
hydrolytic action of the enzyme. Two main factors govern the rate of hydrolysis of the substrate: the ring structure must be a pyranoid, and the configuration of -H and -OH on carbon atoms 1,2,3 and 4 must be similar to that on galactose. Changes at C-6 are tolerated by α-galactosidases. Hence the glycosides indicated in Figure 3 can be hydrolyzed by this enzyme (21).

Kinetic studies of the hydrolytic activity of α-galactosidases on various galactosides or glycosides, which have similar molecular conformation have shown that the Km and Vmax values for different substrates vary greatly and that the hydrolyzability (velocity of the reaction) is not related to the affinity (1/Km) of the enzyme for the substrate(20, 21). The affinity of the enzyme 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 (21).

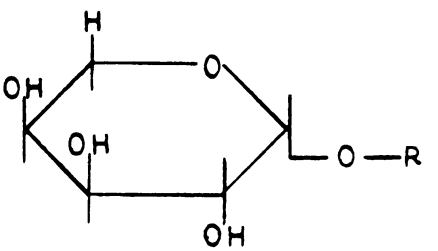
During the hydrolysis of various galactosides, the velocity of the reaction was higher for those galactosides in which the aglycon had aromatic structure than those with alkyl aglycon. In addition it was found that the affinity of α-galactosidases isolated from V. faba seeds and sweet almonds tends to increase, when electron attracting



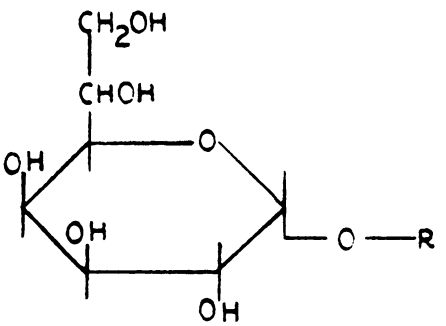
α-D-Galactoside



α-D-Fucoside



β-L-Arabinoside



D-Glycero-α-D-galacto-heptoside

Figure 3. Glycosides hydrolyzed by α-galactosidase.

substituents are present in the phenyl ring of a phenyl-α-D-galactosides, which are used as substrates (Table 1).

Some α-galactosidases can attack galactomannans (12). Almond α-galactosidase can split the internal galactosidic bond of stachyose forming galactobiose and sucrose (21), as indicated in Figure 4.

On the other hand coffee bean α-galactosidase can only cleave stachyose in a stepwise fashion, starting from the non-reducing end (12).

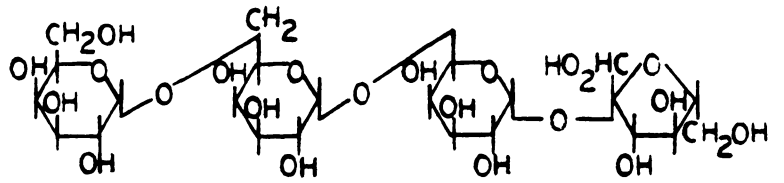
Inhibitors

Most α-galactosidases are inhibited by "sulfhydryl reagents", such as p-chloromercuribenzoate, iodoacetamide and N-ethyl-maleimide. But not all α-galactosidases are -SH enzymes (e.g. the sweet almond α-galactosidase, V. faba).

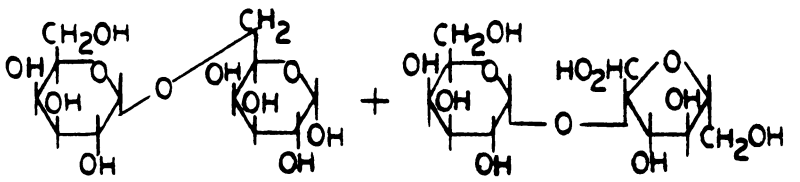
Ag^{+} and Hg^{++} ions exhibited strong inhibition upon α-galactosidases. In all cases α-D-galactose at concentration higher than 10 mM exhibited competitive inhibition. Also some substrates, such as PNPG, above certain concentration can cause decrease of the velocity of the enzymic reaction, which becomes more pronounced as the substrate concentration increases (20).

Table 1. Substrate specificity of α-galactosidase from *V. faba* (20).

Substrate	Km
n-propyl-α-D-galactoside	6.13
ethyl-α-D-galactoside	6.93
phenyl-α-D-galactoside	1.11
m-chloro-phenyl-α-D-galactoside	0.83
p-nitro-phenyl-α-D-galactoside	0.38



Stachyose



Galactobiose Sucrose

Figure 4. Hydrolysis of stachyose by α-galactosidase from almonds.

Mechanism of Action

Few concrete facts are available regarding the mechanism of action of α-galactosidases. So far there have been no studies of the bond fission by α-galactosidases, although by analogy with other glycosidases it is likely that the galactose-oxygen bonds of substrates are cleaved (21). Nuclear magnetic resonance and polarimetry studies have clearly shown with sweet almond α-galactosidase that the liberated galactosyl residues possess the same anomeric configuration as the substrate (16).

The substrate specificity studies on sweet almond and *V. faba* α-galactosidases (20, 42) showed that the electronic nature of the aglycon has a noticeable influence on the rate of enzymic hydrolysis. Thus the hydrolytic reaction could be a result of the presence of basic and acidic groups at the active site. These groups were identified by kinetic studies as carboxyl (deprotonated) and imidazolium (protonated), respectively. On the basis of these results a "two step" mechanism has been postulated for the action of sweet almond α-galactosidase (16). The aglycon is cleaved off by the combined action of carboxyl and imidazolium groups (Figure 5). This is followed by reaction with an acceptor molecule, which may be water or another substrate molecule, resulting in hydrolysis or transfer products.

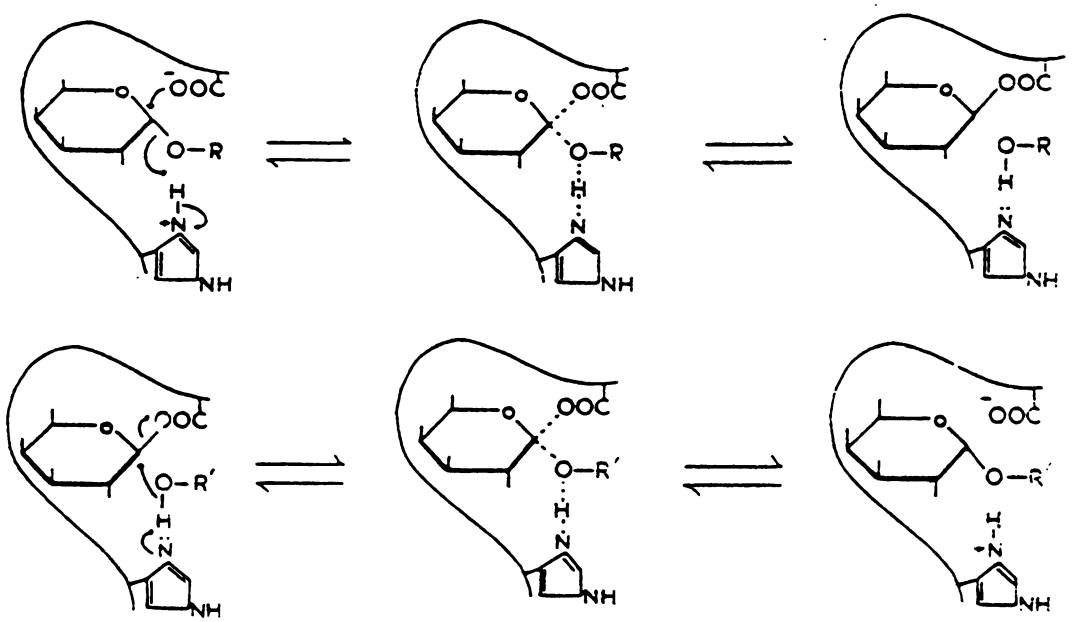


Figure 5. "Two step" mechanism of action of α-galactosidase from sweet almonds.

It is possible that the imidazolium group alone is sufficient to cleave the glycosyl-oxygen bond, with the formation of a carbonium ion at the C-1 of the liberated galactose moiety. The free carbonium ion though could lead to mutarotation products (α- and β-anomeric forms of galactose) in the mixture. For this reason it is believed that the carbonium ion is stabilized somewhere on the active site (probably COO^-) retaining the stereochemical configuration of the liberated galactose.

Physiological Significance and Possible Applications of α-galactosidases

In the plant kingdom galactose containing oligo- and polysaccharides and lipids are ubiquitous (3, 9, 12, 15, 39, 58, 60). Among the oligosaccharides known are the galactosyl sucrose derivatives of raffinose and stachyose, which are found in substantial amounts in the legume seeds. In many seeds there is a concomitant synthesis of these galactosyl-sucrose compounds and an apparent high content of α-galactosidase. It is apparent that, in order for those galactosyl derivatives to accumulate in the seed during maturation, there must be some mechanism, that prevents the interaction between enzyme and substrate; perhaps this is achieved by compartmentalization or an endogenous inhibitor (21). During germination, the α-galactosidase must be involved in the hydrolysis of these oligosaccharides, which serve as soluble

and readily metabolizable energy reserves.

It appears that O_2 is somehow involved in the hydrolytic reaction of the sugars in vivo, as this is inhibited if moistened seeds are kept in a reduced oxygen atmosphere (54).

The disappearance of raffinose and stachyose during germination is not accompanied by the concomitant presence of galactose in the tissues, but only a marked increase in the sucrose content has been observed (21, 29, 39). What could happen in this case is that galactose is immediately phosphorylated, converted into glucose and metabolized. Besides the biochemical and biophysical aspects of α-galactosidase there is another important aspect of this enzyme. This is the potential of this enzyme for industrial or medical applications.

Raffinose is found in variable amounts in sugar beets, which usually increase during storage. The amount of raffinose in sugar beets could come up to 0.15% of raw material (58). In the sugar industry raffinose is known as an obstacle substance for the normal crystallization of the beet sugar. When the raffinose content in the beet molasses is gradually increased to an amount of 6% to 10%, the crystallization of the sugar becomes unprofitable and it is abandoned (41, 58).

If raffinose in the beet juice or beet molasses can be removed or decomposed by some method, it is possible that

the crystallization of the beet sugar will be further improved and consequently the yield of the sugar increased. Many workers attempted successfully the removal of raffinose by incorporating into the molasses crude preparations of α-galactosidase, isolated from various microorganisms. Of all the available sources of α-galactosidase, members of the fungi have been used successfully (9, 32, 38, 46, 59).

In Japan, Suzuki et al. have employed the mycellial α-galactosidase from a fungus, Mortierella vinaceae, in the form of a pellet and incorporated it into the beet molasses to accomplish the hydrolysis of raffinose (58). The microscopical crystal examination of the recovered sugar from the hydrolyzate revealed normal crystal formation, due to the absence of the inhibitory action of raffinose. A similar experiment was attempted in Poland with the same successful results (46).

An economic evaluation allowing for investment and operational costs shows that the α-galactosidase process is profitable, when 1) there is a considerable price difference between sugar and molasses, 2) the factory is isolated, 3) the molasses is of little value, 4) the beet raffinose content is high, 5) the campaign is protracted.

Soybeans are used in increasing amounts, as low cost high quality protein supplement. The potential of soy milk, as a substitute for cow's or human milk has been emphasized during the years. It can be used successfully, in cases

where cow's milk is not available or there is some kind of physical disorder in humans (13, 60).

Soy milk is deficient in the sulfur containing amino acids (36). It also contains considerable amounts of galactosyl sucrose oligosaccharides, which have been implicated as factors responsible for flatulence (56). Various methods have been employed for the removal of these troublesome compounds from the soybeans (soaking, soaking and germination, fermentative process (44), water extraction, ultrafiltration). All these methods are tedious and time consuming.

Enzyme treatment by microbial α-galactosidase offers a promising solution to this problem (13, 43, 55, 60). Sugimoto et al. succeeded in removing all the oligosaccharides from soy milk in three hours, by treating it with 16×10^{-3} units of α-galactosidase per gram of solid material.

Commercially prepared crude enzyme preparation from A. niger was used (57). Microbial α-galactosidases have been used for the removal of the oligosaccharides from soy milk commercially in a hollow fiber reactor (55). Entrapment of α-galactosidase from a fungus in polyacrylamide gel has also been carried out (60). These methods allowed continuous and multiple use of the enzyme.

Finally there is an additional interest in some plant α-galactosidases (coffee beans, tomatoes). Experimental work proved that they can modify the B specificity of intact human

erythrocytes by removing α-1,3-linked galactose residues from cell surfaces (49, 61). A similar reaction was demonstrated by α-galactosidases isolated from figs and soybeans. Pressey working with α-galactosidase isolated from tomatoes concluded that his enzyme may be employed in the future to convert group B erythrocytes to type O (49). This process has drawn considerable interest recently as a way of utilizing unused type B blood (49).

LITERATURE REVIEW

The α-galactosidases of A. niger, Coffee Beans and E. coli

Although oligosaccharides containing α-D-galactosyl groups in their structure, such as raffinose and stachyose, and galactosyl glycerides in lipid fractions, are widely distributed in the plant kingdom, α-galactosidases have not been studied as extensively as β-galactosidases. Microbial galactosidases have been reported in brewers yeast and some strains of bacteria and molds, but detailed information on their characteristics have not been given until recently.

In preliminary studies Sugimoto et al. (57) found that a considerable amount of fungal strains belonging to the genus Aspergillus exhibited powerful abilities to produce galacto-oligosaccharide decomposing enzymes, such as α-galactosidase and invertase.

Some commercial enzyme preparations were derived from the same fungi containing considerable activities of both enzymes and were distributed under various names, such as Molsin (Seishin Pharmacetuical Co., Ltd., Tokyo) from A. satoi and Rhozyme HP-150 (Rhom and Haas Co., Philadelphia) from A. niger.

Some of the research which has been conducted around enzymic preparations from the various species of Aspergillus included mainly various purification and isolation procedures and a few practical applications, mainly limited to experimental levels, employing the use of α-galactosidase for the removal of oligosaccharides from soy milk. Specifically Sugimoto et al. (57) working with a crude commercial preparation from A. satoi determined an optimum reaction temperature for this enzyme at 55°C, an optimum pH between 5.0 and 5.5 and the Km value of this enzyme for melibiose, as substrate, was found to be 3.1×10^{-3} M. Furthermore, investigations by means of thin-layer chromatography indicated that addition of small amounts of this enzyme preparation to soy milk resulted in complete hydrolysis of the galacto-oligosaccharides. McGhee et al. (43) also investigated the removal of flatulence factors from soy milk by employing a crude enzyme preparation from A. awamori, isolated in their laboratory from cultures of this fungi. Gel filtration techniques estimated the molecular weight of this enzyme at 290,000. Their enzyme exhibited maximum activity at 50°C and pH 5.0. The Km values of this α-galactosidase with melibiose were 3.0×10^{-2} M and 3.6×10^{-2} M for raffinose. The molecular weight of this enzyme was estimated to be about 130,000, on the basis of Sephadex and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. This enzyme has the advantage of not being inhibited by the presence of

galactose.

Bahl et al. (5) conducted an extensive study centered around the purification and isolation of the α-galactosidase (among the other glycosidases) from a crude commercial preparation (Rhozyme HP-150). Their work included ammonium sulfate precipitation, pressure dialysis, chromatography and study of the hydrolytic action of the isolated enzyme upon oligo- and polysaccharides, such as melibiose, raffinose, stachyose and galactomannans from guar and locust beans. During the hydrolysis of the latter substrate the enzyme liberated 35% to 40% of the total D-galactose residues. The Km and Vmax values of this enzyme for PNPG was estimated to be 3.5×10^{-4} M and 58.8 μmoles of p-nitro-phenol/min/mg of crude enzyme preparation, respectively.

Lee et al. (40) worked on the isolation of the α-galactosidase from Rhozyme HP-150. Their work included precipitation and isolation procedures by using gel chromatography with Sephadex, ion exchange chromatography on DEAE, electrofocusing and rechromatography of the isolated α-galactosidase on CM-cellulose. The additional procedure of electrofocusing suggested that there were more than one component (isozymes) of α-galactosidase. The observation that different optimum pH values existed agreed with the above suggestion.

The determination of the Km constant for PNPG gave a value of 4.6×10^{-3} M and a distinct substrate inhibition has been reported.

Courtois et al. (12, 17) and Petek et al. (48) included in their carbohydrate studies some experiments for the isolation and investigation of the enzymatic characteristics of α-galactosidases from coffee beans. Their work is the most prominent in the literature review, related to the study of α-galactosidases from this source. Petek and ToDong (48) obtained their enzymatic preparations from ground coffee beans by the method of acetone powder. During the purification and isolation procedures on alumina column, they succeeded in separating two α-galactosidases, which were defined as α-galactosidase I, with optimum pH 5.3 and Km for PNPG 2.2×10^{-3} M, and α-galactosidase II, with optimum pH 6.0 and Km for PNPG 1.1×10^{-3} M. On the contrary the optimum pH during the hydrolysis of melibiose by α-galactosidase I was 3.8 (51a).

Studying the transferase activity of these enzymes the same workers used PNPG as a donor, while D-mannose, D-galactose, sucrose, cellobiose and maltose were used as acceptors. The appearance of compounds such as raffinose and stachyose in the reaction mixture, determined chromatographically, was used as indication of transferase activity.

Courtois et al. studied the mode of hydrolysis of stachyose by α-galactosidase from coffee beans. His conclusion was that this enzyme hydrolyzes stachyose in two stages: first one molecule of galactose is removed and raffinose is formed; the latter is then split into galactose and sucrose

(12).

Studying the action of α-galactosidase from coffee beans upon various galactomannans, isolated from Trifolium repens, Gleditschia triacanthos and some other species, Courtois et al. concluded that this hydrolase splits at similar initial rates α-D-galactosyl units from all galactomannans. At pH 4.6 and at 37°C the Km value was estimated for raffinose to be 5.3×10^{-3} M and 2.5×10^{-3} M for stachyose (12).

α-Galactosidase from coffee beans was also employed to demonstrate the hydrolyzation of galactose molecules from B blood group substance (61). The study of α-galactosidase from E. coli was limited only in the area of biochemical research, as subject of the overall process of induced enzyme formation by certain microorganisms, such as E. coli.

It has been known for some time that the α-galactoside melibiose can induce in E. coli simultaneously the synthesis of α-galactosidase (53). Growth of E. coli on melibiose requires the induced synthesis of α-galactoside permease and α-galactosidase (8). This is achieved by a "melibiose operon" controlling the synthesis of the above proteins (51). It was observed that raffinose utilizing E. coli strains contained β-galactosidase. When these strains were tested for the presence of α-galactosidase, only those grown on melibiose and raffinose showed high activity (26). This indicates that the formation of α-galactosidase in E. coli is

induced, while β-galactosidase has a constitutive character. Cell free extracts from induced E. coli cultures were assayed for α-galactosidase activity with PNPG (8). Enzyme activity was observed only at high protein concentrations and the activity decreased exponentially with dilution. The reason for this is that this hydrolase, unlike other known similar enzymes, requires NAD^+ as a cofactor.

Burstein et al. studying the α-galactosidase from E. coli K12 determined that for optimal activity the enzyme requires Mn^{++} and a high concentration of mercaptoethanol with an optimum pH of 8.1. The Km values of this α-galactosidase for PNPG is 3.0×10^{-3} M and for melibiose 1.0×10^{-2} M. In an older paper the behavior of α-galactosidase was studied in intact induced cells of E. coli B12 (52). Successful extraction of the α-galactosidase in active form from the E. coli B12 cells was not possible. The Km value of this enzyme at all levels of activity was the same, 5.5×10^{-5}M for PNPG. When induced E. coli B12 cultures were subsequently incubated in a growth medium without an inducer, loss of the ability to produce α-galactosidase was observed (no induction). This was indicated by a constant decrease of α-galactosidase activity.

The present study was aimed at obtaining data upon the various physical and kinetic parameters of three α-galactosidases derived from three different sources, A. niger, coffee beans, and E. coli.

Although the previously reviewed communications included excellent experimental procedures, they did not provide much information upon the kinetic constants, Km and Vmax, of these enzymes for various substrates. Four compounds were used as substrates, PNPG, melibiose, raffinose and stachyose. The experiments were centered around the determination of the optimum reaction temperature and pH during the hydrolysis of PNPG and estimation of the Km, Vmax values for all four substrates.

MATERIALS AND METHODS

1. p-Nitro-phenyl-α-D-galactoside (PNPG) free of p-nitrophenol (Sigma).
2. o-Nitro-phenyl-α-D-galactoside (ONPG) free of p-nitrophenol (Sigma).
3. Melibiose, raffinose, stachyose (Sigma).
4. Crude α-galactosidase preparation from A. niger.
5. α-Galactosidase from coffee beans (25 units) in 3.2 M $(NH_4)_2SO_4$ solution, pH 6.0, 10 units/mg of protein (Sigma).
6. α-Galactosidase from E. coli (10 units). Lyophilized powder, 20-40 units/mg of protein (Sigma).
7. β-D-galactose dehydrogenase (25 units) from recombinant E. coli, using Pseudomonas fluorescent gene, in suspension in 3.2 M $(NH_4)SO_4$, pH approximately 6.0, 58 units/mg of protein (Sigma).
8. Buffer solutions: acetate, phosphate, Tris-HCl, prepared according to reference #51 and adjusted to the desired pH level electrometrically.
9. Beckman DU single beam spectrophotometer.

D-Galactose Standard Curve

Two 0.6 mM D-galactose stock solutions were prepared, one in 0.05 M acetate buffer, pH 5.0 and the other in 0.01 M phosphate buffer, pH 7.0. Serial dilutions were prepared from the stock solutions with the following molarities: 0.30 mM, 0.24 mM, 0.18 mM, 0.12 mM, 0.06 mM and 0.03 mM galactose. A 0.6 mM NAD^+ solution was prepared, in 0.1 M Tris-HCl buffer, pH 8.6. Twenty five units of β-D-galactose dehydrogenase were disolved into 2.5 mL of Tris-HCl buffer. This enzyme solution was kept in the freezer at -10°C and was stable for five weeks.

Two mL of each of the previously prepared serial solutions were transferred into stoppered glass tubes, 2.0 mL of the NAD^+ solution were added, mixed well and the absorbance of these mixtures was measured spectrophotometrically against blanks, at 340 nm. Next 10.0 μL (0.1 units of enzyme) of β-D-galactose dehydrogenase solution were added into each tube, the contents were mixed well again and the stoppered tubes were set aside at room temperature (25°C) for 12 hrs. Two series of experiments were conducted, in triplicates, one for the galactose solutions in the acetate and the other for the phosphate buffer solutions. Conditions and procedures were identical during both experiments.

The final pH in the tubes was measured after the addition of NAD^+ solution and was 8.4 for the acetate and 8.5 for the

phosphate tubes. The optimum pH level for the irreversible lactonization of galactose, catalyzed by α-D-galactose dehydrogenase was about 8.6 (28).

The blanks were prepared by mixing 2.0 mL of the corresponding buffer with 2.0 mL of NAD^+ solution and 10.0 μL of deactivated β-D-galactose dehydrogenase. The blanks were prepared immediately after the galactose solutions.

After 12 hours of reaction the absorbance of the NADH in each tube was measured again at 340 nm. The final absorbance was estimated by subtracting the first reading from the last one. The absorbance readings were plotted vs. the galactose concentrations and the line was drawn by the method of least squares polynomials. The coefficients (a,b) and the index of determination (r) were estimated by computer.

p-Nitrophenol Standard Curve

Twenty five mg of p-nitrophenol were diluted into 100.0 mL of 0.05 M acetate buffer, pH 5.0. One hundred and fifty mL of 0.3 N NaOH were added to the previous solution (1.5:1.0 v/v) and the pH of the system was elevated to about 11.5. The above mixture was used as stock solution, containing 0.1 mg p-nitrophenol/mL.

For the preparation of the serial dilutions 5.0 mL, 4.0 mL, 3.0 mL, 2.0 mL and 0.5 mL of stock solution were transferred into 100.0 mL volumetric flasks and made to volume with a mixture of 0.3 N NaOH and acetate buffer

(1.5:1.0 v/v). The absorbance of these solutions was measured at 420 nm. As a blank during the measurements was used the NaOH:acetate (1.5:1.0) mixture.

All measurements were done in triplicate and the extinction coefficient of p-nitrophenol was estimated to be 28.9 units of absorbance per μmole of p-nitrophenol per mL.

α-Galactosidase from Aspergillus niger

Isolation Procedures

A crude enzymic preparation in the form of powder was derived from A. niger Au 37 grown as follows:

Growth Medium (g/100 mL H_2O):

$(NH_4)_3PO_4$	1.00
$(NH_4)_2SO_4$	0.15
$NaNO_3$	0.50
K_2HPO_4	0.01
$MgSO_4 \cdot 7H_2O$	0.08
wheat bran	4.00

Growth conditions (shake flasks):

pH	4.0 - 4.5
Temperature	30°C
Time	5 to 5 days

Harvesting:

Centrifugation for 10 min at 2,000 g, to separate the biomass from the culture medium. Filtration through a membrane filter (1.2 μpore size). The filtrate containing

the enzyme was lyophilized.

Assay of the Enzymatic Activity

An enzyme solution was prepared, containing 1.0 mg of the crude preparation per mL, in 0.05 M acetate buffer, pH 5.0. The substrate used was a 0.05 mM PNPG solution in the above buffer. The assay procedure included mixing 0.5 mL of enzyme solution with 2.0 mL of substrate, let react for 5 min at 55°C. The enzymic reaction was stopped by adding into the tube 9.0 mL of 0.3 N NaOH and the absorbance of the hydrolyzed p-nitrophenol was measured against a blank at 420 nm. The blank contained 2.0 mL of acetate buffer, 0.5 mL of enzyme solution and 9.0 mL of 0.3 N NaOH. One unit of the enzyme activity was defined as the amount of enzyme which hydrolyzed 1.0 μmole of p-nitrophenol per min, under the previously described reaction conditions. The specific activity of an enzyme expresses the number of units per mg of protein. The nitrogen content of this crude enzymic preparation, estimated by the microkjeldahl method (3), was 13.6%. This corresponds to about 84.3% protein content (N% x 6.25). The specific activity of this enzyme was estimated to be 0.06 units/mg of protein.

Optimum Reaction Temperature

For the determination of the optimum reaction temperature the activity of this α-galactosidase was tested at

various temperatures, ranging from 25°C to 75°C. Half mL of the enzyme solution, containing 1.0 mg of crude enzyme crude enzyme preparation/mL of solution, in acetate buffer (pH 4.5), was transferred into stoppered tubes (in triplicate) and incubated for 15 minutes under the following temperatures: 25°, 30°, 36°, 40°, 45°, 50°, 58°, 60°, 63°, 67.5°, 70°, 75°C. At the end of the incubation time 2.0 mL of substrate (0.1 mM of PNPG solution, in 0.05M acetate buffer, pH 4.5) were added into each tube containing the enzyme, mixed well and allowed to react for 5 minutes. The enzymatic reaction was stopped by adding 9.0 mL of 0.3 N NaOH. The enzymatic activity at each temperature was estimated by the hydrolyzed nmoles of p-nitrophenol/min.

Optimum Reaction pH

The effect of pH on the rate of the hydrolysis, when PNPG was used as substrate, was studied with a series of acetate buffers, ranging between pH 3.0 and 6.5. Substrate solutions were prepared in each buffer separately at the tested pH level (substrate concentration was 0.1 mM of PNPG). The enzyme solution was prepared in a lower molarity buffer (0.01 M acetate, pH 4.5), so that the addition of the enzyme solution into the reaction system will not change the final pH. The reaction systems were prepared as previously and the enzymatic reaction was run at 55°C, for 5 min.

Determination of the Hydrolysis Rates

When the hydrolysis rate of PNPG was studied, 42.0 mL of PNPG solution (0.2 mM) in acetate buffer, 0.05 M, pH 5.0, were transferred into a stoppered vessel, 10.5 mL of enzyme solution (1.0 mg/mL) were added, mixed well and allowed to react in a water bath at 55^{o}C. At various time intervals (1, 3, 5, ...30 min) aliquots of 1.25 mL each were taken and transferred into tubes, containing 4.5 mL NaOH each. Before each aliquot was taken the main vessel was shaken, in order to achieve thorough mixing of the contents within it. The absorbance of the hydrolyzates was measured as previously. The hydrolysis rate of PNPG by this enzyme was expressed as nmoles of hydrolyzed p-nitrophenol/mole of PNPG.

When melibiose, raffinose and stachyose were the substrates the reaction rate was determined as follows: 10.0 mM solution for each substrate in acetate buffer (0.05 M, pH 5.0) was prepared. Fifteen mL of each substrate were transferred into a vessel and 15.0 mL of enzyme solution in buffer at a concentration of 5.0 mg of enzyme preparation/mL of solution, were added. The vessels were stoppered, shaken adequately and placed in a water bath at 45^{o}C. All substrate and enzyme solutions were freshly prepared and the vessels were stoppered tightly, to avoid any losses due to evaporation, during the prolonged incubation time.

The hydrolysis of the oligosaccharides was observed for 24 hours. Aliquots of 1 mL each were taken from the reaction mixture at predetermined time intervals (1 1/2, 3, 5, ...20, 24 hours), transferred into stoppered tubes and placed in boiling water for 3 minutes to inactivate the enzyme (3 minutes of boiling was previously determined to be enough for full enzyme inactivation).

The quantitation of the liberated galactose, in the aliquots, was performed as follows. After boiling the aliquots were allowed to cool at room temperature, 4.0 mL Tris-HCl buffer (0.1 M, pH about 8.6) were added into each aliquot and mixed well. Two mL of the previous mixtures were transferred into other tubes containing 2.0 mL of 0.6 mM NAD^+ solution in Tris-HCl buffer, mixed well and their absorbance was measured against blanks at 340 nm. Following that, 10.0 μL of β-D-galactose dehydrogenase solution were added into each tube. The tubes were stoppered, vortexed and let aside, at room temperature to react for 12 hours. Blanks were treated similarly. Finally the absorbance of the contents in each tube was measured again and the amount of galactose was estimated by the difference of the second minus the first absorbance reading, multiplied by a dilution factor of 20.

Determination of the Km and Vmax Values

The determination of the kinetic constants of this enzyme was achieved both by the method of Lineweaver-Burk

double reciprocal and the Eadie-Hofstee reciprocal plots.

When PNPG was the substrate, solutions of this compound in acetate buffer with the following molarities were prepared: 0.1 mM, 0.125 mM, 0.15 mM, 0.2 mM, 0.25 mM and 0.5 mM. Two mL of each substrate solution were transferred into a tube (triplicates), 0.5 mL of enzyme solution were added, mixed well and allowed to react for 3 minutes at 55°C. The reaction was stopped by the addition of 9.0 mL 0.3N NaOH.

The velocity of the reaction was estimated after the quantitation of the hydrolyzed p-nitrophenol and plots were drawn. The method of least squares polynomials was also employed. The a, b and r coefficients are indicated with the figures, in the Discussion section.

The Km and Vmax values for the oligosaccharides were determined as follows. For each substrate a series of solutions, in acetate buffer, with the following molarities were prepared: 2.5 mM, 5.0 mM, 10.0 mM, 20.0 mM, 40.0 mM and 80.0 mM of each oligosaccharide. One mL solution from each substrate concentration was pipetted into each of three tubes. One mL of enzyme solution was added, mixed well and allowed to react at 45°C for 1 1/2 hour for melibiose and raffinose and for 2 1/2 hour for stachyose. At the end of the reaction time the tubes were placed in boiling water for three minutes. For the galactose quantitation the same procedures were followed, as previously. The reaction velocities were used along with the corresponding substrate

concentrations for the derivation of the Km and Vmax values, as with PNPG

Stability Tests

Studying how changes of the ionic strength in the solution medium (buffers) affect the enzymic activity, two solutions of the enzyme were prepared, one in 0.05 M acetate buffer, pH 5.0, and the other in 0.5 M, same pH, containing 1.0 mg of enzyme preparation/mL of solution. The enzymatic activity of each solution was tested during the hydrolysis of two mL of substrate (0.75 mM PNPG solution in the corresponding buffer). Similar reaction conditions were used as previously (10 min at 55°C). In order to study the stability of the enzyme solutions during longer periods of time, two identical solutions in 0.05 M acetate buffer were kept, one under refrigeration (2°-3°C) and the other under freezing conditions (-10° to -12°C). Every two weeks the solutions were taken out and their activity was assayed with PNPG. Studying the thermal stability of this enzyme, two identical reaction mixtures for each oligosaccharide were prepared. One of them was incubated at 45°C and the other at 25°C. After 0, 6, 12, 18 and 24 hrs aliquots were taken from each solution and the enzymatic activity was determined, based upon the estimated amount of hydrolyzed galactose.

Other Enzymes Found in the Enzymic Preparation from A. niger

The presence of some other enzymes, which are known to coexist with α-galactosidase in crude preparations, such as this one, was also examined. Among the most commonly found enzymes is β-galactosidase and particularly invertase.

The activity of β-galactosidase was assayed with o-nitrophenyl-β-galactoside (ONPG). A 0.5 mM solution of this compound, in 0.05 M acetate buffer, pH 4.0 was prepared and 2.0 mL of this substrate solution were mixed with 0.5 mL of enzyme. solution. The reaction was run at 37°C and the hydrolyzed o-nitrophenol was determined qualitatively with NaOH.

The presence of invertase was confirmed by its hydrolytic action upon sucrose. A 20.0 mM sucrose solution was prepared, in 0.05 M acetate buffer, pH 5.5 (at lower pH levels sucrose is subjected to acid hydrolysis) and 2.0 mL of this solution were mixed with 2.0 mL of enzyme solution (5.0 mg/mL of solution). The reaction was run at 37°C, for 24 hours. The presence of glucose and fructose in the hydrolyzate was determined qualitatively by descending paper chromatography. As solvent was used, a mixture of n-butanol, ethanol, water (5:3:2 v/v). The elution lasted 24 hours. For the identification of the sugars the guide strip method was used. To develop the spots, the chromatograms were sprayed with 3% p-anisidine (some hydrochloric acid was added into the p-anisidine solution) and heated for 5 minutes at 105°C.

The presence of invertase was also detected by HPLC but not very successfully. Two mL of a 20.0 mM sucrose solution in distilled water were subjected to hydrolysis by 2.0 mL of enzyme solution in water (5 mg/mL of solution), for 24 hours at 37°C.

Another mixture of sucrose solution with heat inactivated enzyme was prepared and injected into the pump of an HPLC analyzer (by Waters). As carrier solvent was used a mixture of acetonitrile, water and ethanol (85:12:3 v/v), with a flow rate of 2.0 mL/min.

Twenty μL of the sucrose hydrolyzate were injected and the component separation was recorded. The separation was not very successful but the sucrose in the mixture with the deactivated enzyme gave a distinct peak, which was not observed during the elution of the components in the hydrolyzate.

Due to the high amount of protein in the hydrolyzates, before the initiation of both chromatographic techniques described above, protein precipitation and removal was performed by the addition of 10% (w/v) lead acetate and centrifugation at 5,000 xg. The excess of lead acetate was precipitated by 10% (w/v) oxalate and centrifuged out.

Inhibitors

Metal ions, sugars and other inhibitory substances were added to reaction mixtures and their effect upon the enzyme

activity was tested, using a 0.75 mM PNPG solution as substrate. Table 6 in the Discussion section indicates the inhibitors, their final concentration in the reaction mixtures and the relative activity of the enzyme in each case. The reaction mixtures consisted of 1.0 mL substrate solution, 0.25 mL of enzyme solution and the indicated concentration of the inhibitor. The inhibition was expressed as % of the activity demonstrated by control samples.

α-Galactosidase from Coffee Bean

Dialysis

A 0.5 mL suspension of α-galactosidase from coffee beans (5 units) in 3.2 M $(NH_4)_2SO_4$, pH 6.5 was purchased from Sigma. This enzyme preparation was subjected to dialysis against 0.01 M acetate buffer, pH 5.4. The enzyme suspension was transferred quantitatively into a cellulose dialysis tube (2 inch width), which was previously prepared as follows. The tubing was simmered in 1.0 L of 50% ethanol, for 1 hour. Immersion of the tubing, after boiling, again in 50% ethanol for 1 hour followed. Another immersion in 10.0 mM $NaHCO_3$ solution, which was repeated with a change of solution. Immersion in 1.0 mM EDTA for one hour followed. Finally the tubing was rinsed in distilled water for 1 hour. One end was tied into a double knot and so was the other, after the enzyme was transferred into the tube.

The enzyme was transferred with some acetate buffer and the total volume of its solution in the tubing was about 10.0 mL. The dialysis lasted for 20 hours and was performed under continuous stirring in cold room at 2°C. Following the dialysis the enzyme solution was transferred into a 10 mL volumetric flask and was made to volume. This enzyme solution contained about 0.5 units/mL, a total of 5 units.

Assay of the Enzymatic Activity

The reaction mixture consisted of 10.0 mL acetate buffer, 0.2 mL of substrate solution (10 mM PNPG in the same buffer) and 0.5 mL of the dialyzed enzyme solution. The reaction lasted for 10 minutes at 45°C. At the end of the reaction time 10.0 mL of 0.3 N NaOH were added. Blanks were prepared by mixing 10.2 mL of acetate buffer, 0.01 M, pH 5.4, with a 0.5 mL of enzyme solution and 10.0 mL NaOH. Samples were prepared in triplicate. The activity of this enzyme was estimated to be 62.5 units/mL of solution, under the previously described experimental conditions.

Optimum Reaction Temperature

For the determination of the optimum temperature similar procedures were followed as in the case of the α-galactosidase from A. niger. Ten mL of buffer (0.01 M, pH 5.4) were mixed with 0.5 mL of enzyme solution in stoppered tubes and incubated for 15 minutes at various temperatures ranging from 25° to 70°C. The rest of the reaction conditions were kept

the same as during the enzyme assay.

Optimum Reaction pH

The rate of hydrolysis of PNPG by this α-galactosidase was studied with a series of acetate buffers, 0.1 M and pH ranging from 3.3 to 5.75. Similar volumes of substrate, enzyme and NaOH solutions as during the assay experiment were used and the reaction was run at optimum temperature level ($50^{o}C$). The reaction rates were determined based upon the amount of hydrolyzed p-nitrophenol.

Reaction Rates

During the next series of experiments another enzyme solution was used, which after the dialysis contained 1 unit/mL of solution. The reason for diluting this enzyme less was the fact that enzymes maintain their activity longer at higher concentrations.

The procedures for the determination of the reaction rates, during the hydrolysis of the four substrates by this enzyme, are similar to those followed during the reaction rate experiments for α-galactosidase from A. niger. Only the volumes and the concentrations in the reaction mixtures vary.

Thirty three mL of substrate solution (1 mM of PNPG, in 0.01 acetate buffer, pH 5.0) were mixed with 330.0 L of enzyme solution in the above buffer and allowed to react for 35 minutes at $50^{o}C$. One mL aliquots were taken at various

reaction times and the hydrolyzed p-nitrophenol was quantitated spectrophotometrically, after the addition of 3.6 mL of NaOH. The rate of hydrolysis of the oligosaccharides was studied during a 24 hour observation. Three main vessels were prepared, containing the reaction mixture for each oligosaccharide, each having a molarity of 10.0 mM. Thirty mL of each substrate solution were mixed with 600.0 L of enzyme solution. The reaction lasted for 24 hours at 45°C. Aliquots of 1.0 mL each were taken into screw-capped test tubes and boiled for three minutes.

The galactose quantitation was done in a similar way as before. The dilution factor was in this case twice as large (1/10.02), because in the aliquots of α-galactosidase from A. niger the volume of the substrate was 1/2 of the total volume.

Determination of the Km and Vmax Values

The values of the kinetic constants of this enzyme were estimated as in the case of the α-galactosidase from A. niger, for the initial rates of the reaction. The estimation of Km and Vmax values was achieved as previously, by the Lineweaver-Burk and Eadie-Hofstee, after the statistical manipulation of the experimental data. For the determination of the Km and Vmax values for PNPG a series of solutions with the following molarities were prepared: 0.25 mM, 0.5 mM, 0.75 mM, 1.0 mM, 1.5 mM and 2.0 mM.

One mL of substrate solution was hydrolyzed by 5.0 L of enzyme solution for 3 minutes at 50°C. The reaction was stopped by the addition of 3.6 mL of 0.3 N NaOH solution.

For the determination of the kinetic parameters of the oligosaccharides, solutions of melibiose and raffinose were prepared in acetate buffer (0.01 M, pH 5.0), with the following molarities: 2.5 mM, 5.0 mM, 10.0 mM, 20.0 mM, 40.0 mM and 80.0 mM. For stachyose solutions with the following molarities were prepared: 1.875 mM, 3.75 mM, 7.5 mM, 15.0 mM, 30.0 mM and 60.0 mM. An additional solution was prepared for melibiose, with molarity of 1.25 mM, due to substrate inhibition phenomena observed at concentrations of about 70.0 mM of melibiose and above. The reaction mixtures contained 2.0 mL of each substrate and 40.0 µL of enzyme solution (1 unit/mL of solution). The reaction mixtures of melibiose and raffinose were incubated for 1 1/2 hours and those with stachyose for 3 hours. At the end of the reaction time the tubes were boiled for three minutes.

For the galactose quantitation 2.0 mL of Tris-HCl buffer were added into each tube, which contained the melibiose and stachyose solutions and 5.0 mL of the same buffer into each tube containing the raffinose solutions. The rest of the procedures, for the galactose determination, were the same as previously (α-galactoseidase from A. niger). The dilution factors were 5, for melibiose and stachyose and 7,

for raffinose aliquots.

Inhibitors

The inhibitory effect of several compounds upon the enzymatic activity of the α-galactosidase from coffee beans was studied, using PNPG as substrate. To study the inhibitory action of Ag^{+} ions, 2.5×10^{-7} moles of $AgNO_3$ were added to 5.0 mL of active enzyme solution, containing 1.0 unit of enzyme per mL of solution. Next 5 μL of this enzyme solution were incubated with 1 mL of substrate solution (0.75 mM PNPG, in acetate buffer) for 5 minutes at 45°C. Control samples were also tested, containing active enzyme solutions. The enzyme inactivation was determined from the ratio:

$$100 \times \frac{\mu\text{mol of hydrolyzed p-nitrophenol by enz w/ inhibitor}}{\mu\text{mol of hydrolyzed p-nitrophenol by enz w/o inhibitor}}$$

When Hg^{++} ion was the inhibitor, 1.0×10^{-7} moles of $HgCl_2$ were added into 5 mL of active enzyme solution. Experiments for the Hg^{++} inhibition were also performed as previously.

As in the case of the α-galactosidase from A. niger, the effect of KI was tested upon enzyme solutions, inactivated by the addition of heavy metal ions, such as Ag^{+} and Hg^{++}.

α-Galactosidase from E. coli

The α-galactosidase from E. coli was purchased in the form of lyophilized powder, containing 20-40 units/mg of protein. An enzyme solution containing approximately 1 unit/mL was prepared in 0.01 M phosphate buffer, pH 6.5. This enzyme solution was constantly kept in iced water, to prevent rapid loss of its activity. For the assay of this enzyme 1.0 mL of the above phosphate buffer was mixed in a tube with 100 μL of a PNPG solution, with a molarity of 5.0 mM, also prepared in phosphate buffer and 5.0 μL of the enzyme solution.

Optimum Reaction Temperature

The determination of the optimum reaction temperature was carried out by incubating 100 μL of substrate solution (5.0 mM of PNPG in 0.01 M phosphate buffer, pH 6.5), with 1.0 mL phosphate buffer and 5.0 μL of the previously prepared enzyme solution. The reaction lasted for 10 minutes and was run at various temperatures ranging from 25° to 60°C. Prior to the addition of the substrate, the enzyme was incubated along with the buffer at the tested temperature for 10 minutes. The reaction was stopped by the addition of 2 mL of 0.3 N NaOH solution. The blanks used during the spectrophotometric determination of the hydrolyzed p-nitrophenol were prepared by mixing 1.1 mL of phosphate buffer with 5 μL of enzyme solution and 2 mL of 0.3 N NaOH.

Optimum Reaction pH

The effect of pH upon the enzymatic activity of α-galactosidase from E. coli was studied again during the hydrolysis of PNPG. Two different series of buffers were prepared each with a molarity of 0.1 M. For the pH levels between 4.0 and 5.75 a series of acetate buffers was prepared. For the pH range 6.0 through 8.75 a series of phosphate buffers were prepared. The reaction mixtures were prepared as previously and the reaction was run at 45°C.

Reaction Rates

The hydrolysis of PNPG by this α-galactosidase was followed for 36 minutes. Aliquots were taken from the reaction mixture in the same way as before, at various times and the enzymatic activity was plotted vs. the reaction time. Optimum temperature (45°C) and pH (7.0) conditions were kept. The hydrolysis of melibiose and raffinose were followed for 24 hours and that of stachyose for 38 hours. The reaction mixtures were prepared as for the other two enzymes, in three main vessels, one for each substrate. All substrate solutions were prepared with the same molarity, 10 mM, in phosphate buffer (0.01 M, pH 7.0). The volume ratio of the substrate and enzyme solution was 1 mL of substrate/25 μL enzyme solution (1 unit/mL of solution). Aliquots of 1 mL each were taken at various times, depending

upon how fast the reaction was progressing. For example, during the hydrolysis of stachyose the aliquots were taken every 3 hours in the beginning of the reaction and every 5 hours later, in order to yield measurable differences in the amount of hydrolyzed galactose.

Initially a temperature of 45°C was selected for the reaction. The galactose quantitation data though, particularly those from the hydrolysis of stachyose, gave very low values, even after many hours of hydrolysis. For this reason a second experiment was run, only for stachyose, at 25°C and for the same reaction time, which gave better results. For the determination of the galactose concentration in the aliquots, the absorbance readings were multiplied by 20, for melibiose and raffinose, and by 4 for stachyose.

Determination of the Km and Vmax Values

During the determination of the Km and Vmax values for PNPG a series of substrate solutions with the following molarities was prepared: 0.0625 mM, 0.125 mM, 0.250 mM, 0.50 mM, 0.75 and 1.0 mM, in phosphate buffer (0.01 M and pH 7.0). One mL of each substrate solution was mixed with 5 μL of enzyme solution and the reaction was run for 10 minutes at 45°C. The reaction was stopped by the addition of 2 mL of 0.3 N NaOH. Quantitation of the hydrolyzed p-nitrophenol followed.

For the determination of the enzyme kinetic constants for the oligosaccharides, solutions with the following

concentrations were prepared, in the above phosphate buffer: 2.5 mM, 5.0 mM, 10.0 mM, 20.0 mM, 40.0 mM, and 80.0 mM for melibiose and raffinose and for stachyose 1.25 mM, 2.5 mM, 5.0 mM, 10.0 mM, 20.0 mM and 40.0 mM.

The reaction mixtures contained 2 mL of each substrate solution and 50 µL of enzyme solution. The tubes were incubated at 25°C (all three substrates), for 2 hours, for melibiose and raffinose and for 3 hours for stachyose. At the end of the reaction time the tubes were placed in boiling water for three minutes. The galactose determination followed and the absorbance readings were multiplied by 4, for raffinose and stachyose and by 20 for melibiose.

Inhibitors

A few inhibition experiments were conducted, with several inhibitory agents, such as galactose, Ag^{+} and Hg^{++}. Also the effect of KI upon inactivated enzyme solutions was studied. The reaction mixtures consisted of 1 mL phosphate buffer, 50 µL of substrate solution (5.0 mM PNPG in buffer) and 0.25 mL of either active (controls) or inactivated enzyme solution, after the addition of various amounts of inhibitors, as indicated in Table 12 in the Discussion section (1 unit of enzyme/mL of solution).

RESULTS AND DISCUSSION

Standard Curve of Galactose

The enzymatic activity of all three enzymes was determined by a dual approach. When PNPG was the substrate, the amount of liberated p-nitro-phenol was determined. When melibiose, raffinose and stachyose were the substrates, the enzymatic activity was measured by the amount of liberated galactose.

Several methods were tried for the galactose determination: paper chromatography (PC), thin layer chromatography (TLC), high pressure liquid chromatography (HPLC) and an enzymatic method employing the use of β-D-galactose dehydrogenase. The chromatographic methods did not give satisfactory results. The major reason for this was the microquantities of galactose, which had to be determined.

The enzymic methods are more specific, precise and rather simpler, in cases where specificity and accurate determination of microquantities are crucial factors.

For the enzymic determination of galactose one can choose between two methods, depending upon the conditions and the components in the reactivation system. Factors such as pH, temperature and reactants affecting the rate of the

enzymic reaction can greatly influence the results. One of the methods is galactose oxidase, which catalyzes the oxidation of galactose at the sixth carbon atom (49). This is indicated by Figure 6. During this reaction the properties of H_2O_2, produced stoichiometrically are exploited in a manner analogous to the determination of glucose, with glucose oxidation (33). This method, though, was considered improper in this case, because other sugars, such as galactose derivatives (melibiose, raffinose, stachyose) can react appreciably with their terminal galactose moieties, leading to erroneous results (49).

An alternative method is the α-D-galactose dehydrogenase or D-galactose:NAD-1-oxydoreductase (E.C. 1.1.1.48), which catalyzes the conversion of β-D-galactose into D-galactono-lactone in the presence of coenzymes NAD^+ or DPN, as indicated below:

$$\beta\text{-D-galactose}+NAD^+ \longrightarrow \text{D-galactono-}\delta\text{-lactone}+NADH+H^+$$

$$\beta\text{-D-galactose}+DPN \longrightarrow \text{D-galactono-}\gamma\text{-lactone}+DPNH$$

This enzyme is selective only for β-D-galactose, α-L-arabinose, and β-D-fucose. The optimum pH for the reduction of NAD^+ or DPN is between 8.0 and 9.0. In this range the galactonolactone undergoes spontaneous hydrolysis and the reaction is virtually irreversible (21).

The galactose concentration is a function of the absorbance of the formed NADH at 340 nm. The extinction

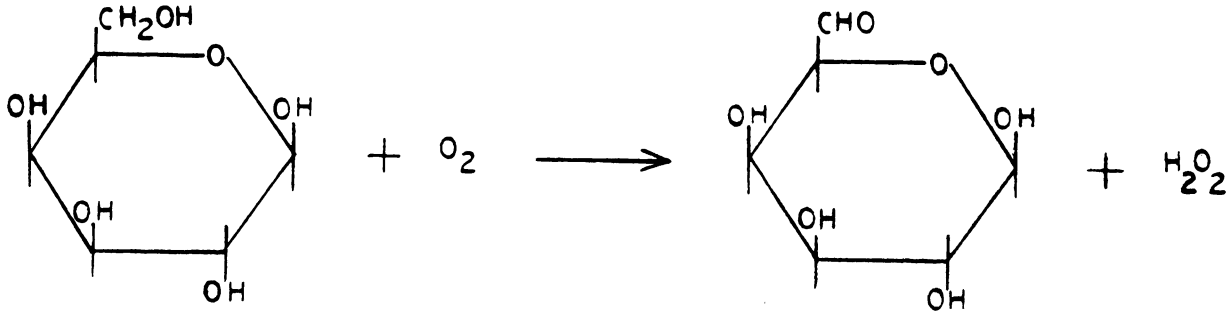


Figure 6. Reaction of galactose oxidase.

coefficient of NADH is 4.25 absorbance units/μmole/mL. Since the formation of NADH occurs stoichiometrically with the galactose lactonization, the amount of galactose is equivalent to the formed NADH (29).

In aqueous solutions D-galactose exists as an equilibrated mixture of various isomers. For this reason it takes a long time for the entire amount of galactose to be lactonized. Mutarotase or aldose-1-epimerase (E.C. 5.1.3.3) can be added to convert all the galactose into the β-D-configuration for rapid enzymatic reaction (6). The addition of mutarotase though can be omitted from the system, if the reaction is allowed to run long enough.

Several researchers who employed the use of this enzyme in their work (26, 27) decided that 1 hour at 37°C would be enough time for the completion of the reaction, without mutarotase. Another factor influencing the reaction time is the number of β-D-galactose dehydrogenase units used in the system. The determination of an adequate incubation time for the β-D-galactose dehydrogenase reaction was achieved after repeated measurements of the NADH absorbance, formed in tubes of samples containing D-galactose, NAD^+ and enzyme. It was found that at room temperature (25°C) an incubation time of 12 hours was sufficient, when 5 μL of a β-D-galactose dehydrogenase solution, containing 0.1 units/mL, were added into the reaction system.

The determination of the galactose standard curve was accomplished by measuring the absorbance of samples with various galactose concentrations ranging from 0.0 to 0.3 μmoles/mL of solution (Figure 7). The absorbance of 0.3 μmoles of galactose/mL was 1.25 A, and was considered to be the highest galactose concentration that should be found in a reaction mixture for accurate readings. The galactose concentration in the experimental samples was adjusted up to this level by various dilutions with Tris-HCl buffer, 0.1 M and pH 8.6.

Two kinds of buffers were used during our experiments, acetate and phosphate. The presence of two different ionic species in the reaction mixtures of galactose dehydrogenase could affect differently the activity of this enzyme. Two series of samples were prepared (in triplicate), one with various galactose solutions in acetate buffer (0.05 M, pH 5.0) and the other in phosphate (0.01 M, pH 7.0). The rest of the reaction conditions were identical. The galactose absorbance readings, corresponding to equal galactose concentrations, were very close (Table 2).

Standard Curve of p-nitro-phenol

The p-nitro-phenol derivatives are very popular compounds, used in many cases, as chromogenic substrates for the colorimetric assays of various enzymes. Pure solutions of PNPG are colorless. During the enzymatic hydrolysis of

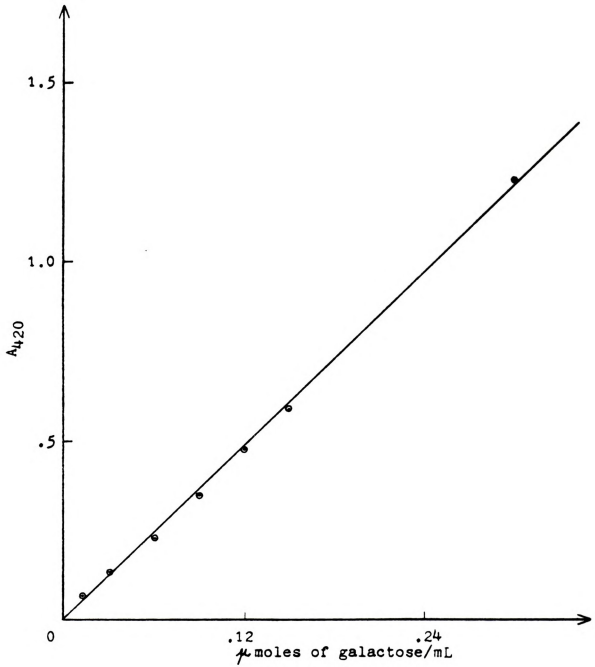


Figure 7. Standard curve for determining galactose.

Table 2. Absorbance of galactose in acetate and phosphate buffers at 340 nm.

Galactose (μmoles/mL)	Acetate buffer	Phosphate buffer
0.300	1.230	1.205
0.150	0.585	0.600
0.120	0.473	0.450
0.090	0.345	0.320
0.060	0.235	0.250
0.030	0.130	0.145
0.015	0.073	0.080

this compound by α-galactosidases, p-nitro-phenol is liberated, which has a pKa of 7.2. This compound in alkaline environment (1 to 2 pH units above its pKa value) develops a deep yellow color suitable for the quantitative measurement of the enzyme activity. Advantages of this method include simplicity, accuracy, sensitivity and fast results (34). The alkaline compounds, used to develop the color of p-nitro-phenol, also stop the enzymic reaction by raising the pH (when 0.3 N NaOH is added, the pH of the reaction mixture reaches 12.8). The mechanism of the chemical reactions occurring during the enzymatic hydrolysis of PNPG by α-galactosidases and the color development in the alkaline environment are shown in Figure 8.

In many cases o-nitro-phenol derivatives are used instead. It seems though that the p-derivative is the compound of preference, because the o-nitro-phenol is steam distilled, due to the formation of intramolecular hydrogen bonds (Figure 9).

The blanks, used during the various spectrophotometric measurements, initially were prepared by mixing equal volumes of PNPG solutions, to those used in the experimental samples, heat inactivated enzyme solution and NaOH. Immediately after mixing the blanks were colorless but with time they developed a deep yellow color. This was due to the gradual liberation of p-nitro-phenol, after the addition of NaOH. For this reason the PNPG solution in the blanks was replaced by the

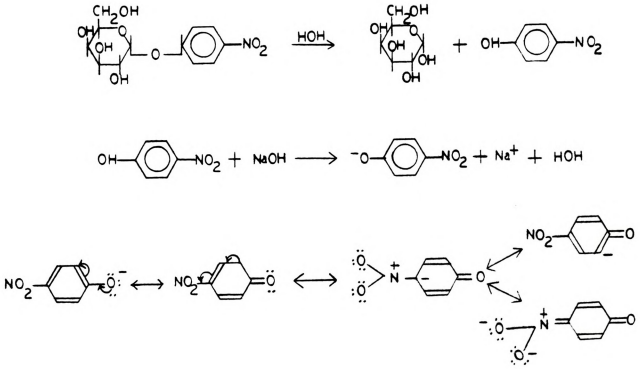


Figure 8. Reactions occurring during the hydrolysis of PNPG by α-galactosidase and color development of the liberated p-nitro-phenol.

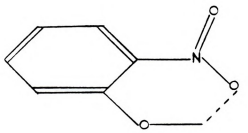


Figure 9. Formation of intramolecular hydrogen bond by o-nitro-phenol.

corresponding buffer. Pure PNPG solutions do not absorb any light at 420 nm, so do the buffers (Figure 10).

Other workers used water, as a reference, in similar experiments.

α-Galactosidase from A. niger

Optimum Reaction Temperature

Figure 11 indicates that the optimum temperature for the hydrolysis of PNPG, by α-galactosidase from A. niger, is 55°C. Between 50° and 60°C the enzyme shows 94 to 100% of its maximum activity. At 65°C the activity drops to 86%. Beyond 65°C an abrupt decrease of the enzymic activity is demonstrated, due to heat denaturation of the enzyme molecule. At 70°C the enzyme loses 65% of its maximum activity in 15 minutes. Holding the enzyme solution for several hours at room temperature also resulted in some loss of activity. For this reason the enzyme was always kept in iced water.

The thermal stability of other α-galactosidases, isolated from various species of the genus Aspergillus, such as A. satoi (57) demonstrated a similar optimum temperature, about 55°C. Another α-galactosidase, from A. awamori (43), was found to be less thermostable, with an optimum temperature at 50°C. This α-galactosidase was inactivated at 55°C in 15 minutes. The enzyme did not lose activity during prolonged incubation at 45°C.

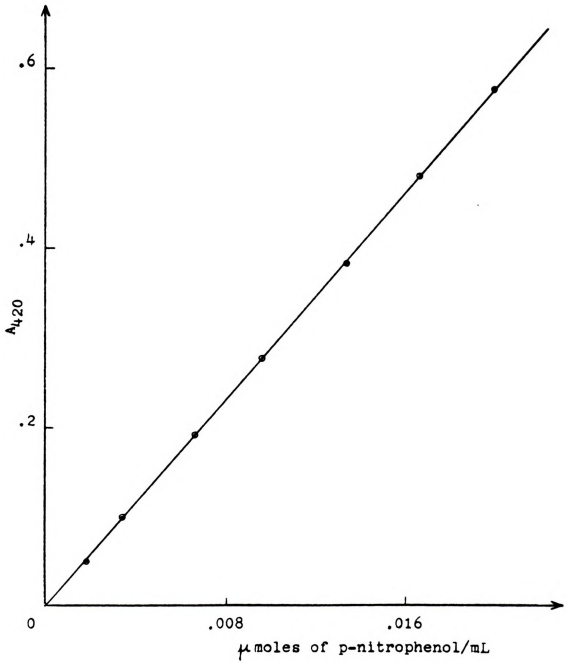


Figure 10. Standard curve for determining p-nitrophenol.

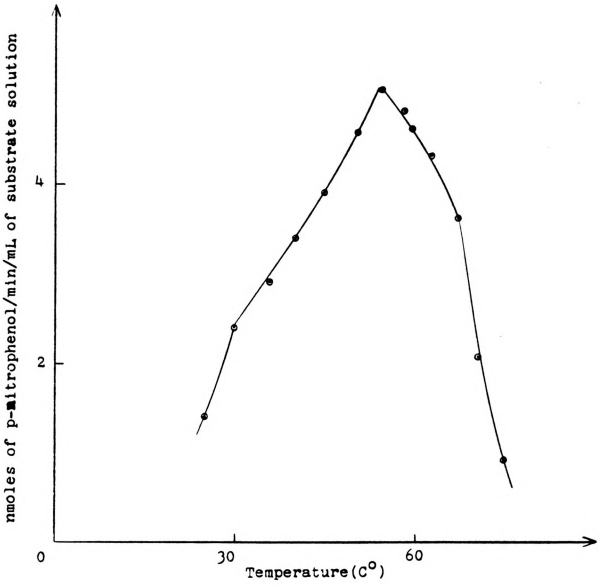


Figure 11. Temperature-activity relationship of α-galactosidase from A. niger with PNPG as substrate (pH 4.5). Reaction mixture: 1 mL substrate solution (0.1 mM PNPG) 0.25 mL enzyme solution (1 mg/mL).

The data in Table 3 makes a comparison of the absorbance readings of two identical samples of the oligosaccharides, incubated at 25°C and 45°C. The reaction mixtures contained 1 mL of substrate solution (10 mM of each oligosaccharide in acetate buffer) and 1.0 mL of enzyme solution (5.0 mg of enzyme preparation/mL of acetate buffer).

Optimum Reaction pH

Figure 12 indicates the relation between the enzyme activity and the pH in the reaction system. The optimum reaction pH is about 5.0. It is apparent that this enzyme favors acidic environments, showing increased activity at the pH range between 3.75 and 5.0. Actually at pH 3.75, where many enzymes show very low activity and some of them are totally inactivated, this enzyme maintains 96% of its apparent activity. The increased activity of this enzyme in acidic environment can be explained by the fact that it is derived from a mold. These microorganisms can tolerate acidic environments well. As a result of this their enzymes also remain active in acidic environments.

Lee et al. (40) determined the optimum reaction pH for the α-galactosidase from A. niger at 4.6, while Bahl estimated it at 4.9 (5). Note that there is a wide optimum pH level, in which the enzyme demonstrated increased activity. This could indicate the presence of more

Table 3. Thermal stability of α-galactosidase from A. niger, during incubation for 24 hours at 25°C and 45°C. A_{340}* of a system containing 1 mL of substrate solution (10 mM melibiose, raffinose or stachyose) and 1 mL of enzyme solution (5.0 mg of enzyme per mL of solution) after 6, 12, 18, 24 hours of reaction.

Substrate	Incubation at 25°C Reaction time (hours)				Incubation at 45°C Reaction time (hours)			
	6	12	18	24	6	12	18	24
Melibiose	9.6	11.6	12.6	13.4	9.7	11.8	12.8	13.4
Raffinose	10.2	13.1	13.5	13.6	10.6	12.8	13.4	13.8
Stachyose	5.2	7.6	8.6	10.0	5.0	7.7	9.0	9.8

*A_{340} of NADH is a measure of galactose determined enzymatically. The A_{340} values represent the original absorbance readings multiplied by a dilution factor of 20.

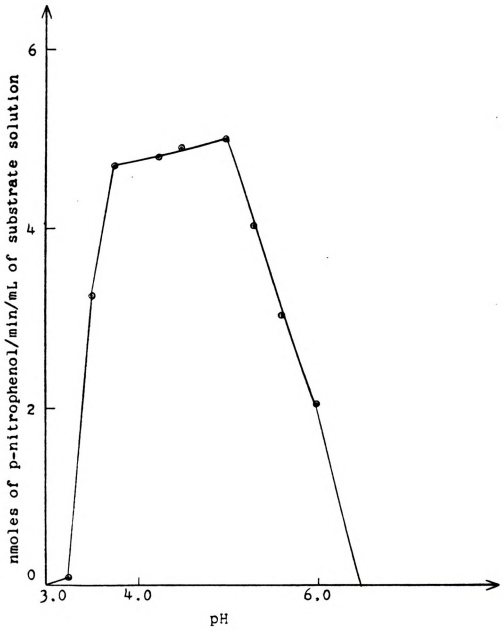


Figure 12. pH-activity relationship for α-galactosidase from A. niger, with PNPG as substrate at 55°C. Reaction mixture: 1 mL substrate solution (0.1 mM PNPG) 1 mL enzyme solution (1 mg/mL).

α-galactosidases in this crude enzyme preparation (40).

Reaction Rates

During the study of an enzyme for the determination of its kinetic constants, Km and Vmax, it is important that the data of the velocity are taken during the steady state of the reaction, when the disappearance rate of the substrate, S (equation 1) is equal to the appearance rate of the product, P. In this way the concentration of the complex ES is constant (by definition) in the steady state (Briggs-Haldane hypothesis, ref. 24).

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E + P \qquad (1)$$

where: E = enzyme concentration
S = substrate concentration
P = product concentration

The steady state is achieved very rapidly after mixing the enzyme with the substrate. Fast reaction techniques have shown that in most cases it is achieved within a few miliseconds and before any finite product has been formed. This means that, when one measures the initial reaction rate of an enzyme by the usual experimental methods, one is actually measuring the steady state of the reaction (24). Figures 13 and 14 show the course of the reaction of α-galactosidase from A. niger, during the hydrolysis of

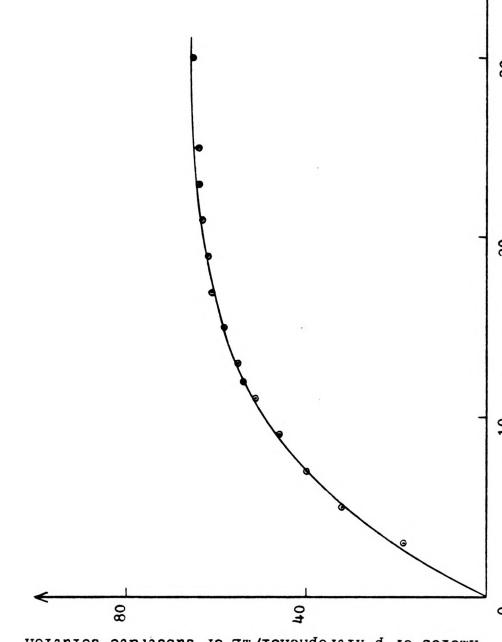


Figure 13. Hydrolysis of PNPG by α-galactosidase of A. niger. Reaction mixture: 1 mL of PNPG solution (0.2 mM PNPG). 0.25 mL of enzyme solution (1 mg of enzyme preparation/mL).

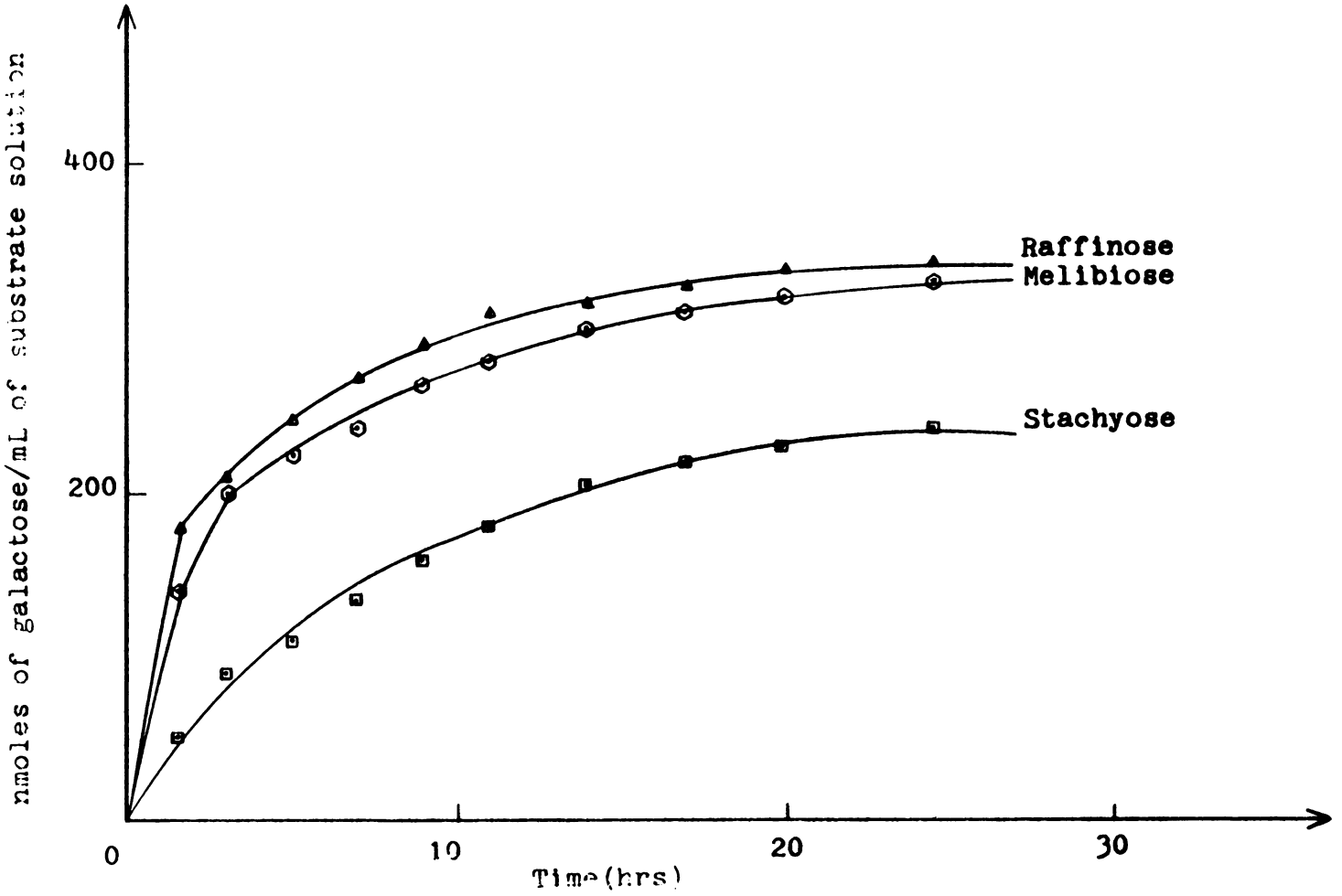


Figure 14. Hydrolysis of melibiose, raffinose and stachyose by A. niger. α-galactosidase. Reaction mixture: 1 mL of substrate solution (10 mL of oligosaccharide), 1 mL of enzyme solution (5 mg/mL).

PNPG and melibiose, raffinose and stachyose. For PNPG the reaction rate plateaus within 12 minutes, while for the oligosaccharides this is not observed until several hours are gone. Among the three oligosaccharides stachyose is hydrolyzed most slowly, while raffinose seemed to be the substrate of preference. Melibiose was initially hydrolyzed at a slower rate than raffinose and reached later the hydrolysis rate of raffinose.

Km and Vmax Values

The determination of the Km and Vmax values was estimated for the initial reaction rates, about 3 minutes for PNPG, 1 1/2 hours for melibiose and raffinose, 2 1/2 hours for stachyose. When PNPG was the substrate, a decrease of the reaction rate was observed, when the substrate concentration in the reaction mixture was higher than 0.75 μmoles of PNPG/mL of reaction mixture. As Figure 15 shows, among the various PNPG concentrations, the reaction rate was higher, when the substrate solution had a molarity of about 0.75 mM. The same observation was made by Dey and Pridham, when they were collecting data during kinetic studies upon the α-galactosidases I and II from Vicia faba (20). These researchers observed that, when PNPG was the substrate, at concentrations above 0.75 mM the simple Michaelis-Menten law was not obeyed. They characterized this phenomenon as "substrate inhibition". One possible explanation for this

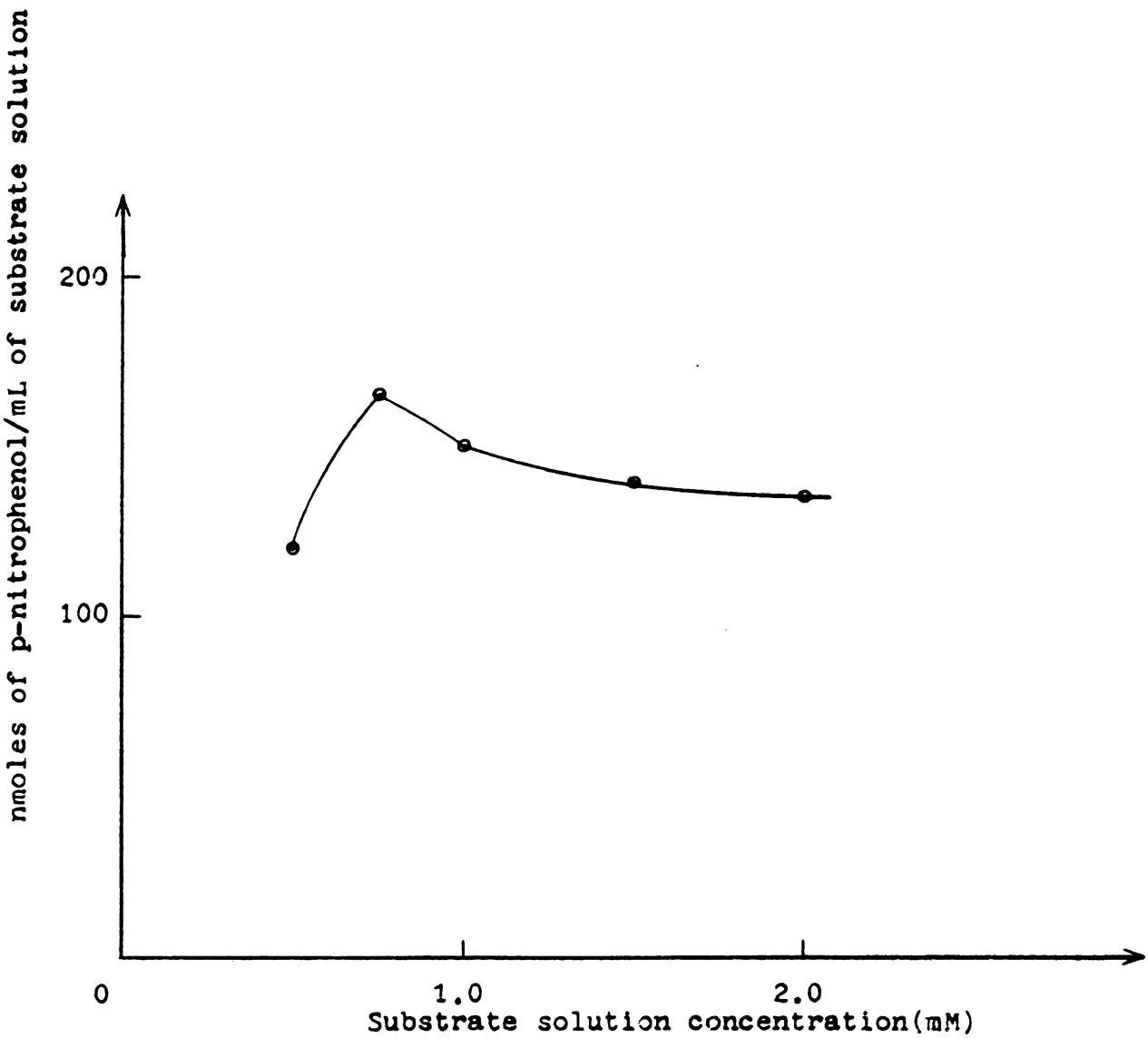


Figure 15. Optimum substrate concentration during the hydrolysis of PNPG by α-galactosidase of A. niger. Reaction mixture: 1 mL of substrate solution, 1 mL of enzyme solution (1 mg enzyme preparation/mL).

is that for the formation of an effective enzyme-substrate complex a single substrate molecule may associate with a binding site on the enzyme. At higher substrate concentrations though a second molecule may associate with the enzyme to form an inactive complex (20), i.e.

$$E + S \longleftrightarrow ES \longleftrightarrow E + P$$
$$\downarrow$$
$$ESS \text{ (inactive complex)}$$

During the hydrolysis of the oligosaccharides, inhibition phenomena were observed with melibiose at concentrations above 0.07 mmoles per mL of reaction mixture. The presence of stachyose at the same concentration in the reaction mixture did not cause any inhibition. If Haldane's theory about the substrate inhibition is correct, then the formation of the inactive compound ESS is not possible, when stachyose is used as substrate. Probably stereochemical limitations at the enzyme's active site do not allow more than one large molecule, such as stachyose, to bind. This substrate inhibition phenomenon is not observed in the various biological systems, due to the small concentrations of substrate and continuous removal of the product, which renders available more enzyme sites.

The determination of the kinetic constants of α-galactosidase from A. niger was based upon the Michaelis-Menten equation. Two modifications of this equation were

used to obtain the Km and Vmax values of the four substrates. One was the well known Lineweaver-Burk equation (2) and the other was the Eadie-Hofstee equation (3).

$$\frac{1}{v} = \frac{Km}{Vmax} \times \frac{1}{S} \times \frac{1}{Vmax} \qquad (2)$$

$$v = Vmax - \frac{v}{S} \times Km \qquad (3)$$

where: v = initial rate of reaction

S = initial substrate concentration

Vmax = maximum reaction rate

Km = Michaelis constant

Figures 16 through 23 indicate the derivation of the Km and Vmax values of the α-galactosidase from A. niger, for the four studied substrates, by both methods. All the lines 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, α and β, along with the indexes of determinations, r, are indicated with the plots. The Km and Vmax values were calculated from the formulas:

$Km = \frac{b}{a}$ $\qquad$ $Vmax = \frac{1}{a}$ $\qquad$ Lineweaver-Burk plots

$Km = -b$ $\qquad$ $Vmax = a$ $\qquad$ Eadie-Hofstee plots

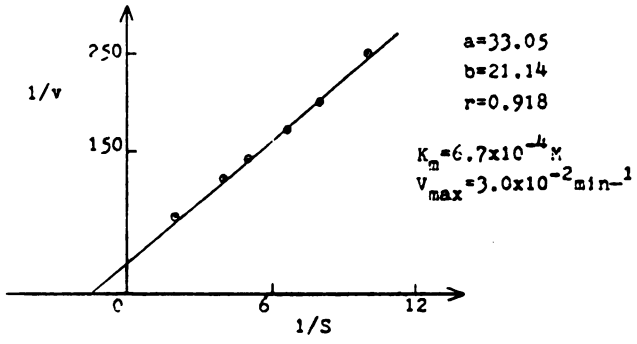


Figure 16. Lineweaver-Burk double reciprocal plot of α-galactosidase of A. niger for PNPG. Reaction mixture: 1 mL substrate solution, 0.25 mL of enzyme solution (1 mg enzyme preparation/mL).

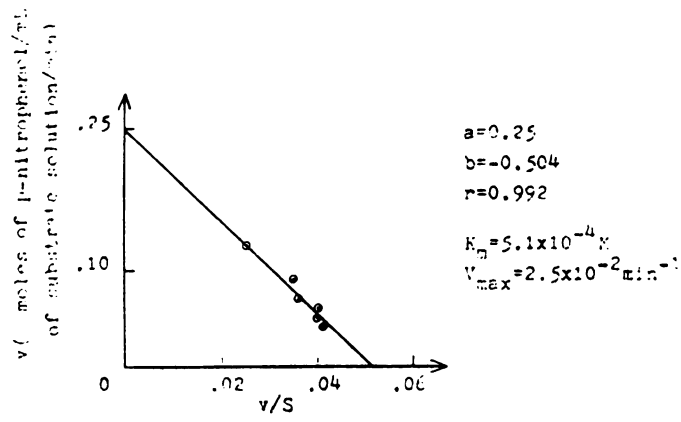


Figure 17. Eadie-Hofstee reciprocal plot of α-galactosidase of A. niger for PNPG. Reaction mixture: 1 mL substrate solution, 0.25 mL enzyme solution (1 mg/mL).

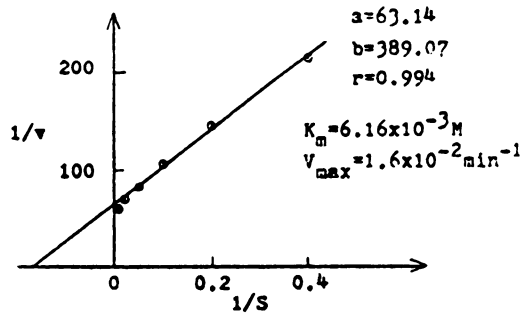


Figure 18. Lineweaver-Burk double reciprocal plot of α-galactosidase of A. niger for melibiose. Reaction mixture: 1 mL of substrate solution, 1 mL of enzyme solution (5 mg of enzyme preparation/mL).

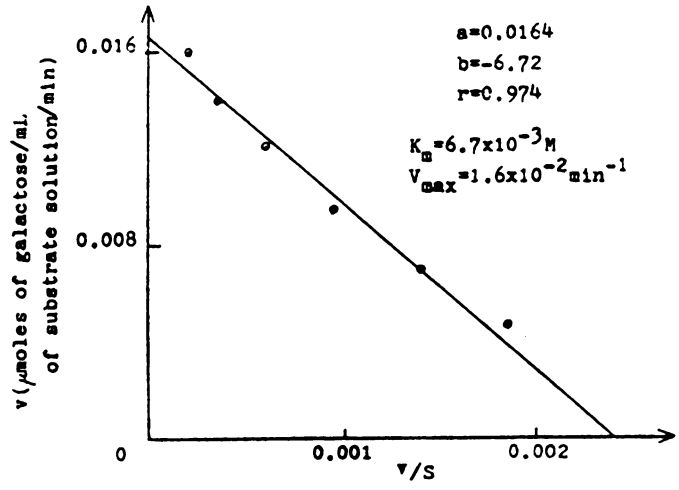


Figure 19. Eadie-Hofstee reciprocal plot of α-galactosidase from A. niger for melibiose. Reaction mixture: 1 mL substrate solution, 1 mL enzyme solution (5 mg/mL).

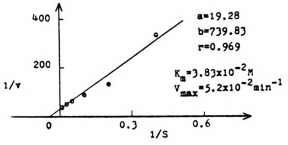


Figure 20. Lineweaver-Burk double reciprocal plot of α-galactosidase of A. niger for raffinose. Reaction mixture: 1 mL of substrate solution, 1 mL of enzyme solution (5 mg of enzyme preparation/mL).

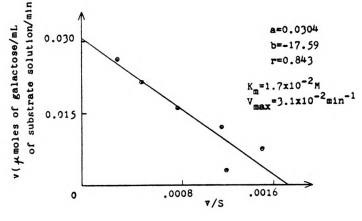


Figure 21. Eadie-Hofstee reciprocal plot of α-galactosidase of A. niger for raffinose. Reaction mixture: 1 mL of substrate solution, 1 mL of enzyme solution (5 mg enzyme preparation/mL).

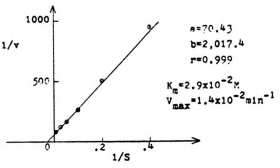


Figure 22. Lineweaver-Burk double reciprocal plot of α-galactosidase of A. niger for stachyose. Reaction mixture: 1 mL of substrate solution, 1 mL enzyme solution (5 mg enzyme preparation/mL).

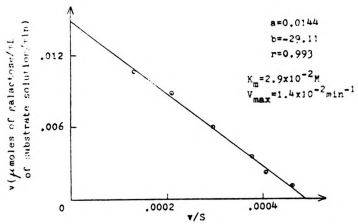


Figure 23. Eadie-Hofstee reciprocal plot of α-galactosidase from A. niger for stachyose. Reaction mixture: 1 mL substrate solution, 1 mL enzyme solution (5 mg/mL).

The above formulas were derived from equations (2) and (3).

Table 4 gives the Km and Vmax values of α-galactosidase from A. niger for PNPG, melibiose, raffinose and stachyose, as they were estimated by both methods. As one can see the Km and Vmax values change independently, among the various substrates. Comparing the Km and Vmax values obtained for each substrate by the two methods, one may conclude that, when the indexes of determination (r) are closer to 1.0 the estimated values of the kinetic constants estimated by both methods are almost the same. This enzyme had higher affinity (1/Km) for PNPG.

Stability

Stability tests indicated that this α-galactosidase is more stable than the enzymes from coffee beans and E. coli. Dissolving the enzyme in 0.5 M acetate buffer caused a 10% increase of the enzyme activity, compared to that in 0.05 M acetate buffer. The enzyme solutions kept under refrigeration and freezing both demonstrated 99% of their original activity during the first month. After 1 1/2 months the refrigerated solution indicated only 65% of each original activity, while the frozen one retained 90% of it. In two months the refrigerated solution had lost all its activity, while the frozen one still retained 75% of it.

Table 4. Kinetic constants of α-galactosidase from A. niger for 4 substrates determined by the Lineweaver-Burk (L-B) and Eadie-Hofstee (E-H) methods.

Substrate	Km (moles)		Vmax (μmoles/mL) substrate/min)	
	L-B	E-H	L-B	E-H
PNPN	6.7×10^{-4}	5.1×10^{-4}	3.0×10^{-2}	2.5×10^{-2}
Melibiose	6.2×10^{-3}	6.7×10^{-3}	1.6×10^{-2}	1.6×10^{-2}
Raffinose	3.8×10^{-2}	1.8×10^{-2}	5.2×10^{-2}	3.0×10^{-2}
Stachyose	2.9×10^{-2}	2.9×10^{-2}	1.4×10^{-2}	1.4×10^{-2}

Other Enzymes

The experimental procedures for the determination of the presence of other enzymes, in this crude α-galactosidase were not very detailed and accurate. Yet they revealed some information about enzymes that can be expected to be found in this crude α-galactosidase preparation. The experiments with NPG indicated the presence of β-galactosidase. The chromatographic analysis of the sucrose hydrolyzates, by TLC and HPLC, revealed the presence of invertase.

Inhibition

Table 5 presents data taken during the inhibition experiments. EDTA and citrate, known as chelating agents, did not cause significant inhibition. This led to the assumption that there are not any activating metals at the active side of this enzyme. On the other hand this enzyme seemed to be sensitive to heavy metals, such as Ag^{+} and Hg^{2+}. Although this sensitivity indicates participation of thiol groups (-SH) during the enzymic catalysis (20), typical thiol specific reagents, such as iodoacetamide and chloro-mercury-benzoate, were not tested to confirm the presence of this thiol group related inhibition. The addition of Ag^{+} and Hg^{2+} caused a considerable amount of inhibition, even without incubation, and within 2 hours the enzyme solutions were completely inactivated.

Table 5. Inhibitors of α-galactosidase from A. niger.[a]

Inhibitor	Amount of inhibitor (moles) in reaction mixture	% Inhibition
EDTA	2.5×10^{-4}	15
Citrate	2.5×10^{-5}	30
$HgCl_2$	2.5×10^{-5}	100 (without incubation)
$AgNO_3$	2.45×10^{-5}	100 (after 2 hrs incub)
$AgNO_3$	4.9×10^{-8}	50 (without incubation)
Galactose	3.0×10^{-6}	100
Galactose	3.0×10^{-5}	26

[a]Reaction mixture: 1 mL of substrate solution (0.5 mM PNPG), 0.25 mL enzyme solution (1 mg of crude α-galactosidase preparation per mL of solution), 0.5 mL of inhibitor solution.

Experiments with KI indicated that the addition of this compound into enzyme solutions, which were inactivated by Ag^{+} and Hg^{++} ions, regenerated their activity, to a degree relative to the amount of KI in the reaction mixtures. This compound added into enzyme solutions before the introduction of the inhibitory metals protected the enzyme. Ag^{+}-inactivated enzyme solutions were not regenerated by the addition of KI at concentrations higher than 10^{-4} M. Table 6 presents data upon the regenerating effect of KI at various concentrations.

Table 7 shows that adding 5.5×10^{-5} moles of KI into the reaction mixtures resulted in 90-100% restoration of the enzyme activity. Adding 5×10^{-5} moles of EDTA into 5 mL of enzyme solution inactivated by Ag^{+} or Hg^{++} resulted in 75% regeneration of the enzyme. Treatment with citrate was not effective at all. When cysteine was added to similarly inactivated enzyme, testing the enzyme activity immediately after the addition of cysteine did not indicate any activity restoration. Fourty minutes later, though, 90% of the original activity was restored, when Ag^{+} was the inhibitor, and 80% in the case of Hg^{2+}. The same observation was made by Dey and Pridham (20).

Galactose caused considerable inhibition in amounts above 10^{-6} moles. Adding 3.5×10^{-5} moles of galactose in the reaction system caused 100% inhibition. At concentrations 3.0×10^{-6} M galactose caused 86% inhibition. Addition

Table 6. The regenerating effect of KI upon α-galactosidase from A. niger inactivated by Ag^{+} and Hg^{2+}. % inhibition and relative amounts of KI (moles) in the reaction mixture.

KI (moles)	None	2.5×10^{-5}	5.0×10^{-5}	7.5×10^{-5}
Control	0	0	2	5
$HgCl_2$	88	34	0	1
$AgNO_3$	15	32	0	1

Reaction mixtures. Control: 1 mL substrate solution (0.5 mM PNPG), 0.25 mL enzyme solution (1 mg of crude α-galactosidase preparation per mL of solution), 1 mL of distilled water, or 1 mL KI solution.

with $HgCL_2$: 1 mL substrate solution, 0.25 mL enzyme solution, 1 mL of $HgCl_2$ solution (2.5×10^{-7} moles of $HgCl_2$), 1 mL KI solution.

with $AgNO_3$: 1 mL substrate solution, 0.25 mL enzyme solution, 1 mL $AgNO_3$ solution (2.5×10^{-7} moles of $AgNO_3$), 1 mL KI solution.

Table 7. Regeneration of α-galactosidase from A. niger inactivated by Ag^{+} and Hg^{2+} expressed as % of control. Effect of KI, EDTA, citrate and cysteine.

Inhibitor 2.5×10^{-7} (moles)	KI 5.5×10^{-5} (moles)	EDTA 5.0×10^{-5} (moles)	Citrate 5.0×10^{-5} (moles)	Cysteine 5.5×10^{-3} (moles)
$AgNO_3$	100	75	0	90
$HgCl_2$	90	-	-	80

Reaction mixture: 1 mL substrate (0.5 mM PNPG), 0.25 mL solution, 1 mL inhibitor solution, 1 mL KI, EDTA, citrate, or cysteine solution.

KI into the reaction system prior to the addition of galactose did not prevent the inhibitory effects of galactose. Dey and Pridham (20) reported that the addition of small amounts of galactose into their system protected the enzyme (α-galactosidase from Vicia faba) from inactivation by heavy metals. Similar experiments with α-galactosidase from A. niger did not have the same results. This might lead to the assumption that the action center of KI is not located at the active site of the enzyme.

The regenerating effect of KI needs further study to elucidate its action upon this and other enzymes. For example KI restored 80% of the activity of β-galactosidase (A. niger) after a 100% inactivation by $HgCl_2$, when PNPG was the substrate. It is also worth repeating this experiment with oligosaccharides.

The action of K^+ and I^- ions separately upon the enzyme molecule is worth investigating. K^+ have been previously reported to act as activators of α-galactosidases (21).

α-Galactosidase from Coffee Beans

Optimum Reaction Temperature and Stability

Figure 24 shows that the α-galactosidase from coffee beans demonstrated maximum activity at a temperature range between 45°C and 50°C. At 60°C the enzyme retained only 80% of its maximum activity and at 70°C only 26% of it.

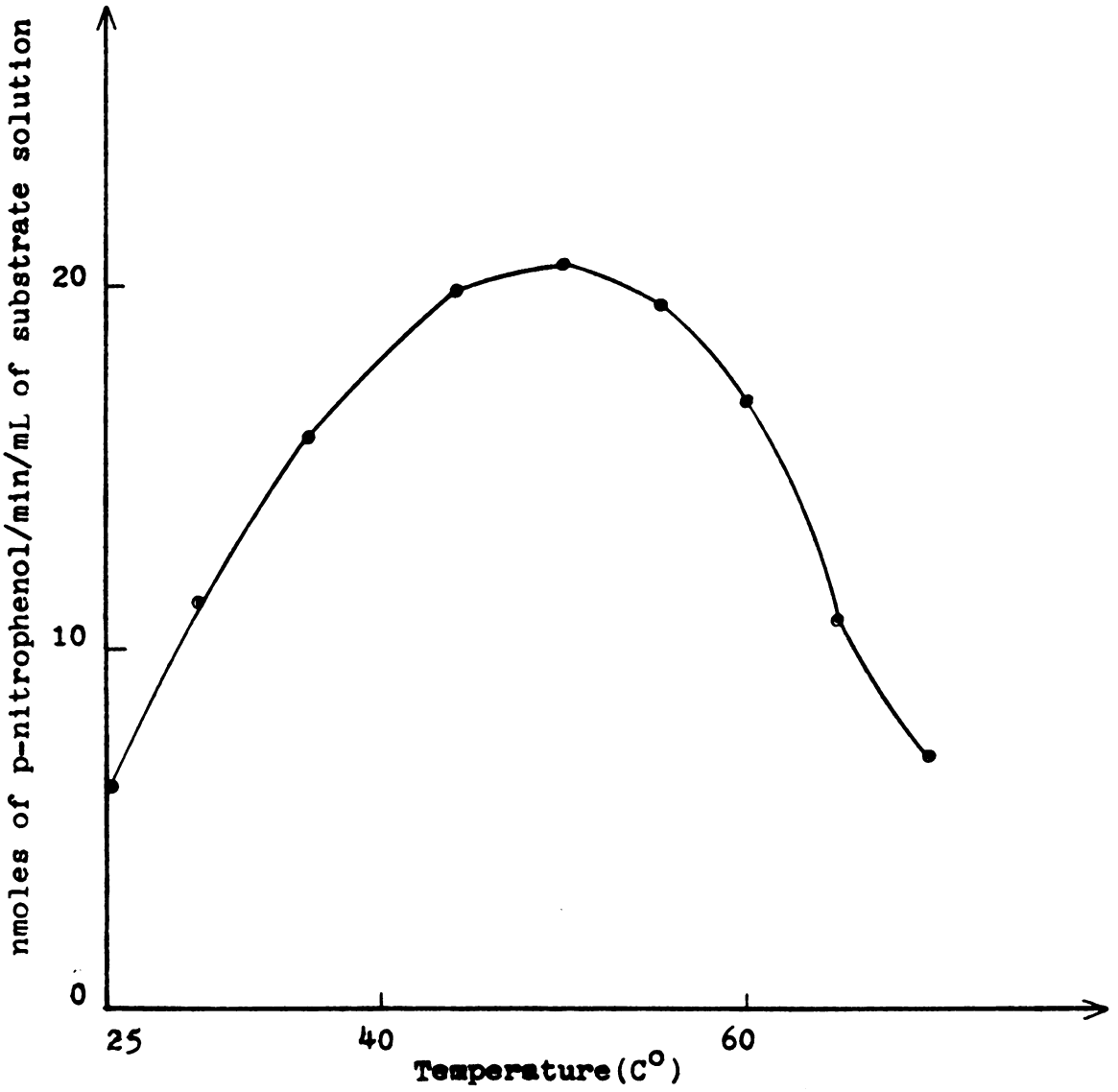


Figure 24. Temperature-activity relationship of α-galactosidase from coffee beans with PNPG as substrate, pH 4.5. Reaction mixture: 10 mL acetate buffer, 0.5 mL enzyme solution (0.5 units/mL) 0.2 mL substrate solution (10 mM PNPG).

The above data indicate that the α-galactosidase from A. niger is more thermostable than that from coffee beans. On the other hand though during a long incubation period at 45°C this enzyme showed increased activity. Table 8 presents data of the enzyme activity, during incubation for 24 hours, at 25°C and at 45°C.

The α-galactosidase from coffee beans demonstrated higher storage stability than that from A. niger. Solutions of this enzyme in 0.01 M acetate buffer, pH 5.0, containing 1 unit/mL of solution demonstrated 95% of their original activity, after two months in the freezer. This increased stability of the enzyme from coffee beans could be contributed to a higher degree of purity and concentration. The solutions of the enzyme from A. niger had a concentration of 0.06 units/mL.

Optimum Reaction pH

Figure 25 shows that the optimum pH for this enzyme is at 5.0. Petek and ToDong isolated two α-galactosidases from coffee beans, I and II, and determined their optima pH at 5.3 and 6.0 respectively, using phenyl-α-D-galactoside as substrate. The pH-activity curve of the α-galactosidase from coffee beans had a sharp peak at the optimum pH level, in contrast to that from A. niger (Figure 12).

Table 8. Thermal stability of α-galactosidase from coffee beans during incubation for 24 hours at 25°C and 45°C. A_{340}* of a system containing 1 mL of substrate solution (10 mM melibiose or raffinose or stachyose) and 10 µL of enzyme solution (1 unit per mL of solution), after 6, 12, 18, 24 hours of reaction.

Substrate	Incubation at 25°C Reaction time (hours)				Incubation at 45°C Reaction time (hours)			
	6	12	18	24	6	12	18	24
Melibiose	3.8	6.9	7.0	7.5	4.2	7.6	8.1	8.4
Raffinose	6.2	7.5	8.0	9.0	6.9	8.3	9.0	9.9
Stachyose	2.9	4.1	5.0	5.8	3.3	5.0	5.6	6.0

*A_{340} of NADH is a measure of galactose determined enzymatically. The A_{340} values represent the original absorbance readings multiplied by a dilultion factor of 10.02.

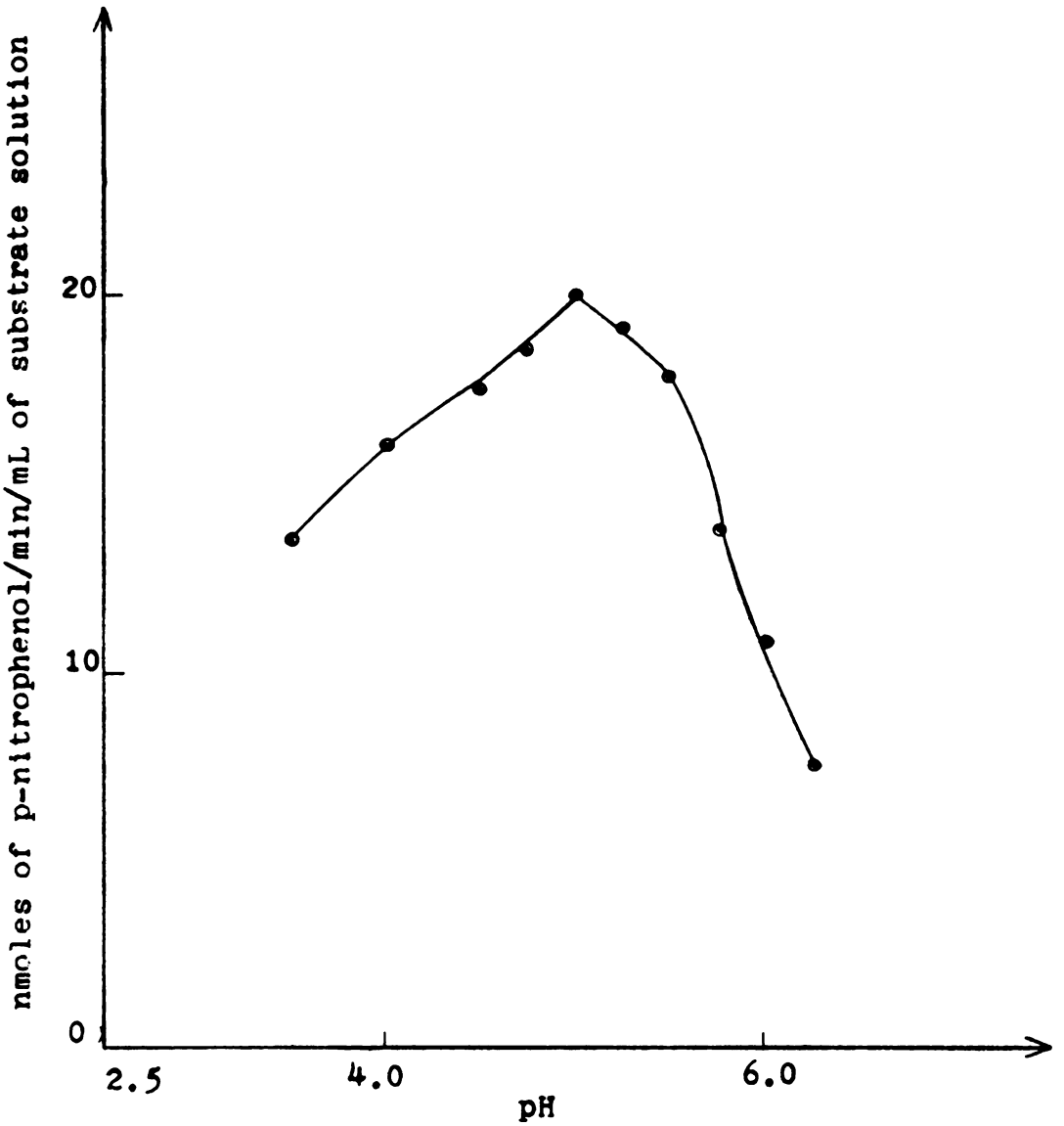


Figure 25. pH-activity relationship for α-galactosidase from coffee beans, with PNPG as substrate at 50°C. Reaction mixture: 10 mL acetate buffer, 0.5 mL enzyme solution (0.5 units/mL), 0.2 mL substrate solution (10 mM PNPG).

Reaction Rates

Figures 26 and 27 represent graphically the hydrolysis on PNPG and the oligosaccharides (melibiose, raffinose, stachyose). When PNPG is the substrate, the hydrolysis reaction plateaus after approximately 30 minutes of incubation, under optimum conditions of temperature and pH. When the oligosaccharides are the substrates, the reaction of their hydrolysis levels off after approximately 24 hours of incubation. Raffinose seems to be the substrate of preference, while stachyose was hydrolyzed at the slowest rate.

Phenomena of substrate inhibition were also observed with this enzyme when PNPG at a concentration above 2.0 mM was used in a reaction mixture containing 0.005 enzyme units/mL. The inhibition was manifested in terms of slower reaction rates. Similar inhibition phenomena were observed, when melibiose was the substrate, at concentrations above 70 mM (0.02 enzyme units/mL).

Km and Vmax Values

Figures 28 through 35 show the Km and Vmax values along with the Lineweaver-Burk double reciprocal and Eadie-Hofstee reciprocal plots. The values of the kinetic constants were determined as those for the α-galactosidase from A. niger. The substrate preference of this enzyme for raffinose was indicated by its Vmax value for this substrate,

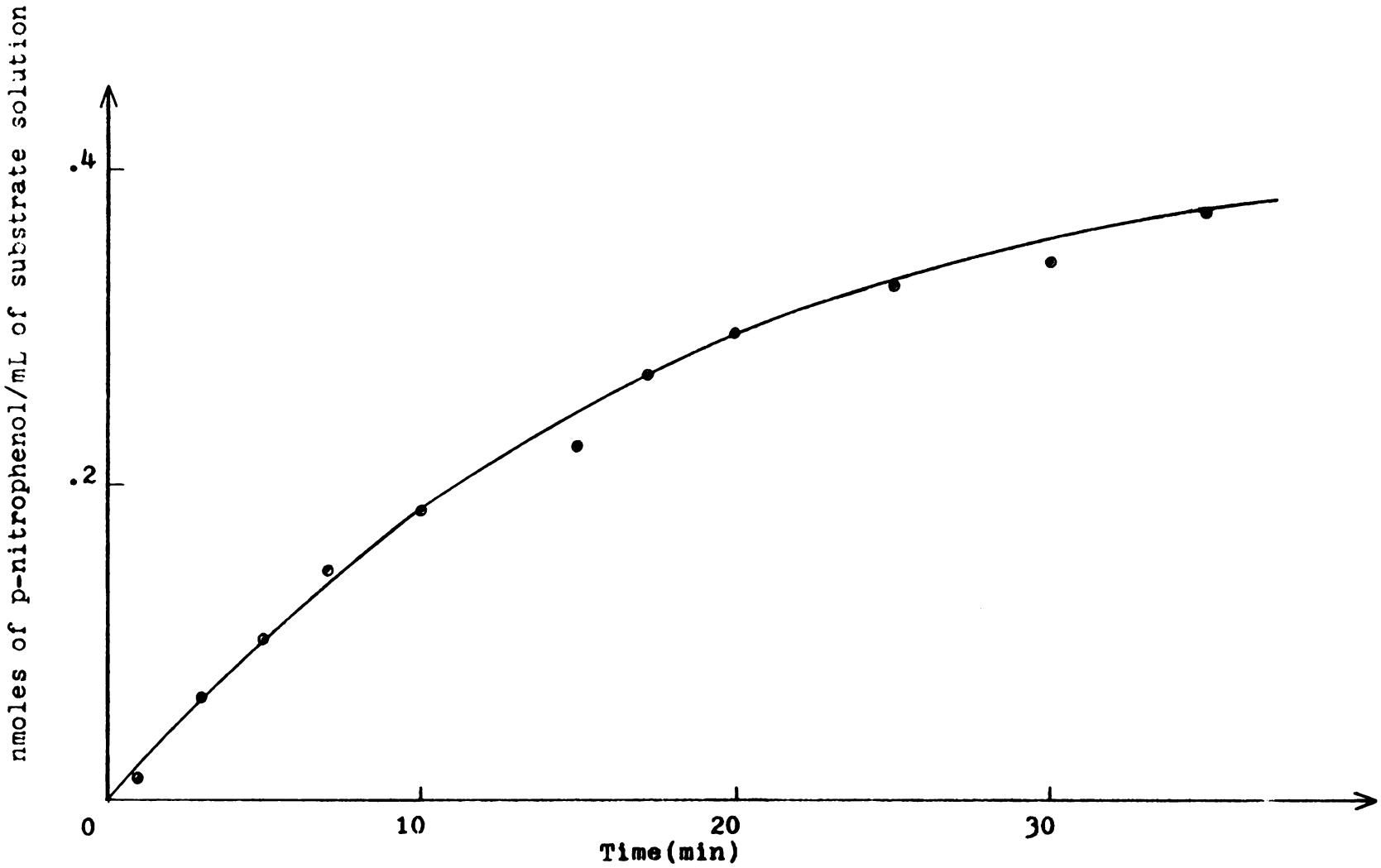


Figure 26. Hydrolysis of PNPG by α-galactosidase from coffee beans. Reaction mix: 1 mL substrate solution (1 mM PNPG) 10 µL enzyme solution (1 unit/mL).

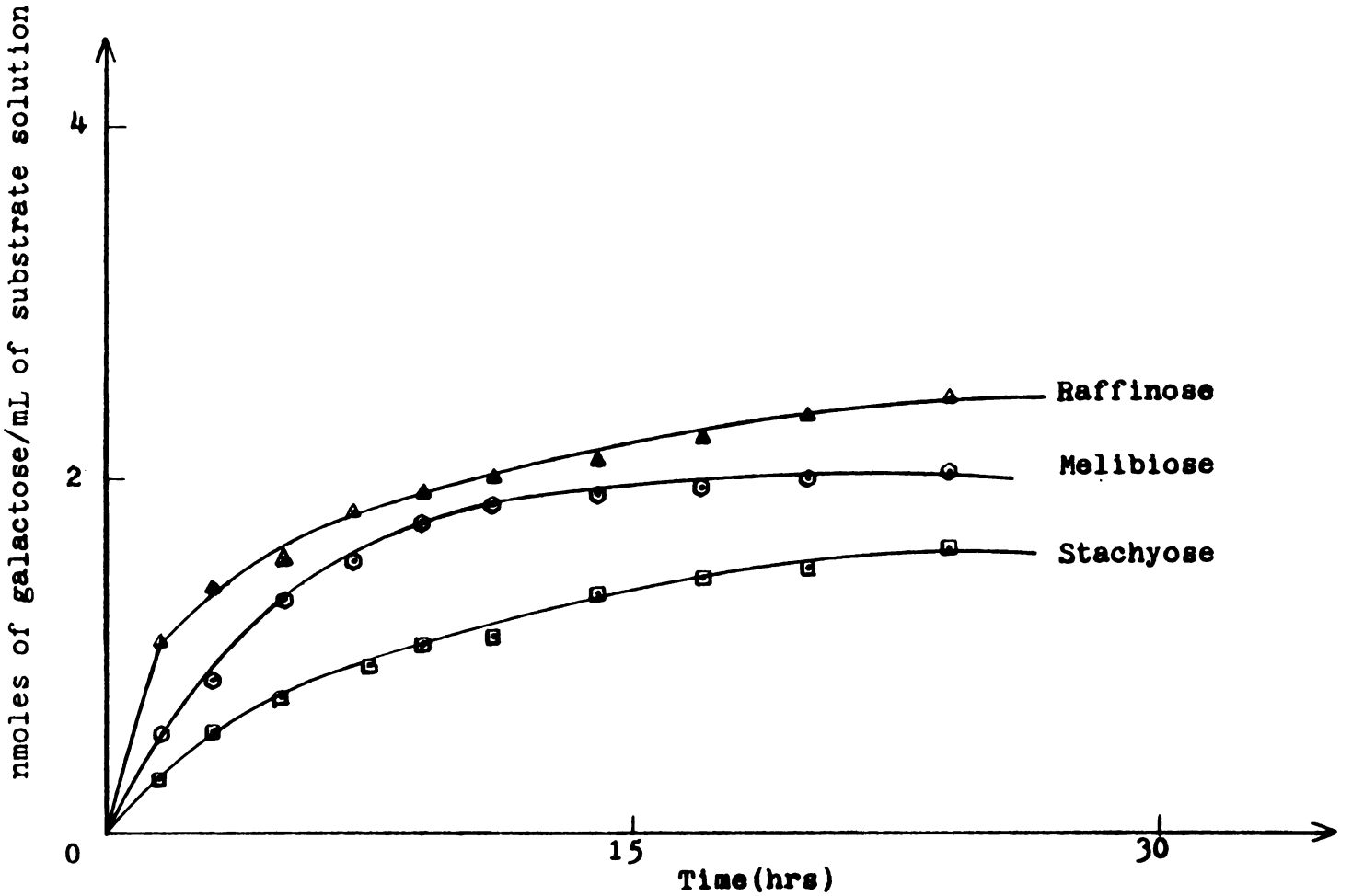


Figure 27. Hydrolysis of melibiose, raffinose, stachyose by α-galactosidase from coffee beans. Reaction mixture: 1 mL substrate solution (10 mM oligosaccharide), 20 μL enzyme solution (1 unit/mL).

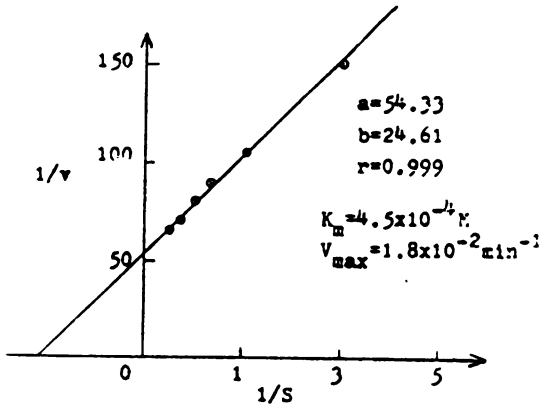


Figure 28. Lineweaver-Burk double reciprocal plot of α-galactosidase from coffee beans for PNPG. Reaction mixture: 1 mL substrate solution, 5 µL enzyme solution (1 unit/mL).

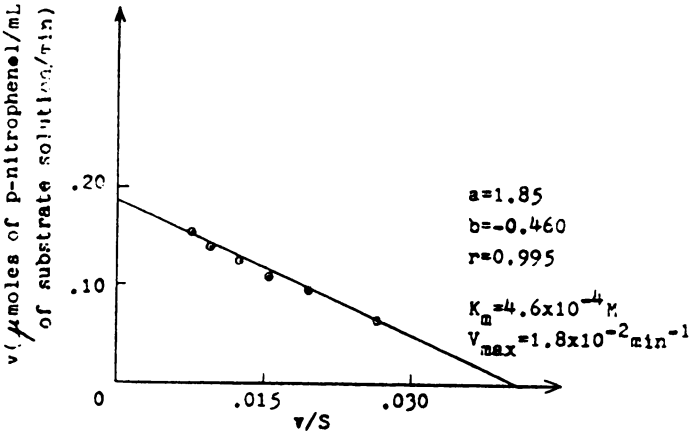


Figure 29. Eadie-Hofstee reciprocal plot of coffee bean α-galactosidase for PNPG. Reaction mixture: 1 mL substrate solution, 5 µL enzyme solution (1 unit/mL).

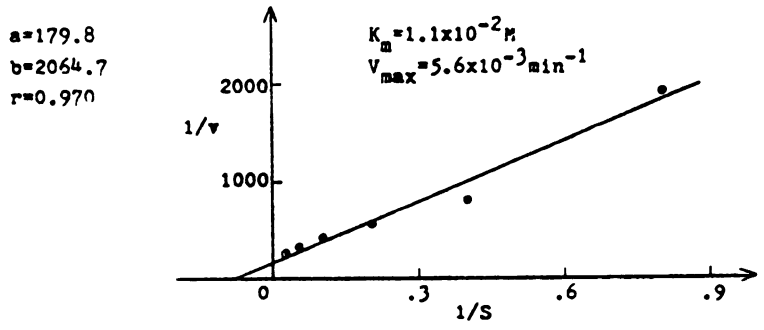


Figure 30. Lineweaver-Burk double reciprocal plot of coffee bean α-galactosidase for melibiose. Reaction mixture: 1 mL substrate solution, 5 µL enzyme solution (1 unit/mL).

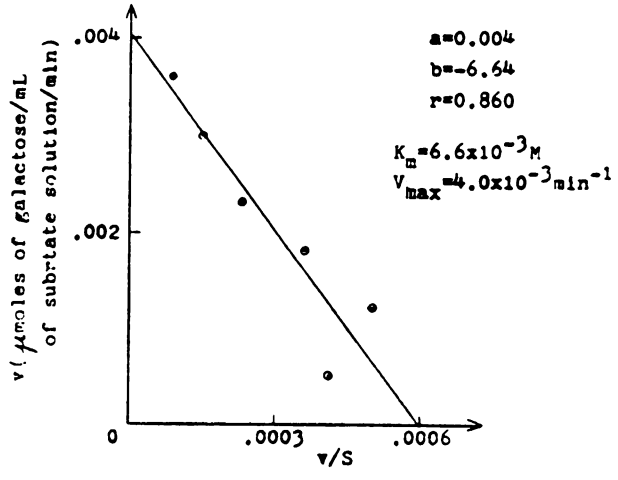


Figure 31. Eadie-Hofstee reciprocal plot of coffee bean α-galactosidase for melibiose. Reaction mixture: 1 mL substrate solution, 5 µL enzyme solution (1 unit/mL).

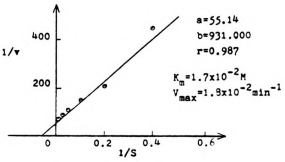


Figure 32. Lineweaver-Burk double reciprocal plot of α-galactosidase of coffee beans for raffinose. Reaction mixture: 1 mL substrate solution, 5 μL enzyme solution (1 unit/mL).

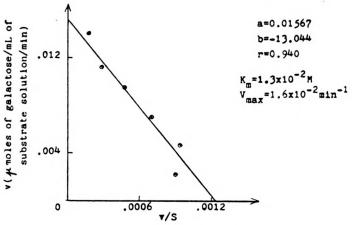


Figure 33. Eadie-Hofstee reciprocal plot of coffee bean α-galactosidase for raffinose. Reaction mixture: 1 mL substrate solution, 5 μL enzyme solution (1 unit/mL).

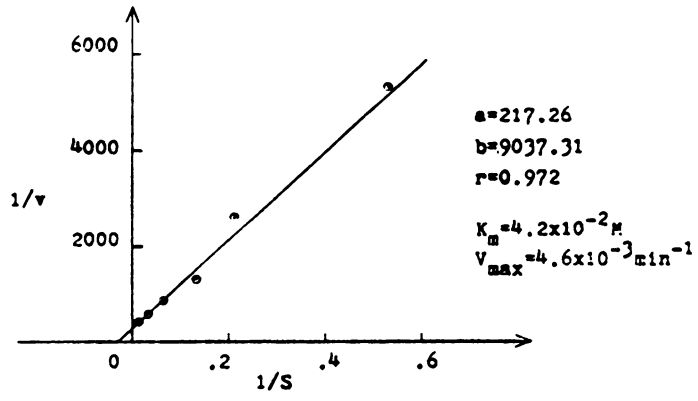


Figure 34. Lineweaver-Burk double reciprocal plot of coffee bean α-galactosidase for stachyose. Reaction mixture: 1 mL substrate solution, 5 μL enzyme solution (1 unit/mL).

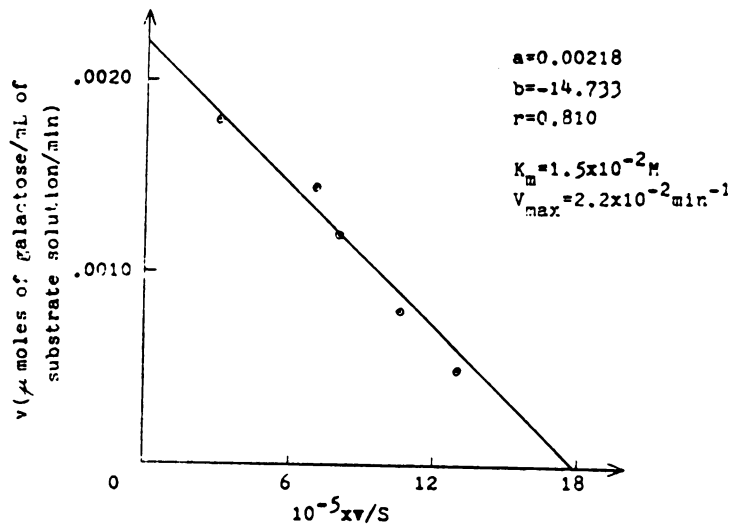


Figure 35. Eadie-Hofstee reciprocal plot of coffee bean α-galactosidase for stachyose. Reaction mixture: 1 mL substrate solution, 5 μL enzyme solution (1 unit/mL).

which had the highest value among those for the three oligosaccharides. The enzyme also demonstrated a substrate preference for the synthetic compound PNPG. The Vmax values for PNPG and raffinose were similar. The enzyme affinities (1/Km) for these substrates varied (Table 9).

Inhibition

Ag^{+} and Hg^{2+} caused inhibition, when they were added at low concentrations into solutions of α-galactosidase from coffee beans. Hg^{2+} caused stronger inhibition than Ag^{+} and the addition of KI was less effective in restoring the activity than in the case of α-galactosidase from A. niger. Addition of KI restored the coffee bean enzyme activity by 90% when Ag^{+} was used as inhibitor, and to 85% when Hg^{+} was the inhibitor. Table 10 indicates some inhibitors of the coffee bean α-galactosidase, their final concentration and % inhibition.

α-Galactosidase from E. coli

Stability, Optimum Reaction Temperature, Reaction Rates

The α-galactosidase from E. coli was a very unstable enzyme. Solutions in 0.01 M phosphate buffer, pH 7.0 containing 1 unit of enzyme per mL, kept under refrigeration at 2-3°C lost in 24 hours 45% of the original activity. In 48 hours 70% of the activity was gone and in 72 hours 80%. Due to this instability the absorbance readings during the

Table 9. Kinetic constants of α-galactosidase from coffee beans for 4 substrates determined by the Lineweaver-Burk (L-B) and Eadie-Hofstee (E-H) methods.

Substrate	Km (moles)		Vmax (moles/mL of substrate/min)	
	L-B	E-H	L-B	E-H
PNPG	4.5×10^{-4}	4.6×10^{-4}	1.8×10^{-2}	1.8×10^{-2}
Melibiose	1.1×10^{-2}	6.6×10^{-3}	5.6×10^{-3}	4.0×10^{-3}
Raffinose	1.7×10^{-2}	1.3×10^{-2}	1.8×10^{-2}	1.6×10^{-2}
Stachyose	4.2×10^{-2}	1.5×10^{-2}	4.6×10^{-3}	2.2×10^{-3}

Table 10. Inhibitors of α-galactosidase from coffee beans.

Inhibitor	Amount of inhibitor in the reaction mixture (moles)	% Inhibition
$AgNO_3$	5.0×10^{-8}	85 (15 min)
$AgNO_3$	5.0×10^{-8}	100 (60 min)
$HgCl_2$	2.0×10^{-8}	95 (15 min)
EDTA	2.0×10^{-3}	0
Galactose	1.2×10^{-2}	95

Reaction mixture: 1 mL substrate solution (0.75 mM PNPG), 5 μL enzyme solution, inhibitor.

various spectrophotometric measurements from experiments performed under identical conditions were different.

The addition of NAD^+ and Mn^+ and/or mercaptoethanol neither improved the enzyme stability nor optimized its activity. Enzyme solutions kept in the freezer for 2 1/2 months retained 90% of their initial activity. Figure 36 represents the relation between the temperature and the enzyme activity, expressed as nmoles of liberated p-nitrophenol/min/mL reaction mixture. This α-galactosidase demonstrated maximum activity at 45°C, during the hydrolysis of PNPG. Between 45°C and 50°C the enzyme exhibited 90% to 100% of its maximum activity. At 60°C only 10% of its activity remained, while the other two α-galactosidases, from A. niger and coffee beans, retained 94% and 80% of their activity, respectively.

Although incubation of PNPG with this enzyme at 45°C for 10 min indicated a very high enzymatic activity, the enzyme lost a substantial amount of its activity when it was incubated at that temperature for longer periods. This sensitivity of the enzyme was demonstrated by data taken during prolonged incubation periods for the hydrolysis of the oligosaccharides. Thermal stability experiments with two identical mixtures, one with melibiose and another with raffinose as substrates, were conducted at two different temperatures, 45°C and 30°C. The results showed that for melibiose the ratio of the reaction rates at 45°C

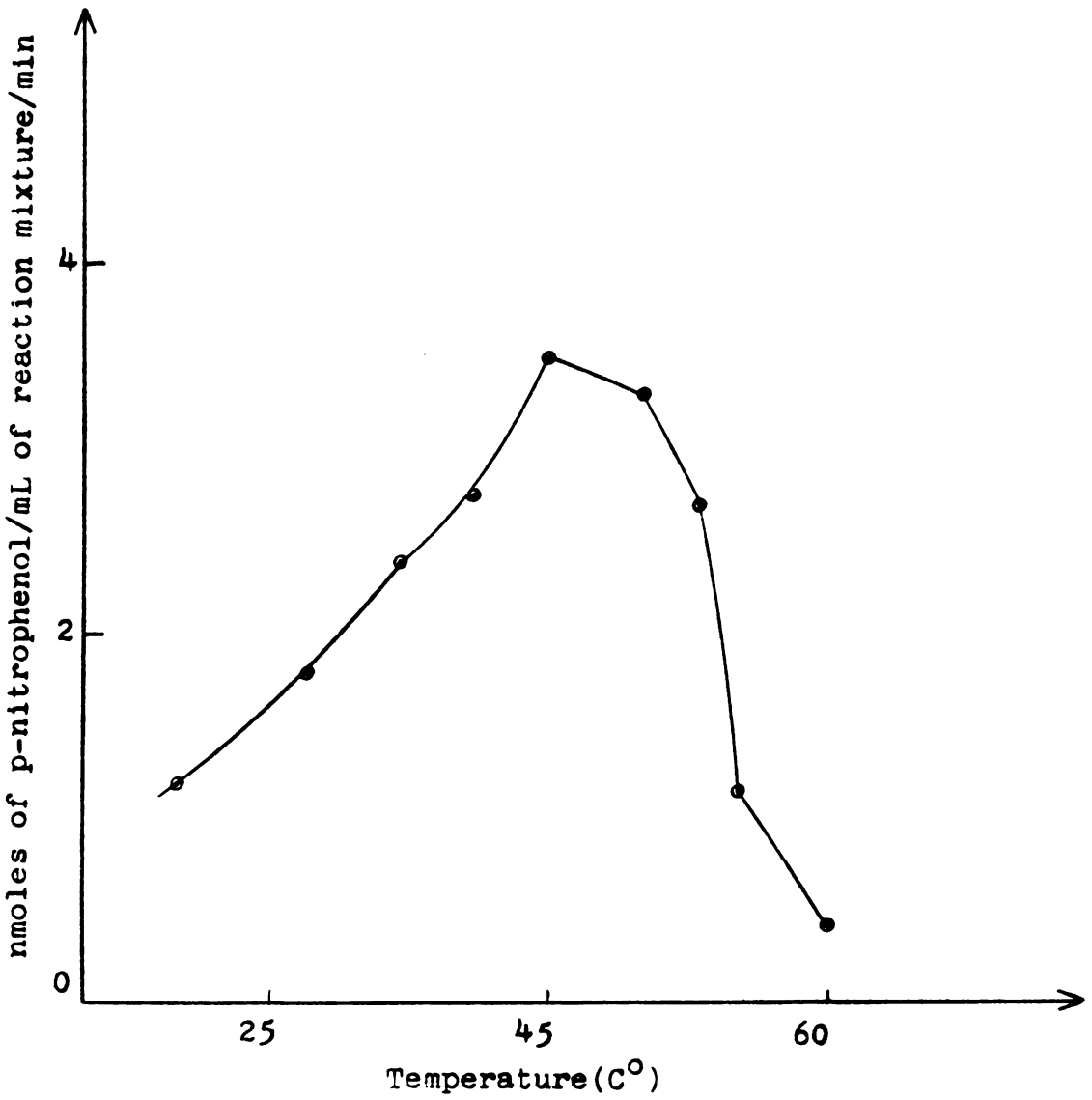


Figure 36. Temperature-activity relationship of α-galactosidase from E. coli, with PNPG as substrate, at pH 6.5. Reaction mixture: 1 mL phosphate buffer, 100 µL substrate solution (5 mM PNPG), 5 µL enzyme solution (1 unit of enzyme/mL solution).

to that at 25°C was 1:2 and for raffinose 1:4. Raffinose was hydrolyzed more slowly than melibiose. The latter was the substrate of preference for this enzyme. Stachyose was hydrolyzed much more slowly, compared to the other two oligosaccharides. This is clearly indicated by Figure 39, where the hydrolysis rates of the three oligosaccharides were plotted vs. the reaction times. Raffinose at concentrations above 80 mM demonstrated the same inhibition phenomena, as melibiose did with the coffee beans α-galactosidase (the enzyme concentration in the reaction mixture was 0.005 units).

Substrate inhibition phenomena were observed with PNPG as substrate at concentrations higher than 2 mM. The rate of hydrolysis of PNPG plateaued after 30 minutes (Figure 38) while that of melibiose after about 12 hours, of raffinose after 18 hours, and of stachyose after about 10 hours (Figure 39).

Optimum Reaction pH

Figure 37 shows how the enzyme activity changes at various pH levels. It is apparent, that at those pH levels, where the previously examined enzymes demonstrated maximum activity (pH between 4.5 and 5.0) this enzyme does not have any activity at all.

Since phosphate buffers do not have good buffering capacity at pH levels below 6.0, a series of acetate buffers

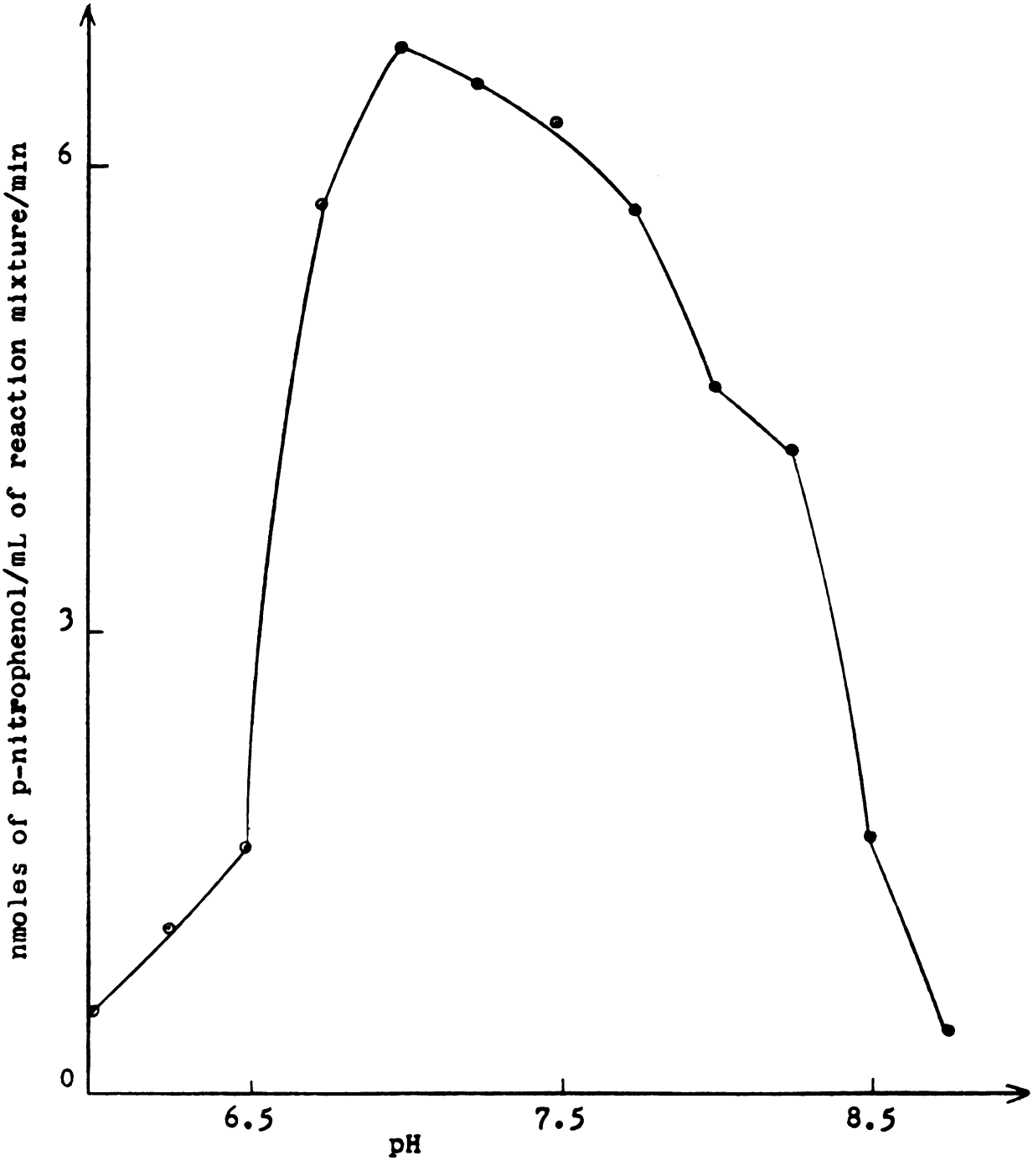


Figure 37. pH-activity relationship of the α-galactosidase from E. coli, under optimum temperature (45°C). Reaction mixture: 1 mL phosphate buffer, 100 µL substrate solution (5 mM PNPG), 5 µL enzyme solution (1 unit/mL).

was prepared to cover the pH range from 5.00 through 5.75 during the pH experiments.

The difference of ionic species in the buffers did not affect the enzyme activity. At pH of 6.75 through 7.75 the enzyme activity shows a sharp increase and then it decreases slowly, as the pH of the reaction mixture becomes more alkaline.

It is obvious that this enzyme favors slightly alkaline media. The optimum pH is 7.0. At pH 8.5 the enzyme has almost the same activity as at pH of 6.5. A change of the pH by 0.5, in the acidic range, below the optimum level, decreased the enzyme activity as much as a change of the pH by 1.5 above the optimum level, in the alkaline range.

The behavior of the enzyme at the various pH levels is compatible with the fact that it was derived from bacteria. Most bacteria can not tolerate acidic environments. In fact the optimum pH for the growth of E. coli is 7.0.

Km and Vmax Values

Table 11 shows that the kinetic constants of this enzyme also change independently, between the four substrates. The Vmax for melibiose had the highest value and that of stachyose had the lowest. The Km values for these substrates were similar. Figures 40 to 47 show the Lineweaver-Burk and Eadie-Hofstee plots pertaining to the E. coli α-galactosidase.

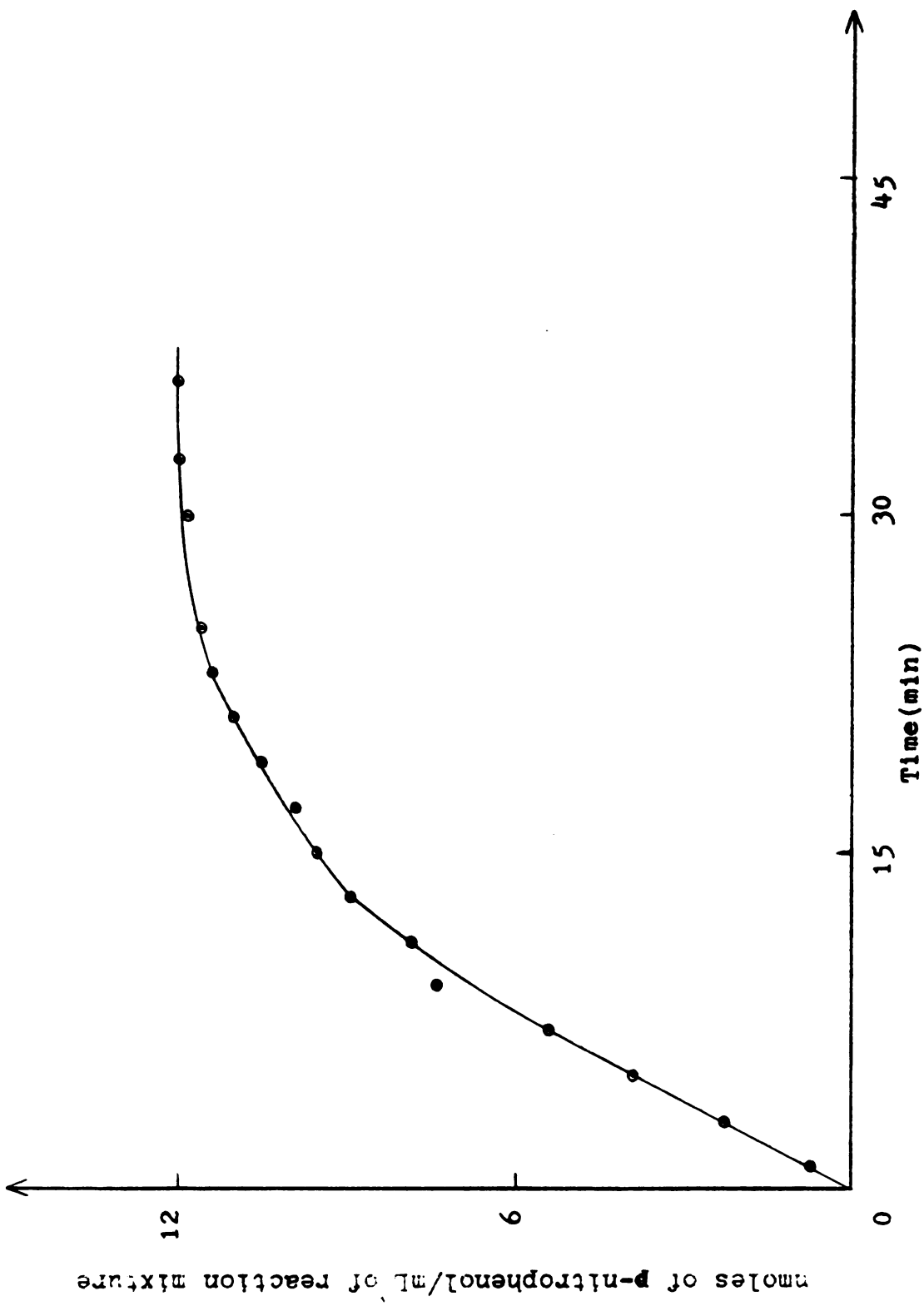


Figure 38. Hydrolysis of PNPG by α-galactosidase from E. coli, at optimum conditions of pH and temperature. Reaction mixture: 1 mL phosphate buffer, 100 µL substrate solution (5 mM PNPG), 5 µL enzyme solution (1 unit/mL).

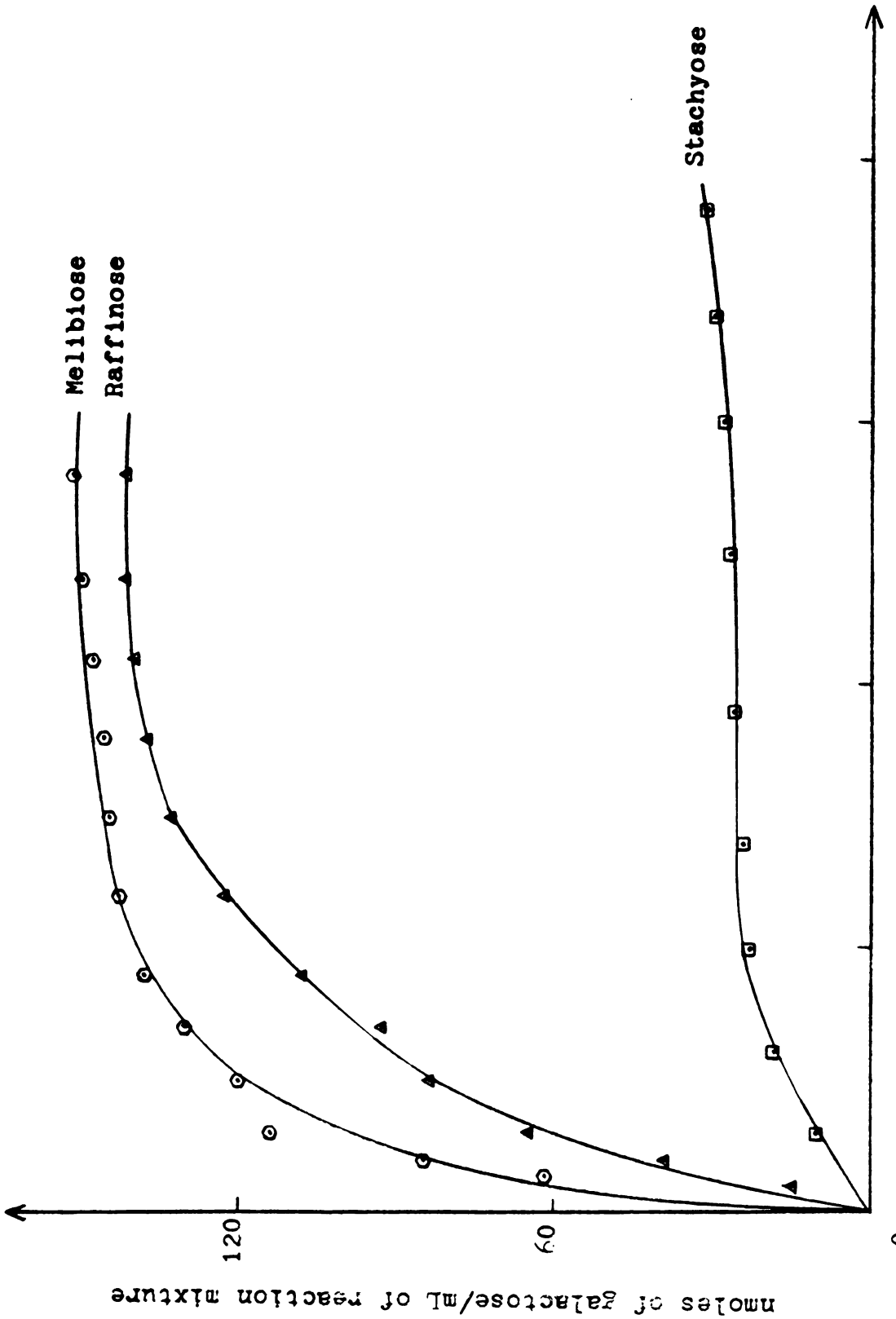


Figure 39. Hydrolysis of melibiose, raffinose, stachyose by α-galactosidase from E. coli at optimum conditions of temperature and pH. Reaction mixture: 1 mL substrate solution (10 mM oligosaccharide), 25 µL enzyme solution (1 unit/mL).

Table 11. Kinetic constants of α-galactosidase from E. coli, determined by the Lineweaver-Burk (L-B) and Eadie-Hofstee (E-H) methods.

Substrate	K_m,M		Vmax (moles/mL of substrate/min)	
	L-B	E-H	L-B	E-H
PNPG	2.8×10^{-4}	2.6×10^{-4}	3.3×10^{-3}	3.2×10^{-3}
Melibiose	2.1×10^{-2}	1.9×10^{-2}	3.1×10^{-2}	3.0×10^{-2}
Raffinose	1.2×10^{-2}	1.2×10^{-2}	9.5×10^{-3}	9.5×10^{-3}
Stachyose	2.1×10^{-2}	1.3×10^{-2}	2.6×10^{-3}	1.9×10^{-3}

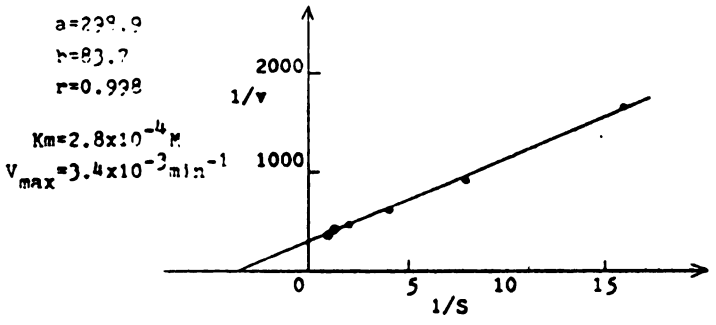


Figure 40. Lineweaver-Burk double reciprocal plot of α-galactosidase from E. coli for PNPG. Reaction mixture: 1 mL substrate solution, 25 µL enzyme solution (1 unit/mL).

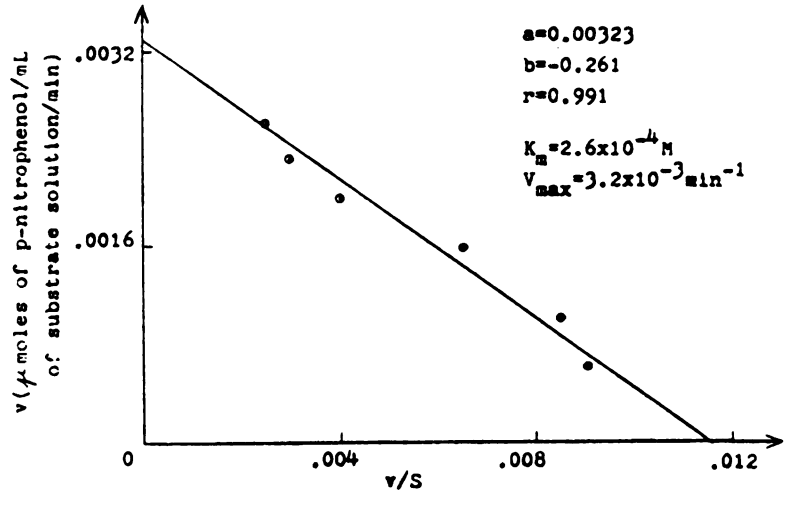


Figure 41. Eadie-Hofstee reciprocal plot of α-galactosidase from E. coli for PNPG. Reaction mix: 1 mL substrate solution, 25 µL enzyme solution (1 unit/mL).

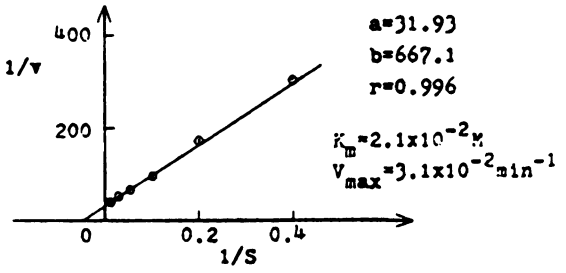


Figure 42. Lineweaver-Burk double reciprocal plot of α-galactosidase from E. coli for melibiose. Reaction mixture: 1 mL substrate solution, 25 µL enzyme solution (1 unit/mL).

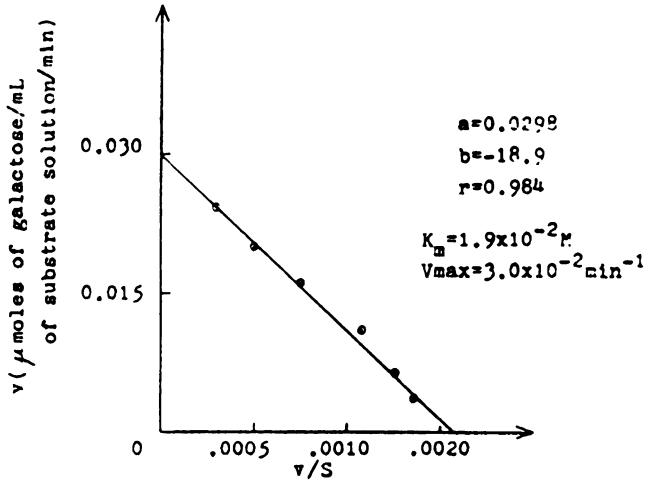


Figure 43. Eadie-Hofstee reciprocal plot of α-galactosidase from E. coli for melibiose. Reaction mixture: 1 mL substrate solution, 25 µL enzyme solution (1 unit/mL).

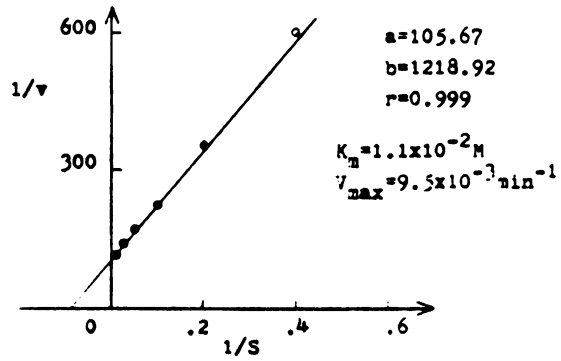


Figure 44. Lineweaver-Burk double reciprocal plot of α-galactosidase from E. coli, for raffinose. Reaction mixture: 1 mL substrate solution, 25 µL enzyme solution (1 unit/mL).

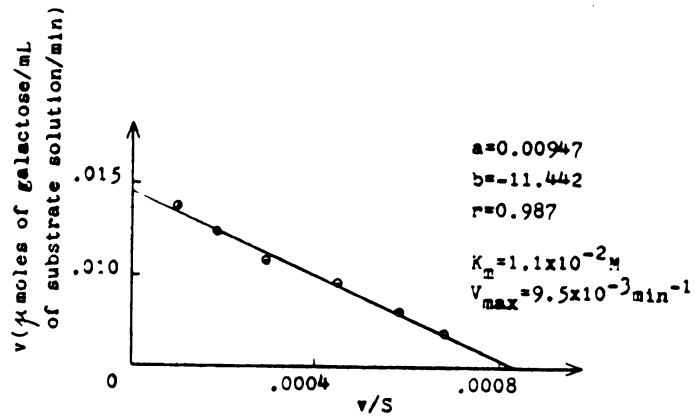


Figure 45. Eadie-Hofstee reciprocal plot of α-galactosidase from E. coli for raffinose. Reaction mixture: 1 mL substrate solution, 25 µL enzyme solution (1 unit/mL).

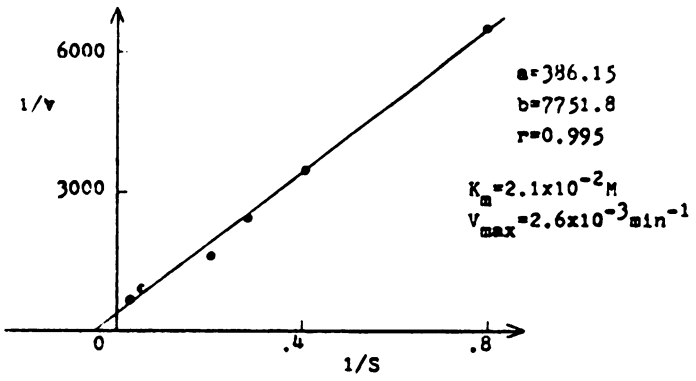


Figure 46. Lineweaver-Burk double reciprocal plot of α-galactosidase from E. coli for stachyose. Reaction mixture: 1 mL of substrate solution, 25 µL enzyme solution (1 unit/mL).

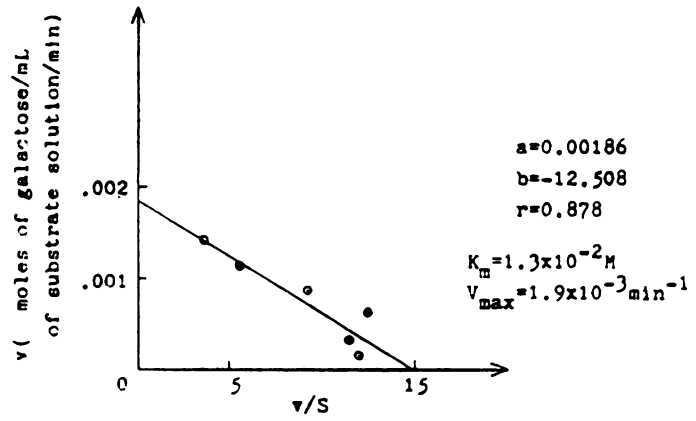


Figure 47. Eadie-Hofstee reciprocal plot of α-galactosidase from E. coli for stachyose. Reaction mixture: 1 mL substrate solution, 25 µL enzyme solution (1 unit/mL).

On some Eadie-Hofstee plots, for the three enzymes, particularly those of stachyose, a curvature can be noticed between the points. This could be due to the presence of more than one α-galactosidases, in the enzymic preparations.

Inhibition

Few experiments were conducted, to study the inhibitory action of various compounds on the activity of the α-galactosidase from E. coli, using PNPG as substrate (Table 12). The addition of KI into enzyme solutions, inactivated by Ag^{+} and Hg^{++} did not restore the activity. On the contrary, KI added to active enzyme solutions caused inhibition (Table 12).

The behavior of the α-galactosidase from E. coli during the addition of KI, along with its optimum pH represent some of the major differences between this enzyme and those from A. niger and coffee beans.

Table 12. Inhibitors of α-galactosidase from E. coli, their amounts in the reaction mixtures (moles) and caused inhibition, expressed as % of control activity.

Inhibitors	Amount (moles)	% Inhibition
KI	2.5×10^{-6}	98
$HgCl_2$	1.0×10^{-7}	100
$AgNO_3$	2.5×10^{-6}	100
EDTA	2.5×10^{-5}	0
Galactose	1.5×10^{-6}	95

Reaction mix: 1 mL phosphate buffer, 50 µL substrate solution (5 mM PNPG), 0.25 mL enzyme solution (1 unit/mL), inhibitor.

CONCLUSIONS

The α-galactosidases from A. niger and coffee beans demonstrated a resemblance in their behavior, when their activity was tested at various pH levels and temperatures.

They both seemed to be quite thermostable and tolerant to acidic environments. Their kinetic constants indicated that both hydrolyzed raffinose at a faster rate than the other two oligosaccharides, under optimal conditions of temperature and pH. Stachyose was hydrolyzed faster by the α-galactosidase from coffee beans than the enzyme of the other sources.

Stability tests showed that solutions of both enzymes in buffers, with proper pH, maintained a substantial amount of their activity, when they were kept under refrigeration and even more under freezing.

Inhibition studies indicated that both enzymes are inhibited by heavy metals. Their activity was restored by the addition of proper amounts of KI into the reaction mixtures. Due to their stability and hydrolytic characteristics, these α-galactosidases could be successfully employed in various commercial applications.

The α-galactosidase from E. coli showed different behavior from that of the other two enzymes. This enzyme

was very unstable (particularly in solutions). It was more active in slightly alkaline environments. During the kinetic studies of this α-galactosidase a strong substrate preference for melibiose was observed. The hydrolysis rate of stachyose was very slow, compared to the other two α-galactosidases. When this enzyme was inactivated by heavy metals, KI did not restore its activity. The curvature noticed in some of the Eadie-Hofstee plots may indicate the simultaneous action of two enzymes on the substrate.

The enzymatic characteristics of the α-galactosidases from A. niger, coffee beans and E. coli are summarized on Tables 13, 14 and 15. The indicated Km and Vmax values are those determined by the Lineweaver-Burk method.

Table 13. Summary of the optima temperatures, pH of α-galactosidases from A. niger, coffee beans and E. coli.

Enzyme	Opt. Temp. (°C)	Opt. pH
A. niger	52	5.0
Coffee beans	50	5.0
E. coli	45	7.0

Table 14. Summary of the Km values of α-galactosidases from A. niger, coffee beans and E. coli (M).

Enzyme	PNPG	Melibiose	Raffinose	Stachyose
A. niger	6.7×10^{-4}	6.2×10^{-3}	3.8×10^{-2}	2.9×10^{-2}
Coffee beans	4.5×10^{-4}	1.1×10^{-2}	1.7×10^{-2}	4.2×10^{-2}
E. coli	2.8×10^{-4}	2.1×10^{-2}	1.2×10^{-2}	2.1×10^{-2}

Table 15. Summary of the Vmax values of α-galactosidases from *A. niger*, coffee beans and *E. coli*, in min^{-1}.

Enzyme	PNPG	Melibiose	Raffinose	Stachyose
A. niger	3.1×10^{-2}	1.6×10^{-2}	5.2×10^{-2}	1.4×10^{-2}
Coffee beans	1.8×10^{-2}	5.6×10^{-3}	1.8×10^{-2}	4.6×10^{-3}
E. coli	3.3×10^{-3}	3.1×10^{-2}	9.5×10^{-3}	2.6×10^{-3}

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