COMPARATIVE BIOLOGICAL STUDIES OF AMYLASE FROM MALTED SORGHUM (SORGHUM VULGARE) AND PEARL MILLET (PENNISETUM TYPHOIDES) AND MICROORGANISMS

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DECLARATION

I hereby declare that this thesis is a record of my own research and has not been submitted for the award of a degree at any other university. Informations and excerpts from both published and unpublished work of others have been acknowledged in the text.

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CERTIFICATION

This thesis entitled "Comparative Biochemical Studies of α-Amylase from Malted Sorghum and Millet and Microorganisms" by Chukwujekwu, Bernard Nnamdi meets the regulations governing the award of the degree of M.Sc. Biochemistry of Ahmadu Bello University, and is approved for it's contribution to knowledge and literary presentation.

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DEDICATION

To Ogbuiji and Mrs Akwuo Ezeabali Chukujekwe for your avowed goals, my brothers and sisters for the love and prayers and to God for guidance.

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ABSTRACT

α-Amylase is of great importance to the brewing industry because of it's vital role in the modification of the grain and in mash saccharification. The α-amylase of sorghum and millet malt were purified to apparent homogeneity by a combination of charcoal and heat treatment, ammonium sulphate precipitation and sephadex G150-120 gel chromatography. Polyacrylamide gel electrophoresis of the partially purified enzymes revealed single protein bands.

The effect of pH and temperature on the hydrolysis of gelatinised starch by bacterial, fungal, malted sorghum and millet α-amylases gave 6.0, 4.6-4.8, 5.0-5.5 and 4.5, and 70-75°C, 50°C, 60°C and 65°C as the pH and temperature optima respectively. All the enzyme preparations retained full activity after a 30 minute-heat treatment at 50°C. The calculated energy of activation per mole of the bacterial, fungal, sorghum, and millet α-amylases were 6.57kcal, 5.4kcal, 10.05kcal and 7.43kcal between 20° and 50° while molecular weight determined on SDS-gel electrophoresis gave 48,991 ± 1595, 47,140 ± 1022, 52,666 ± 1114, and 46,698 ± 397 daltons respectively.

Of the ions tested on the enzyme activity, Cu^{2+} was a potent inhibitor of the cereal α-amylases whereas Fe^{3+}, Al^{3+} and Zn^{2+} were mildly inhibitory. Cl^- had no effect on the activity of the cereal and fungal enzymes but it stimulated the bacterial α-

amylase. While Cu^{2+}, Fe^{3+} and Al^{3+} were potent inhibitors of the fungal α-amylase, they midly inhibited the bacterial α-amylase. Mg^{2+} and Zn^{2+} were without effect on both microbial enzymes. When stored at freezer, refridgerator and room temperatures for 28 days, all the enzyme preparations were very stable at frozen state but very unstable at room temperature except for the fungal α-amylase that retained over 50% of initial activity. The Km values for the bacterial, fungal, malted sorgium and millet α-amylases were 2.10mg, 3.55mg, 0.156mg and 1.56mg starch/ml respectively..

From the effect of pH on activity of bacterial, fungal, malted sorghum and millet α-amylases, groups with pK values of about 4.7 and 7.3; 3.8 and 5.6; 3.5 and 7.1; and 3.6 and 5.5 respectively were identified as forming the catalytic site. An investigation of the group with pK 7.3, 5.6, 7.1 and 5.5 showed that N-Bromosuccinimide inhibited enzyme activity, p-chloromercuribenzoate had little or no effect while acetic anhydride lowered enzyme activity.

Although much attention is directed at sorghum grain presently, as the probable substitute to barley, there is the need for intensive study of the millet grain as it's α-amylase has been found to possess some desired brewing properties.

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CHAPTER ONE

INTRODUCTION

Cereal grains are the most important source of food for man and farm animals (Chukwura, 1989). Notable among these are wheat, barley, maize, sorghum, millet, rye, rice and oat. Sorghum (Sorghum bicolor (L) Moench or Sorghum Vulgare) and Pearl Millet (Pennisetum americanum (L) Leeke or Pennisetum typhoides (Burm.) Stapf or C.E. Hubbard) are grown extensively for human consumption in India and Africa especially in semi arid areas (Belaia and Varriano-Marston, 1981).

In Nigeria, the strong drought resistant nature of millet endears it to the people living in regions bordering the sahara desert. This is because millet grows well in areas too dry for sorghum and other cereals to thrive thereby assuring the farmers of adequate yield under conditions considered to be unfavourable (Oyenuga, 1968; Obilana, 1981).

Africans traditionally, employed sorghum and millet in both malted and unmalted form. However, most of their use as food involves a steeping or fermentation period prior to cooking (Novellie, 1977).

In the brewing industry, cereal grains form the primary raw material for beer making. The cereal, barley, is the traditional brewhouse raw material especially in Europe and America. This eminent position occupied by barley as the prime ingredient in beer production is due to, among others, (a) the ease with which

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barley germinates, (b) the presence of husk which facilitates filtration of wort, (c) presence of appropriate flavour and colour precursors, and (d) the provision of water soluble components suitable for yeast nutrition (Prentice et al, 1971).

The introduction of the structural adjustment programme (SAP) and the ban on importation of barley malt by the Federal government in January 1988, threw a most awesome challenge to the brewing industries. This situation provided the much needed impetus for a change in the pattern of raw material utilization. Breweries, then adopted sorghum grain, for beer production. The adoption of sorghum for brewing was based on the successful use of malted sorghum for the production of a South African beer, "Kaffir beer" or Sorghum beer (Novellie, 1977), the promising results from experimental sorghum brewing trial (Okafor and Aniche, 1980), the well established use of sorghum as beer adjunct in the United States and Mexico (Aisien, 1988), and the popular use of sorghum in the Northern part of Nigeria for the production of local alcoholic beverages - "Burukutu" and "Pito" (Arri, 1989). Although much attention has been paid to sorghum grains, millet grains have been reported to be capable of producing malt whose diastatic power is very much comparable to that of sorghum malt (Novellie, 1977). However, the small size of the millet grains poses serious technical problems in large scale modern malting plant (Novellie, 1977).

Beer has been defined as a fermented, hopped, malt beverage (Prosarenz, 1975). In line with this definition, the three most

important ingredients for beer production are therefore malt, hops and yeast. The production of an adequately modified malt is achieved by the action of the amylases (α and β) among other enzymes.

The α-amylase is very important in both malting and brewing because, it is the major enzyme in cereal grains capable of degrading intact starch granules (MacGregor, 1980). Therefore an adequate level of α-amylase is a desired property of a good quality malt. Kernels of sound "resting" sorghum grain have little or no trace of α-amylase. As a result, the enzyme is formed mainly during malting (Novellie, 1977; MacGregor, 1980). Unlike sorghum grain, "dry" millet grain has been reported to possess α-amylase activity (Nwankwo and Gpokn, 1986).

The α-amylase of barley and barley malt have been well studied while those of sorghum and millet have received much less attention. It therefore becomes necessary to study the enzyme from these sources, especially those of our locally produced grains. This study was therefore embarked upon to establish:

(i) The similarities and differences in the kinetic properties of α-amylases from malted sorghum, millet, and barley and from micro-organisms.

(ii) The operational temperature stability of α-amylases from malted sorghum and millet, and from micro-organisms since high temperature operation is necessary for brewing especially with sorghum grains.

(iii) The effect of pH variation on the saccharogenic ability of

the α-amylases.

(iv) The molecular weight of α-amylase from these sources.

(v) the optimum condition for storage of α-amylase from these sources.

(vi) The functional groups involved in the catalysis of these α-amylases.

(vii) The affect of ions especially metallic ions on the activity of these α-amylases.

CHAPTER TWO

REVIEW OF LITERATURE

2.1 ORIGIN OF SORGHUM AND MILLET

Cereal crops were cultivated in many parts of the old world, some 10-15 thousand years before the christian era (Mann, 1946). The fact that old biblical accounts associated famines and food shortages with failure of the cereal crops supports the argument that cereals were one of the earliest domesticated plants (Leonard and Martin, 1963). It is generally believed that cultivated sorghum originated in East-central Africa (Leonard and Martin, 1963; Doggett, 1977; Obilana, 1985) from where it migrated westwards into West African region and became domesticated in the savanna and semi-arid areas (Obilana, 1985).

However, there are conflicting reports on the origin of millet. Many botanists consider Pennisetum to have originated in Africa and subsequently got introduced into India (Rachie and Majumdar, 1980), while others hold the view that pennisetum was first domesticated in India and later was transported to Africa (Rachie and Majumdar, 1980). Krishnaswamy (1951; 1962) studied the taxonomy and cytology of several Pennisetum species from Africa and other parts of the world and concluded that pearl millet originated in Africa.

2.2 PRODUCTION

Sorghum is the fifth most widely grown crop in the world

after barley, rice, maize and wheat (Palmer et al. 1989a). About 60 million metric tonnes of sorghum are produced per annum (FAO, 1990). This quantity on a world-scale, places sorghum like oats and rye as a minor cereal crop. However one potential character boosting its importance is its ability to grow well in the semi-arid regions of the world (Palmer et al. 1989a). The main producing countries include the United States of America (U.S.A), China, India, Argentina, Nigeria, Mexico and Australia. Nigeria produces about 5 million tonnes of sorghum annually (Palmer et al. 1989a; FAO, 1990). World-wide hectarage of all kinds of millet nearly equals that of sorghum, however, the yield is only one-half of sorghum grain production per hectare (Badi et al. 1976; FAO, 1990). The world's most important producers of millet are China, India, Nigeria, the USSR, Ethiopia, Niger, Chad, Mali and Tanzania (Rachie and Majmudar, 1980). The estimated annual production of millet in Nigeria is 3.5 million metric tons (FAO, 1990). Sorghum is the most important cereal crop grown in Nigeria accounting for about 50% of the total cereal production. It also occupies about 46% of the total land devoted to cereal production (Egharevba, 1979). Millet is second to sorghum in importance in Nigeria (Nwasike and Egharevba, 1981).

2.3 SOIL AND CLIMATIC CONDITIONS

Sorghum and millet can be grown in a wide range of soils and climates and are of special importance in the semi-arid regions of the world because of their short growing seasons (Schery,

1963). In Nigeria, sorghum and millet are grown in the savanna ecological zone located between latitude 8°N and 13°N (Obilana, 1981). This zone also supports the cultivation of other cereals such as rice, maize and wheat and cow peas with which sorghum and millet may be inter-cropped (Egberavba, 1979).

Millet is second to sorghum in importance in the sudan savanna areas but supersedes sorghum in the sahel savanna because of its stronger ability to withstand heat, draught and low soil fertility (Nwasike and Egberevba, 1981). These cereals are not affected by soil pH which is generally low in Africa (Novelile, 1977), and they also respond well to fertilization and irrigation even though they are draught resistant (Novelile, 1977; Egberevba, 1979). Abichandi and Bhatt (1965), investigating the effects of varying levels of soil salinity on some varieties of sorghum and millet, found that the pennisetum spp are generally more salt tolerant than sorghum.

2.4 USES OF SORGHUM AND MILLET

Presently, about 95% of the total harvested sorghum grains are consumed as human food, leaving about 5% for feed and industrial outlets (Aisien, 1989). Sorghum and millet consumption are mainly in the form of flour or paste. These grains are processed into "ogi" or "akamu" or "kamu", a thin porridge made from wet-milled fermented sorghum or millet grains and "tuwo" - a thick, molded or shaped porridge processed from dry-milled, non-fermented whole grain flour (Obilana, 1981.

akamu is the most common porridge eaten by Nigerians (from coast to the extreme northern boundaries of the country). Sorghum and millet akamu are very popular in the savanna where they are mostly grown whereas maize akamu is more popular in the southern forests and derived savanna. Fumo forms the major diet of the people of the northern parts of Nigeria (Obilana, 1981).

One of the major unfermented food drinks of sorghum and millet grains very popular among the people of northern Nigeria is "kunu" which is often spiced to produce different flavours (Obilana, 1985). Other dishes that are sometimes made from these grains include deep-fried snacks, (masa), steamed dumplings (eko or agidi) and "fura" which is often served with milk ("nono").

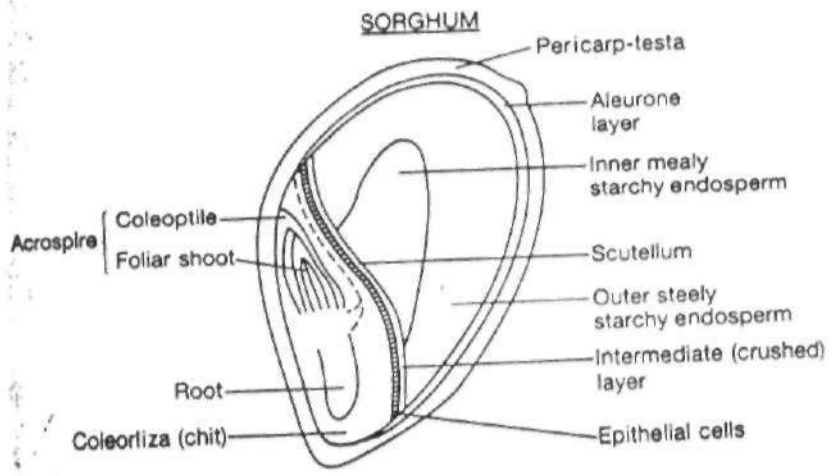
Locally, malted sorghum and millet grains are processed into two main indigenous alcoholic beverages - "burukutu" and "pito" (Obilana, 1985; Arti, 1989). These drinks are most popular among the people of the middle belt.

2.5 THE STRUCTURE OF THE GRAIN

Unlike malting barley that is endosperted, sorghum and millet are almost round. The millet grains are about one-third as large as the sorghum grain (Badi et al, 1976). The gross morphology of normal pearl millet kernel resembles that of sorghum kernel (Sullins and Rooney, 1976).

2.5.1 Pericarp-Testa

Sorghum and Millet grains do not possess husk. The pericarp-testa of these "naked" seeds contain small starch


SORGHUM
Pericarp-testa
Aleurone layer
Inner mealy starchy endosperm
Acrospire
Coleoptile
Foliar shoot
Scutellum
Outer steely starchy endosperm
Intermediate (crushed) layer
Root
Coleorliza (chit)
Epithelial cells

grannies and a high polyphenol pigment content (Hip and Burns, 1969, 1971; Palmer et al. 1989a). The pigment of some varieties are so unpalatable to some birds that they are now referred to as the "bird proof" varieties. These pigments especially the proanthocyanidins react with proteins causing them to precipitate in brewers wort or in beer (Bridal, 1986). In order to reduce this incidence of protein complexation, the tannins or polyphenols are adsorbed to compounds like polyvinylpolypyrrolidone (PVPP) (Palmer et al. 1989a) or activated decolourising charcoal (Bates et al. 1967).

2.5.2 Aleurone Layer

The aleurone layers of pearl millet and sorghum grains comprise a single layer of cells in contrast to the aleurone of barley which is usually three cells in thickness except over the scutellum where it is a single layer (Bedi et al. 1976; Sullins and Rooney, 1976; Palmer et al. 1989a). The aleurone cells of these grains contain storage lipids and proteins.

2.5.3 Embryo

Frequent references to the need to preserve whole grain sorghum and millet flours in airtight containers in order to minimise rancidity serves as a reminder of the high lipid content of these grains (Lorenz and Dilsaver, 1980). Most of these lipids are located in the scutellum of the embryo. Sorghum and Millet grains also contain B-group vitamins such as thiamin,

niacin and riboflavin (Opoku et al. 1981, Palmer et al. 1989a). These are thought to be located in the embryo (Palmer et al. 1989a). The localization of most of the mineral content of these grains in the embryo has been reported by Varriano-Marston and Hoseney (1979), Opoku et al. (1981), and Palmer et al. (1989a). The millet kernel contains a higher proportion of embryo than sorghum and this higher germ accounts for the higher oil content of millet grain than sorghum (Sullins and Rooney, 1976).

2.5.4 Starchy Endosperm

The starchy endosperm is the largest tissue of both grains (Palmer et al. 1989a). It consists mainly of starch granules, storage proteins and cell wall materials. The starch granules and storage proteins (prolamins and glutelins) are usually enclosed in endosperm cells which are of two types - inner and outer endosperm.

The cells of the inner endosperm are loosely packed and are refered to as soft or opaque or mealy endosperm whereas the outer area of the endosperm is tightly packed therefore, it is said to be hard, or translucent or steely (Badi et al. 1976; Palmer et al. 1989a). The soft endosperm of millet contains no protein bodies. This is in contrast to the soft-endosperm of sorghum grain (Badi et al. 1979).

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2.6 AMYLASES

The amylases are hydrolytic enzymes which act on starch.

glycogen and derived polysaccharides, hydrolysing the α, 1-->4 glycosidic linkages (Fischer and Stein, 1960). The amylases are subdivided into two groups, endo - and exo-amylase based on the nature of their hydrolytic attack (French; 1960). Those that attack the most internal bonds of the substrate are classified as endoamylases while those that attack alternative bonds from the non-reducing ends of the substrate are refered to as exoamylases.

Alternatively, the amylases are either called α- or β-amylase. α-Amylase (α, 1-->4 glucan-4-glucanohydrolase, E.C. 3.2.1.1) hydrolyses the substrate yielding dextrins or maltose whose reducing group carbon (C_1-carbon) has an α-configuration. Therefore, α-amylase produces a retention of configuration whereas the maltose produced by β-amylase (α, 1-->4 glucanmaltohydrolase, E.C. 3.2.1.2) possesses a β-configuration at the reducing end. Thus the reaction catalysed by this enzyme proceeds with inversion (Gray, 1971; Thume et al, 1971). Therefore the prefix α or β conveys information about product stereochemistry and not substrate specificity.

The hydrolysis of substrate by α-amylase has been reported (Robyt and French, 1963) to proceed in two stages; the initial random attack producing dextrins followed by a much slower, more or less patterned attack, yielding maltose.

The involvement of α-amylase in the modification of barley grain was observed over a century ago by the brewing scientist Horace Brown who refered to this enzyme as a "parasite" produced by the barley embryo, which digested the endosperm for its own

benefit (Palmer, 1979). Though his view on the primary source of the enzyme has been reviewed, his observation remains remarkable.

2.7 α-AMYLASE FORMATION

The "dry", "resting", sound kernels of many cereal grains contain little or no α-amylase (Novellie, 1960; Dyer and Novellie, 1966; MacGregor, 1980; Kwanhwo and Opoku, 1986). The desired level of this enzyme in grains is achieved experimentally by the malting process (Daiber and Novellie, 1968; Pomeranz, 1975; El-Farra et al, 1976; Novellie, 1977; MacGregor, 1978a; 1980; Palmer, 1979; Glennie et al, 1983; Palmer et al, 1985; 1989b). This large increase in the level of α-amylase following germination is widely accepted to be due to de novo synthesis of the enzyme (Van Onkelen and Verbeek, 1969), probably from amino acids released from the proteolysis of storage protein of the tissues (embryo) (Filner and Varner, 1967). Yomo and Jinuma, (1966) reported that this synthesis is specifically under the control of gibberellins (GAs) or gibberellin-like substances synthesized de novo in the embryo. As a result of the inability of GA to stimulate α-amylase production in the sorghum endosperm, as observed with barley (Clutterbuck and Briggs, 1973) it was then suggested that sorghum α-amylase is synthesised in the embryo from where it diffuses into the endosperm (Daiber and Novellie, 1968).

This induction of de novo hydrolytic enzyme synthesis in cereal grains by GA is said to be a post-germinative phenomenon

(Chan, 1975). Chan (1975) found that following GA treatment of the aleurone cells, there was a lag-phase of approximately 8-10 hours before enzyme secretion occured. The implication of this is that the synthesis and secretion of α-amylase are not a primary effect of GA action.

Studying the biochemical events during the lag phase, Jones (1969a, b), showed that rough endoplasmic reticulum (RER) was very prominent during the early hours of germination. This observation was in line with those of Evins (1971) and Evins and Varner (1971; 1972) who reported that both endoplasmic reticular membrane and ribosome synthesis as well as polyribosome formation are stimulated within 2-4 hours of GA_3 administration. It also confirms with the speculation of Johnson and Kende (1971) and later Koehler and Varner (1973) that the induction of hydrolase synthesis by GA was dependent on the elaboration of membranes especially those of the endoplasmic reticulum (ER) for the attachment of ribosomes. This hypothesis was supported by immunochemical evidence on α-amylase localization (Jones and Chen, 1976).

Although GA had no effect on the synthesis of total RNA in the aleurone tissue (Jacobsen and Zwar, 1974), it has been found to specifically increase the level of poly(A) rich RNA species (Jacobsen and Zwar, 1974). Using a cell free translating system from wheat embryos, Higgins et al (1976) showed that this poly(A) rich RNA is the messenger RNA (mRNA) for α-amylase and that the increase in this mRNA begins 5-6 hours after GA treatment.

2.6 TRANSPORT AND SECRETION OF SYNTHESIZED ENZYME

Varner and Mense (1972) reported that α-amylase secretion in barley seeds is an energy dependent process closely coupled to protein and RNA synthesis. Usually this secretion of proteins from Eukaryotic cells follows one of the two routes: the regulated and the constitutive pathways (Farquahr, 1985).

Evidence that the secretion of α-amylase in cereal grains follows the constitutive pathway is overwhelming. Electron microscopy of chemically fixed tissues (Jones, 1969a) does not show the presence of condensing vacuoles or secretory granules. Similarly, autoradiography after radiolabelling with amino acids (Chen and Jones, 1974) failed to show the accumulation of secreted α-amylase within secretory granules of the aleurone cells.

The negation of the possibility of the presence of α-amylase in preformed state was ruled out by density labelling experiment with H_2O^{18} (Filner and Varner, 1967). The close coupling of α-amylase synthesis to their secretion (Higgins et al. 1982) and the presence of α-amylase only in the ER and the golgi apparatus (GApp) (Gubler et al. 1986) further confirm the constitutive secretion of α-amylase.

That the secretion of α-amylase follows the constitutive pathway is further verified by the effect of inhibitors of protein synthesis such as cycloheximide. This compound blocks α-amylase synthesis (Chrispeels and Varner, 1967) but only produces complete inhibition of its secretion after 3-4 hours (Higgins et

al. 1967; Manley and Ewar, 1986). This observation led to the suggestion that this delayed inhibition of the enzyme secretion is due to an intracellular pool of newly synthesized α-amylase that supports secretion for 3-4 hours in the absence of protein synthesis (Warner and Mense, 1972; Moll and Jones, 1983).

The involvement of ER and GApp in the transport of newly synthesized α-amylase was confirmed by the use of the drug, Monensin (Heupke and Robinson, 1985, Melroy and Jones, 1986). Monensin, an inhibitor of the secretory process, produced a pronounced dilation of GApp and cisternae. Melroy and Jones (1986) then concluded that the α-amylase synthesized on RER was transported to the plasma membrane via the GApp.

α-Amylase secretion by bacterial cells is analogous to that obtained in the cereal grains. Like in the cereals there is no evidence that vesicles play any role in bacterial α-amylase secretion. However, vesicle transport has been demonstrated in fungi and saccharomyces species (Lampen, 1976).

The release of the secreted α-amylase molecules in barley into the surrounding medium was found by Warner and Mense (1982) to be dependent on the presence of ions such as Calcium, Magnesium and Potassium.

2.9 GRAIN MODIFICATION

Grain modification involves the breakdown of β-D-glucans, pentosans, proteins and starch granules of the endosperm during malting. The solubilised reserve materials of the endosperm

are the primary constituents of brewer's extract. Therefore the quality and quantity of this extract are controlled by the level of hydrolytic enzymes that develop during the germination process (Palmer et al. 1985). Since the levels of these enzymes influences the extract quality, the quality of the finished malt is therefore a function of the rate and pattern of enzyme release into the endosperm. The implication of this, is that the modification of the grain is dependent on the growth of viable embryo (Briggs, 1973).

Glennie et al. (1983), observed that modification of sorghum starts from the endosperm-scutellar interface from where it progresses into the floury endosperm. Simultaneously, there was slight modification of the peripheral endosperm adjoining the aleurone layer. It was therefore suggested (Glennie et al. 1983) that the enzymes responsible for this modification must have originated in the scutellum rather than the aleurone layers in constrast to that observed in barley (Cluttertuck and Briggs, 1973). Koehler (1981) similarly, reported that over 90% of α-amylase activity occured in the embryo of the germinating sorghum. Okamoto et al (1980) also reported that the scutellar epithelium was the formation site of hydrolases in barley, wheat, rye, oats and maize.

The modification of sorghum starch is characterised by extensive pitting of the granules (Glennie et al. 1983), rather than by the surface erosion characteristic of barley's small starch granules (MacGregor, 1980). However the modification of

protein bodies follows one of the two patterns: internal digestion or external digestion (Ashton, 1976).

2.10 KILNING

During the malt kilning, moisture level of the grain which had risen to about 40-46% at the end of the steeping period (Lloyd, 1988; Ikediobi, 1989)is reduced to about 2-6%, yielding malt in which all biologic activities have been terminated and the enzyme levels required for brewery mashing retained (Lloyd, 1988).

The sequential biochemical changes occuring during kilning are classified into three phases (Lloyd, 1988). The initial phase, the germinative stage, involves essentially, the removal of "free water". This brings the moisture level down to about 20%, while enzyme synthesis continues and the rate of reactions increases. In the second stage described as the enzymic phase, the moisture level drops to 10%. Although the rate of enzyme-catalysed reactions increases as the malt temperature increases, inactiva-tion of some enzymes also takes place. Finally, in the third or chemical or "bound water" stage, colour, flavour and other minor constituents of the malt are accelarated. This process often called "malt curing" is accompanied by more loss in enzyme activity (Narziss, and Knoitze, 1977).

2.11 ASSAY METHODS

α-Amylases fragment large substrates, decreasing the average chain length with concomitant decrease in viscosity, loss of iodine staining capacity, and appearance of new reducing ends

(Fischer and Stein, 1960; Thome et al, 1971). Therefore α-amylase activity measurements are designed to monitor any one of the above phenomena.

Since the substrate possesses many potential points of cleavage, the appearance of product (new reducing group) is prefered for the study of α-amylase kinetics (Dixon and Webb, 1964). The production of new reducing end is most frequently followed by either the 3,5-dinitrosalicylate (Bernfeld, 1955; Fischer and Stein, 1960; Robyt and Whelan, 1965), or alkaline ferricyanide (Robyt and Bemis, 1967; Robyt and French, 1967), or alkaline copper (Nelson, 1944, Dygert et al, 1965) method.

The simplest oxidation method involves the reduction of 3,5-dinitrosalicylic acid, though it has been reported (Thome et al, 1971) to be the least desirable as the measurement is subject to the chain length of the oligosaccharide carrying the reducing group and interferance by calcium ion used to stabilise the enzyme (Robyt and Whelan, 1965).

The best reagent reported (Dygert et al, 1965)for detailed α-amylase kinetics study is the copper neocuproine reagent. This reagent has the advantage of high sensitivity and relative insensitivity to a wide range of contaminating substances. Its major disadvantage is the demanding experimental proceedure.

The decrease in viscosity (Greenwood et al, 1965) and iodine staining capacity (Manning and Campbell, 1961; Mestecky et al, 1969) serve as other valuable parameters for α-amylase assay. Their major advantage lies in their sensitivity to initial random

α-amylase attack. However, they are insensitive to repetitive multiple attacks (Robyt and French, 1967).

An innovation in α-amylase activity measurement involves the use of insoluble chromogenic substrates (Klein et al. 1969; Thoma et al. 1971). The chief merits of this method include its simplicity and stability. This method can be used conveniently to assay for α-amylase activity in the presence of β-amylase (Thoma et al. 1971).

2.12 PROPERTIES OF α-AMYLASE

2.12.1 MULTIPLE FORMS

Many investigators (Frydenberg and Nielsen, 1965; Van Onkelen and Verbeek, 1969; MacGregor et al. 1971; MacGregor, 1977) have reported the presence of multiple forms or "isoenzymes" of α-amylase as a result of the introduction of methods of ion-exchange chromatography, gel electrophoresis and isoelectric focussing into the study of α-amylase. Hence they concluded that the α-amylase from cereals is heterogenous.

The sorghum α-amylase was separated into four subfractions by chromatography on calcium phosphate column (Botes et al. 1967). Based on the close similarity in both physical, chemical and kinetic properties of these subfractions, it has not been ascertained whether these enzymes exist as micro-heterogenous mixtures in the sorghum malt or that this property is an artifact arising from some purification step (Botes et al. 1967). There is little agreement on the number of α-amylases present in

barley. Values ranging from one to five components have been reported (Frydenberg and Nielsen, 1965; Mitchell, 1972). From millet malt, three different forms were found by Belsin and Varriano-Marston (1981). While the possibility of artifact formation has been considered for sorghum malt enzyme, the investigators of the millet enzyme were of the opinion that such possibility is unlikely considering the widely varying properties of these forms especially their molecular weight (Belsin and Varriano-Marston, 1981).

2.12.2 [illegible]

Fischer and Stein (1960) classified α-amylases as metalloenzymes. They reported that calcium ions are required by these enzymes for stability as well as activity. They also reported that α-amylase contains at least one-gram atom of calcium ions per mole of enzyme protein. One of the earliest demonstrations of the involvement of calcium ions in α-amylase activity and stability was the removal of calcium from the enzyme by chelation with ethylenediaminetetraacetic acid (EDTA) and electrodialysis (Stein et al, 1964; Stein et al, 1964). The removal of calcium ions resulted in both reversible and irreversible inactivation (Greenwood and Milne, 1968b). This irreversible inactivation was ascribed to proteolytic degradation of these enzymes since calcium-deficient enzymes were found to be very susceptible to protease digestion (Fallis et al, 1959).

Though calcium atoms are required for activity, there is no indication that calcium atoms participate directly in the

formation of enzyme-substrate complex. Fischer and Stein (1960), suggested that calcium, by forming a tight metal-chelate structure, performs the dual function of maintaining the protein in the proper configuration needed for biologic activity and stabilising the secondary and tertiary structures. Thus calcium confers on the α-amylase molecule, its compact architecture.

Drawing inference from the effect of various chemicals on sulfydydryl groups, Thume et al (1971), suggested that the sulfydydryl groups on α-amylase molecule is one of the calcium binding groups. The replacement of calcium by other metals such as Strontium, Magnesium and Barium resulted in some level of activation whereas heavy metal ions such as Mercury, Silver, Copper and Lead have inhibitory effect (Hsia et al, 1964). But because of the ability of α-amylases to selectively concentrate calcium ions in the presence of other metals, the assertion that other metals are activating agents have not gained wide acceptance (Thume et al, 1971).

2.12.8 MOLECULAR WEIGHT

The α-amylase from diverse sources have been reported (Fischer and Stein, 1960) to have a molecular weight in the neighbourhood of 50,000 daltons. Botes et al, (1967), found that the purified sorghum malt α-amylase isoenzymes have molecular weights of between 46,200 and 51,500 daltons.

The molecular weight obtained by sodium dodecylsulphate (SDS)-gel electrophoresis of α-amylase from malted pearl millet shows a wide variation. Whereas Baisia and Varriano-Marston (1981)

reported three distinct bands at molecular weights of 31,000, 46,000 and 53,000 for α-amylase purified by glycogen complex formation. Abdul-Hussain and Varriano-Marston (1982), obtained a single protein band at molecular weight of 48,000 for the same enzyme purified by the same method. The molecular weight of the same enzyme purified by the starch-column procedure showed two bands, one at 31,000, the other at 22,000 daltons (Beleia and Varriano-Marston, 1981). This lower molecular weight of α-amylases from starch column purification supports the hypothesis that only certain α-amylase isoenzymes are adsorbed on starch or complexed with glycogen (Beleia and Varriano-Marston, 1981).

The molecular weight of malted wheat enzyme was shown to range from 41,500 to 42,500 daltons (Ikarchuk and Kruger, 1974) whereas α-amylase from immature wheat had molecular weights of between 52,000 and 54,000 (Marchylo et al, 1976). While the molecular weight of the barley malt enzyme calculated from osmotic pressure measurements had earlier been put at 59,500 ± 900 (Schwimmer and Balls, 1949). Rodaway (1978) reported a molecular weight of 41,400. Generally, the molecular weight of most cereal α-amylases range from 20,000 to 57,000 daltons (Ikarchuk and Kruger, 1974).

The molecular weights of microbial α-amylases closely approximate to those of cereals. Junge et al, (1959) and Stein et al (1960), and Sirisinha and Allen (1965a)extrapolating from sedimentation velocity, diffusion constant and partial specific

volume measurements gave the molecular weight of Bacillus subtilis (B. subtilis) and Aspergillus oryzae (A. oryzae) α-amylases as 48,700 and 51,750 daltons respectively. Other molecular weight values given include 61,000 and 58,000 daltons for acid-stable α-amylase from Aspergillus Niger (A.Niger) Thoma et al. 1971; Kvesitadze et al. 1978), 48,000 daltons for Bacillus stearothermophilus (B. stearothermophilus) enzyme (Srivastava, 1987) and 60,000 daltons for α-amylase from Aspergillus Aureus (A. Aureus) (Tanaka et al. 1976).

In contrast to the value reported by Junge et al. (1959), native crystalline α-amylase from B. subtilis was found earlier to possess a sedimentation constant which corresponds to molecular weight of 100,000 (Manzi et al. 1957). This high molecular weight of the native enzyme was rationalised as being due to the existence of the enzyme in dimeric form and that this monomer-dimer transformation occurs through the binding of a zinc ion (Fischer and Stein, 1960; Stein et al. 1960). This dimerization phenomenon seems to be peculiar to the B. subtilis enzyme as it has not been demonstrated with any other α-amylase (Thoma et al 1971).

Although most microbial α-amylase possessed molecular weights similar to those of plants, a few organisms produce α-amylase of higher molecular weight, for example Stukalin et al. (1988) reported a molecular weight of 74,000 for a thermostable α-amylase produced by a strain of B. subtilis, B. subtilis 1621.

2.12.4 EFFECT OF pH ON ENZYME ACTIVITY

The effects of pH on the activity of α-amylases from many sources have been reported. Cereal malt enzymes are most active in slightly acidic to moderately acidic solutions (Caldwell and Adams, 1950). Data on optimum pH of activity of purified α-amylases show that most of the cereal α-amylases possessed pH optima in the range of 4.0 to 6.0 (Dube and Nordin, 1961; Bates et al. 1967; Tkachuk and Kruger, 1974; Fuwa et al. 1975a; Marchylo et al. 1976; MacGregor, 1976b; Belein and Varriano-Marston, 1981; Abdul-Hussain and Varriano-Marston, 1982). This acid tolerance of cereal α-amylases may be of immense importance in the preparation of some fermented foods with low pH value (El-Tinay et al. 1979). Although some of these enzymes exhibit optimum pH at about 4.0, they are quickly inactivated at pH 3.5 and below, probably reflecting the instability of the enzyme at lower pH values (Dube and Nordin, 1961).

The microbial α-amylases show a much wider spectrum of optimum pH of activity. A group of these enzymes exhibited some remarkable similarity with cereal amylases (Markovitz et al. 1956; Manning and Campbell, 1961; DePinto and Campbell, 1968; Tanaka et al. 1976; Ghai and Ghahal, 1980). Another group showed maximum activity at about neutral pH (Campbell, 1955; Good and Hartman, 1970; Chiang et al. 1979; El-zainki and Hamza, 1979; Ingledew and Wilson, 1982; Srivastava, 1987; Stukaitis et al. 1988). Recently, a new strain of Bacillus, B. licheniformis and B. coagulans producing thermostable α-amylase with optimum pH of

activity in alkaline pH have been reported (Medda and Chandra, 1980).

A recuring feature in the pH profile in most of these reports (Campbell, 1955; Markovitz, et al, 1956; Manning and Campbell, 1961; Bates et al, 1967, DePinto and Campbell, 1968; Tkachuk and Kruger, 1974; Fuda et al, 1975a; Marchylo et al, 1976; Chiang et al, 1979; Ghai and Chahal, 1980; Belsia and Varriano-Marston, 1981; Abdul-Hussain and Varriano-Marston, 1982) is the presence of a wide pH optima.

Another characteristic which appears not to be common with α-amylases is the occurence of double pH optima as the only reported case was by Acharya (1984).

2.12.5 EFFECTS OF TEMPERATURE ON α-AMYLASE ACTIVITY AND STABILITY

The effects of temperature on α-amylase activity and stability has been well reported in the literature. The temperature of maximum activity was given as 55°-60°C for barley malt (Fuda et al, 1975b), 55°C for barley malt α-amylase 1 (MacGregor, 1978b) and α-amylase from immature barley (MacGregor et al, 1974). Earlier, Acharya (1984), comparing the properties of the sorghum grain and barley malt enzymes gave an optimum temperature of 45°C for barley malt and 50°C for sorghum malt α-amylases respectively.

The optimum temperature of activity of α-amylase from millet malt purified by both starch-and glycogen-complex formation was reported as 55°C (Belsia and Varriano-Marston, 1981) and 45-55°C

(Abdul-Hussain and Varriano-Marston, 1982). A review of these values shows that there is a great similarity in the temperature optimum of these cereal α-amylases. However, a slightly higher value of 60°-62°C has been reported for the wheat enzyme (Tkachuk and Kruger, 1974).

Reports on temperature optima of mold α-amylases consistently indicate that these enzymes are thermolabile, exhibiting their optimal activity at about 40°C, and become inactivated above this temperature (Markovitz et al, 1956; El-zalaki and Hamza, 1979; Ingledew and Wilson, 1982). Most bacterial α-amylases act maximally at temperatures ranging from 55°C to 90°C (Campbell, 1955; Manning and Campbell, 1961; Medda and Chandra, 1980; Srivastava, 1987; Stukalin et al, 1988). Although these enzymes are very active at their optimal temperature, they are however, unstable, retaining between 50-70% of their initial activity after a thirty-minute to one-hour heat treatment at these temperatures (Tkachuk and Kruger, 1974; MacGregor, 1978b).

The effect of temperature on the rate of hydrolysis of starch by α-amylase of various origin when plotted in the usual Arrhenius manner, yields activation energy (Ea) of the enzyme over that temperature range. With some exceptions, most literature reports for plant α-amylase Ea values are in the range of 7.1 - 8.6 kcal/mole/degree (Greenwood and Milne, 1968a). Fischer and de Montmollin, (1951) reported Ea of 7.05 kcal/mole/degree for barley malt enzyme, while MacGregor, (1978b), obtained 9.7kcal/mole/degree for barley malt

α-amylase-1. Similarly, Botes et al. (1967) obtained 12.0 kcal/mole/degree for sorghum α-amylase. Also 8.7 kcal/mole/degree has been reported for malted wheat α-amylase (Thachuk and Krugar, 1974).

Generally, the microbial α-amylases possess a slightly higher Ea compared to those from plants. 10.5kcal/mole/degree was reported for A oryzae (Fischer and de Montmollin, 1951); 14.4 kcal/mole/degree (0-15°) and 8.5kcal/mole/degree (15-40°) for B. saccharophila (Markovitz et al. 1956); 15.0kcal/mole/degree (0-12°) and 11.0 kcal/mole/degree (12-55°) for B. subtilis (Menzi et al. 1957) 14.0 kcal/mole/degree for B. stearothermophilus (Manning and Campbell, 1961) and 12.5 kcal/mole/degree for B. macerans (DePinto and Campbell, 1968).

2.12.6 MICHAELIS CONSTANT

The literature is replete with varying Michaelis constant (Km) values for α-amylases from various sources. One of the earliest reported Km value was the 0.6mg starch/ml obtained by Markovitz et al. (1956) for B. saccharophila α-amylase. Campbell and his coworkers, studying the properties of α-amylase from B. stearothermophilus and B. Macerans reported 1.0mg and 3.33mg starch/ml respectively for the enzymes from these organisms (Manning and Campbell, 1961; DePinto and Campbell, 1968).

Botes et al. (1967) investigating the properties of malted sorghum α-amylase, obtained a Km value of 1.57×10^{-2} mole glycosidic bond per litre with gelatinised starch as substrate. Similarly, the Km of the three isoenzyme forms of

immature hard red spring wheat enzyme were estimated to be α-1, 0.25mg starch/ml, α-2, 0.533mg starch/ml, and α-3, 0.235mg starch/ml (Marchyto et al, 1976). The Km values reported for other α-amylases are 0.364 mg starch/ml (Ingledew and Wilson, 1982) for S. alluvius, 5.88mg starch/ml (Ghai and Chahal, 1980) for A Flavus (F-14) and 1.05mg starch/ml (Srivastava, 1987) for B. stearothermophilus.

Generally, the Km of α-amylases of plant, animal and microbial origin, for their starch substrate has been reported (Manning and Campbell, 1961) to range from 0.18 to 0.65 mg starch/ml. Earlier the Km of α-amylases from human saliva, hog pancreas, B. subtilis, A. oryzae, P. saccharophila and barley malt were given as varying from 1.0 to 5.0 x 10^{-3} mole glycosidic bond/litre under a variety of experimental conditions between 20° and 30°C (Fischer and Stein, 1960).

2.12.7 ACTIVE SITE STUDIES

A large volume of literature describing the modification of side chains of amylase exists. Practically every possible reactive side chain residue in a protein has been implicated in α-amylase activity after submission to harsh and unspecific reagent treatment such as nitrous acid, fluoronitrobenzene, ketene, acetic acid, iodine and diazobenzesulfonic acid (Little and Caldwell, 1942; 1943; Greenwood et al, 1965a). Since modification often induces minor but significant changes in the conformation of the protein molecule, these experiments do not pinpoint the cause of inactivation. Therefore the most

definitive experiments are those that yield negative results (Thoma et al. 1971). In line with this argument, Amos et al. (1956) concluded that the inability of iodoacetamide, p-mercuribenzoate, and phenylmercuric chloride to inactivate hog pancreatic α-amylase, and to only partially destroy the activity of cereal and human salivary amylases is an indication that the sulfyhydryl group does not participate in the enzymes' catalysis.

As a result of shortage of reagents displaying high selectivity towards certain classes of side chains, the influence of pH on the activity of enzymes is often employed in the assignment of pK values and therefore the catalytic amino acids. Greenwood et al. (1965a) and Thoma et al (1971) assigned the catalytic function of α-amylases to carboxyl and imidazole groups from pH data.

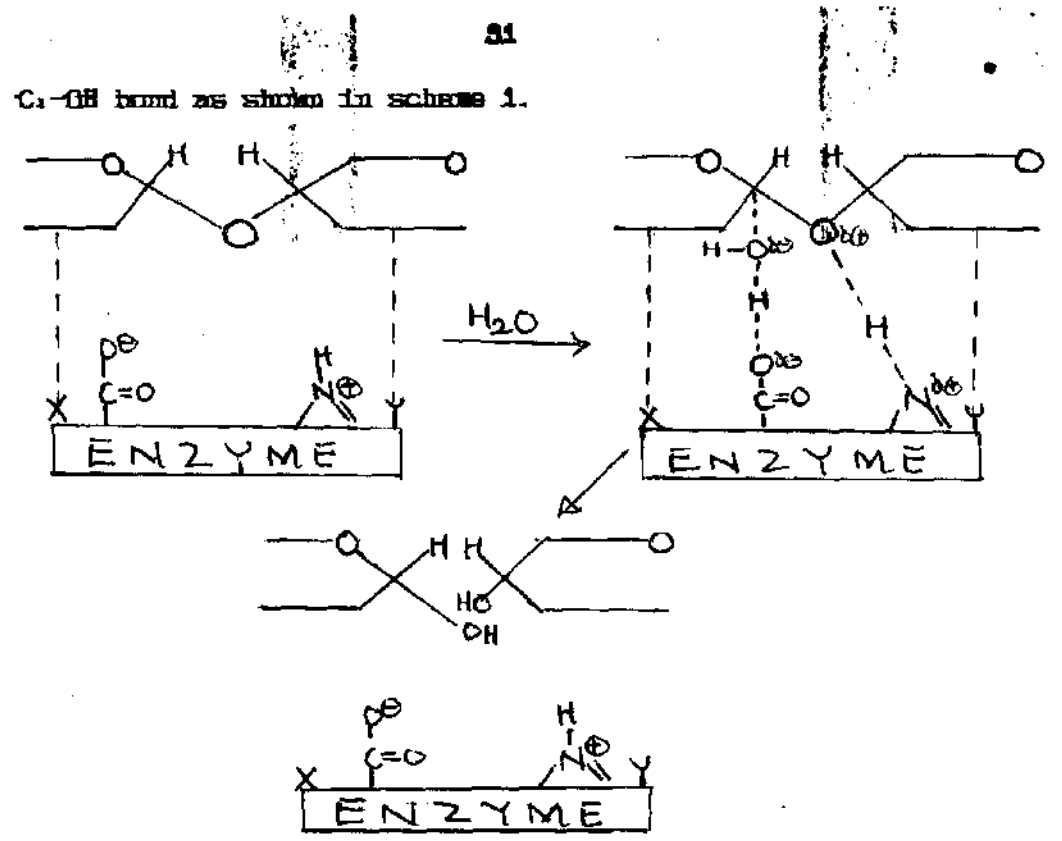
2.13 MECHANISM OF CATALYSIS

The α-amylases are so named because their catalytic action is accompanied by the retention of the configuration at the C_1 of D-glucose (Greenwood and Milne 1968b). The proposed catalytic agents involved in α-amylase catalysis are carboxyl anion and imidazolium cation (Thoma et al. 1971).

Three possible mechanisms have been suggested (Greenwood and Milne, 1968b) to explain the retention of the C_1 configuration. These are the front side displacement, the double displacement and the stereospecific hydration of the carbonium ion.

In the front displacement (a type of S_Ni mechanism), the C_1-O-C_4 bonds break simultaneously with the formation of the new

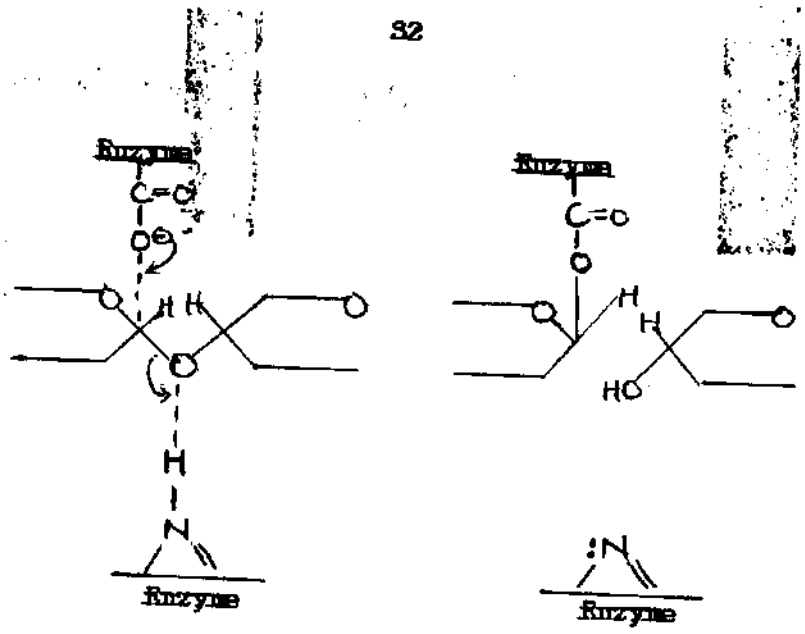
C_1–OH bond as shown in scheme 1.

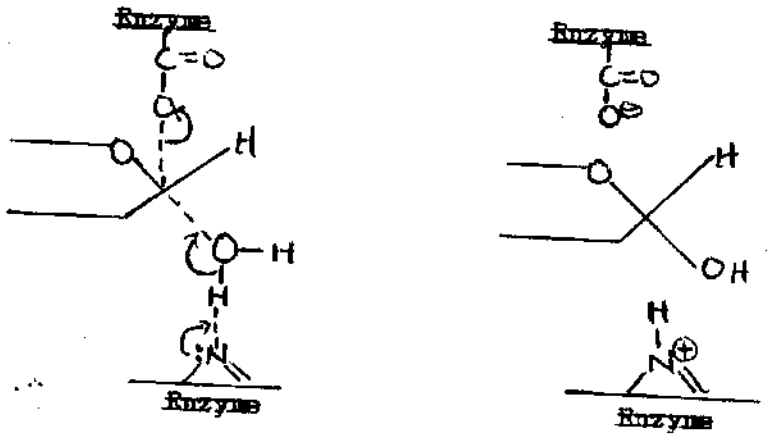


Scheme 1

In the double-displacement mechanism, which involves two S_N2 substitutions, the C_1–O–C_4 bond breaks with the formation of an enzyme-substrate complex. This is then followed by its hydrolysis to give the product.

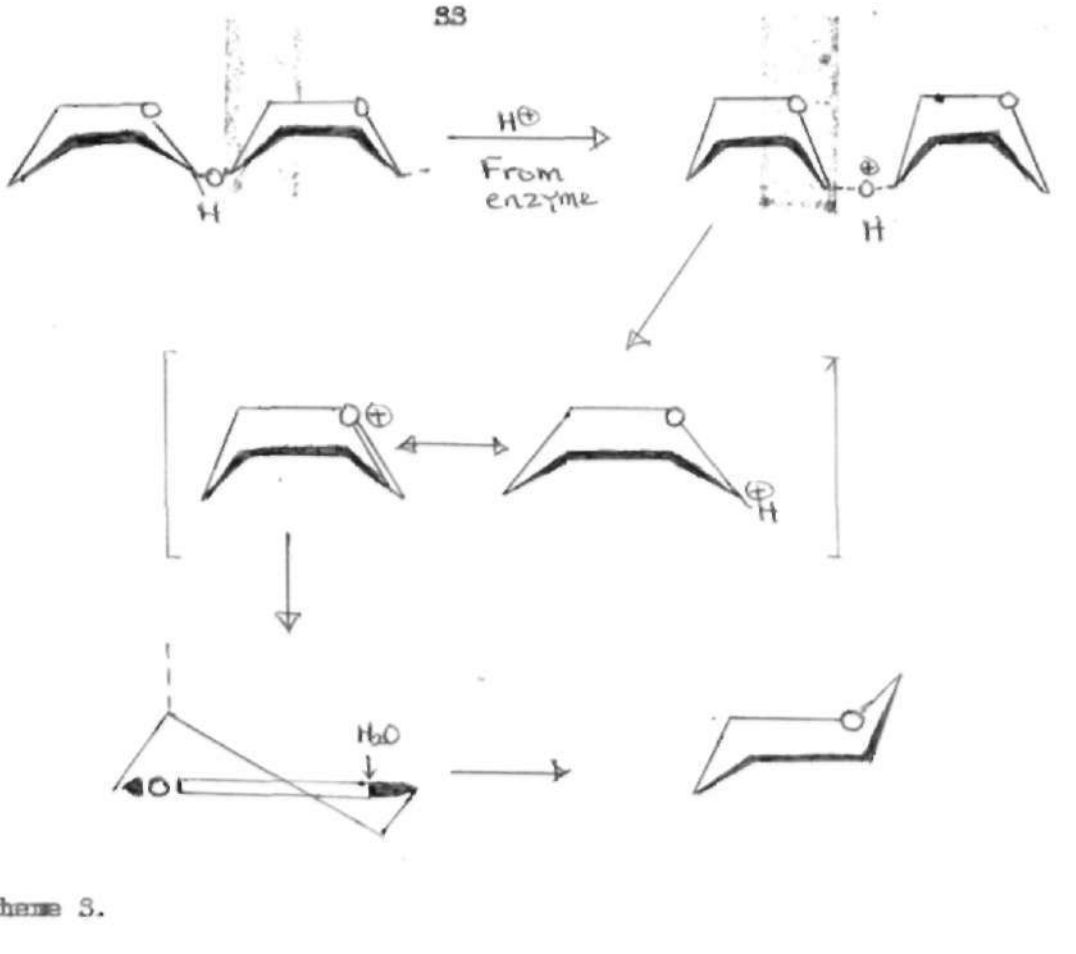
In these schemes, the carbonyl anion acts as an attacking nucleophile on the C_1 position of the substrate and this attack is aided by protonation of the linkage by the general acid (imidazolium ion) (Whitaker, 1972). In the deglucosylation reaction of scheme 2 (double displacement mechanism), the unprotonated imidazole group assists as a general base to remove a proton from water leaving the OH^- to attack C_1 position of the glycosyl-enzyme intermediate.





Scheme 2.

In the third mechanism (an $S_{N}1$ substitution), the ring-oxygen atom becomes protonated leading to the breakage of C_1-O bond. The enzyme then guides the water molecule stereospecifically to one side of the carbonium ion as the product is formed.



Scheme 3.

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CHAPTER 3

MATERIALS AND METHODS

3.1 Sample Collection:

White sorghum (farz-fara) and millet grains were purchased from a local market in Samaru, Zaria.

3.2 MALTING

This was carried out as described by Novellie (1962).

3.2.1 STEEPING

The grains (500g) were washed several times with running tap water to remove stalks, floats, sand and other extraneous matter. They were then steeped for 24 hours at room temperature (25°-30°C) in distilled water. The water was changed three times. To ensure mold free grains, the grains were treated with a 0.2% solution of formaldehyde (1.5 litres) for 40 minutes.

3.2.2 GERMINATION

The steeped grains were germinated on trays and covered with wet jute bags for 5 days. During this period, the germinating grains were turned twice a day, morning and evening and watered.

3.2.3 KILNING

The germinated grains were dried at 50°C for a period of 2 days in a laboratory oven.

The rootlets were then removed from the dry grains by rubbing

the sprouted, dried grains between the palms and sifting the materials through a 0.5mm mesh.

3.2.4 MILLING

The dried de-rooted grains were milled to pass through 0.5mm mesh. The powder obtained was sieved with a (0.3mm) test sieve to produce a fine powder.

3.3 ENZYME EXTRACTION AND PURIFICATION

3.3.1 ENZYME ASSAY

The enzyme was assayed according to the method of Bernfeld (1955) as described by Worthington (1978). The enzyme solution (0.1ml) was introduced into a series of numbered test tubes. The blank contained distilled water (0.1ml). At 1minute interval, 1.0% starch substrate solution (0.9ml) was added to each tube and then incubated in a water bath at 30°C for 10 minutes. Dinitrosalicyclic acid colour reagent (1.0ml) was then added to each tube and heated in a boiling water bath for five minutes. The tubes were then cooled, distilled water (10ml) added and the absorbance read against the blank at 540nm.

The amount of reducing sugar produced was calculated from a standard curve prepared from maltose standard (0.2-1.0mg/ml) solution. (Appendix 3). One unit of enzyme activity is defined as that amount of enzyme that can liberate one micromole of reducing group (calculated as maltose) per minute at 30°C and pH

4.8 under the specified conditions. This assay method was used throughout the study.

3.3.2 DETERMINATION OF PROTEIN CONTENT

The protein content of the enzyme solutions and pooled fractions was determined by the method of Lowry et al. (1957) as described by Cooper, (1977) using the folin-ciocalteau reagent and bovine serum albumin (BSA) as standrad (Appendix 4). The colour formation depends on the reaction of alkaline copper with the protein, and the reduction of the phosphomolybdate by the aromatic amino acids particularly tyrosine and tryptophan in the protein. The protein concentration of the unknown samples were calculated from a calibration curve (Appendix 4).

3.3.3 EXTRACTION

The enzyme was extracted according to Belein and Warriano-Marstun, (1961) by stiring 200g of the meal into 500ml of a 0.1M acetate buffer pH 4.8 containing 0.01M calcium chloride for 3 hours. The slurry was then centrifuged for fifteen (15) minutes at 8,000g and the precipitate washed twice with the extracting buffer (100ml) before being discarded. The supernatants were pooled together and filtered through Whatman No 1 filter paper. Calcium chloride to a final concentration of 0.2% was then added to the pooled supernate.

3.3.4 CHARCOAL AND HEAT TREATMENT

Preliminary tests to establish the optimum condition for this step was carried out (Figs. 1 and 2). The pH of the extract was then adjusted to 6.0 with 2M sodium hydroxide and decolourising charcoal added to give a 5% (Sorghum) and 4% (Millet) mixture. The mixture was then divided into 100ml-fractions and heated at 70°C for fifteen minutes with continous shaking. The suspension was cooled under running tap water. The charcoal and coagulated proteins were removed by a combination of centrifugation at 9,000g for 15 minutes and filtration.

3.3.5 AMMONIUM SULPHATE PRECIPITATION

Pilot run indicated that most of the millet and sorghum malt α-amylase are precipitated by 50% and 60% ammonium sulphate saturation respectively (Table 3). Enough solid ammonium sulphate was then gradually added to the heat treated extract to give 45% (millet) and 55% (sorghum) saturation. The suspension was allowed to stand for one hour in an ice bath with occassional stirring, and then centrifuged at 10,000rpm on Beckman J-2B refrigerated centrifuge, for ten minutes at 4oC. The precipitate was redissolved in a 0.1M Tris buffer pH 7.0 containing 0.2% calcium chloride.

3.3.6 GEL FILTRATION ON SEPHADEX G150-120

The redissolved ammonium sulphate precipitated enzyme, 2ml for sorghum and 20ml for the millet enzyme was applied to a (2.6 x 40cm) sephadex G150-120 column at room temperature.

The gel was prepared according to Flodin and Killander (1962). Sephadex G150-120 (9g) was soaked in excess buffer (400ml) consisting of 0.1M Tris-HCl pH 8.0 in 0.2M sodium chloride for 24 hours at room temperature. The fine particles giving the supernatant turbid appearance were removed by decantation. The column was first equilibrated with 0.1M Tris buffer pH 7.0 containing 0.2% calcium chloride before the sample was applied. The flow rate for sorghum α-amylase elution was 18ml/hr while 28ml/hr was recorded for the millet α-amylase.

3.3.7 POLYACRYLAMIDE GEL ELECTROPHORESIS

Polyacrylamide gel electrophoresis was performed according to Davis (1964) as described by Sargent and George (1975). The acrylamide concentration of 7.5% was used for the running gel while the stacking gel was 3.0%. The running gel consists of 13.5ml of acrylamide (14.25g) and bis-acrylamide (0.75g) dissolved in 100ml of distilled water, 6.25ml of gel buffer containing 24ml of 1.0M HCl and 3.63g of Tris dissolved in 100ml of distilled water, to which is added 0.06ml of N,N,N'N'-tetramethylethylenediamine (TEMED as activator) and 4.0ml of ammonium persulphate (as initiator). While the stacking gel was made from 10.0ml of 10g acrylamide, 2.5g of bis-acrylamide in 100ml of distilled water and 5.0ml of Tris-phosphate buffer (pH 5.5) constisting of 4.95g Tris, 39ml of 1.0M phosphoric acid in 100ml of distilled water. To the mixture, 0.05ml of TEMED was added followed by 3.0ml of ammonium persulphate.

The gel mixtures were mixed properly and poured into the gel tubes, first the running and then the stacking gel. The enzyme sample (0.05ml) was applied on top of the stacking gel followed by a drop of 0.1% bromophenol blue in 50% glycerol. The electrode buffer 0.01M tris-glycinate (pH 7.0) was used for the electrophoretic run. The electrophoresis was performed at a constant current of 5mA per gel at room temperature for 3 hours. After the electrophoresis, some of the gels were removed and placed in a solution of 1% starch for 30 minutes to detect α-amylase activity while the other tubes were stained with a 0.25% coomassie brilliant blue in methanol, Trichloroacetic acid, and water (5:1:5 v/v/v) mixture over night. The gels were then destained with successive changes of a 7% acetic acid solution and photographed.

3.4 PREPARATION OF MICROBIAL ENZYMES

3.4.1 PREPARATION OF ROHALASE AT (BACTERIAL α-AMYLASE)

The stock enzyme solution (2.5ml) (Rohm enzyme technology)was diluted 20 times with 0.1M Acetate buffer pH 4.8 containing 0.2% calcium chloride to a 50ml solution. This solution was diluted 10 times before use.

3.4.2 PREPARATION OF ROHALASE MS (FUNGAL α-AMYLASE)

The enzyme preparation (0.1g) (Rohm enzyme technology) was dissolved in 0.1M acetate buffer (100ml) (pH 4.8) containing 0.2% calcium chloride. This was diluted 20 times before use.

3.5 DETERMINATION OF THE pH PROFILE

To determine the optimum pH of activity, the enzyme preparations were assayed as a function of pH using gelatinised soluble starch as substrate (0.9ml). The activity was determined using 0.1M acetate buffer (pH 3.0-6.0) and 0.1M tris buffer for pH values between 6.5 and 9.0 (Bates et al. 1967). Each buffer solution contained 0.2% calcium.

3.6 EFFECT OF SUBSTRATE CONCENTRATION

The substrate concentration was varied over the range of 0.02 to 0.10mg/ml of starch solution. The effect of substrate concentration on α-amylase activity was carried out as outlined in enzyme assay. The kinetic constants, Km and Vmax for the enzymes were determined from Lineweaver-Burk plots.

3.7 EFFECT OF TEMPERATURE ON ENZYME ACTIVITY

The effect of temperature on the rate of starch hydrolysis was studied at 20°, 25°, 30°, 35° 40°, 45°, 50°, 60°, 70°, 80° and 90°C as described by Stauffer (1989). The enzyme preparations (0.1ml) were added to starch substrate solution (0.9ml) and incubated at each temperature for 30 minutes.

3.8 THERMOSTABILITY OF THE ENZYMES

Equal volumes of the enzyme (0.2ml) were incubated for thirty (30) minutes, at 30°, 40°, 50°, 60°, 70°, 80° and 90°C in a water bath as described by Stauffer, (1989). They were then cooled to

room temperature and remaining activity assayed as previously described in assay using gelatinised starch as substrate.

3.9 STORAGE STABILITY OF THE ENZYMES

Solutions of the purified enzymes were kept at room temperature, refridgerator and deep freezer for one month (28days). Aliquots of the enzyme (0.1ml) were assayed every 3-4 days for the level of enzyme activity as described in the assay method.

3.10 EFFECT OF IONS ON ENZYME ACTIVITY

The effects of some metallic ions - Aluminium (Al^{3+}), Zinc (Zn^{2+}), Iron (Fe^{3+}), Copper (Cu^{2+}), Magnesium (Mg^{2+}) and Chloride ion (Cl^{-}) on α-amylase activity were investigated at two ionic concentrations, 1.0mM and 2.5mM. The enzyme activity was assayed using solutions of salt of these metals made in deionised water.

3.11 DETERMINATION OF MOLECULAR WEIGHT

The procedure of Weber and Osborn (1969) was employed. Acrylamide concentration of 10%T was used for the running gels. The gel was prepared from 13.5ml of a mixture of 22.2g acrylamide and 0.6g bis-acrylamide in 100ml of distilled water, 15.0ml of gel buffer containing 20.4g Na_2HPO_4 (anhydrous), 8.82g $NaH_2PO_4.2H_2O$ and 2.0g sodium dodecyl sulphate (SDS) dissolved in 1000ml distilled water. To this mixture was added 0.025ml of N,N,N',N'-tetramethylethylenediamine (TEMED, as activator) and 1.5ml of 1% Ammonium persulphate (APS, as initiator). These were

properly mixed and then poured into the gel tubes. The tubes were layered with 0.5ml of distilled water and allowed to polymerise for 15 minutes.

Each sample (0.15ml) was dissolved in 1.0ml of the treatment buffer containing 1% SDS, 1% 2-mercaptoethanol, 0.01M phosphate buffer (pH 7) and incubated for 30 minutes at 60°C. Treated samples (0.3ml) were mixed with 0.04ml of 10% glycerol, 0.02ml of mercaptoethanol, and 0.02ml of 0.5% bromophenol blue. 0.04ml of each was carefully delivered to each of the gel tubes after discarding the water layer.

Diluted buffer solution (650ml) (1:2 dilution) was poured into the lower buffer reservior while the upper buffer reservoir was filled with 250ml (1:3 dilution) buffer solution. 1.0ml of 0.05% bromophenol blue was added to the upper buffer. The electrophoretic system was run for 6 hours with a constant current of 5mA per tube at ambient temperature.

After electrophoresis, the gels were removed from the running tubes by the irrigation needle and placed in small tubes filled with the staining solution prepared by dissolving 1.25g of coomassie brilliant blue in a mixture of 454ml of 50% methanol and 46ml of trichloroacetic (TCA) overnight after recording the solvent front. The gels were then rinsed and placed in destaining solution of 7% (v/v) glacial acetic acid solution.

The distance migrated by each protein was then recorded and the relative mobility of each protein calculated. The relative mobilities of marker proteins consisting of Trypsin, Pepsin, Egg

albumin, and bovine serum albumin (BSA) were then plotted against the logarithm of their molecular weights (Figure 25). The molecular weights of the α-amylase samples were then calculated from the standard curve (Appendix 8).

3.12 ACTIVE SITE STUDIES

3.12.1 EFFECT OF N-BROMOSUCCINIMIDE (NBS)

The modification of the enzyme was carried out as described by Sirisinha and Allen, (1965b). Equal volumes (5.0ml) of the enzyme and 1.0×10^{-4}M solution of NBS made in 0.1M acetate buffer (pH 4.0) were mixed at 0°C. The reaction was terminated after 90 minutes by the addition of 1×10^{-4}M sodium thiosulphate solution (5ml). The enzyme activity was then assayed. The control experiment contained buffer in place of the NBS.

3.12.2 EFFECT OF P-CHLOROMERCURIBENZOATE (PCMB)

This was carried out as described by Riordan and Vallee (1967). A solution of 6.0×10^{-4}M PCMB (5.0ml) was slowly added to an equal volume of the enzyme solution and the mixture allowed to stand for 1 hour at 0°C. An aliquot (0.1ml) of the modified enzyme protein solution was assayed for the level of enzyme activity while 0.1M acetate buffer pH 4.8 (5.0ml) was used in place of PCMB in the control experiment.

3.12.3 EFFECT OF ACETIC ANHYDRIDE

Saturated sodium acetate solution (10ml) was added to an equal volume of the enzyme solution. The mixture was cooled in an ice bath and treated with acetic anhydride, 10µl, 15µl, 23µl and 60µl respectively for sorghum, millet, bacterial and fungal α-amylases in drops at 0°C in 1 hour course. The mixture was then left for another hour and small volumes (0.1ml) were removed and assayed for enzyme activity as described by Fraenkel-Conrat (1957).

CHAPTER 4

RESULTS AND DISCUSSION

EXTRACTION AND PURIFICATION

The purification of α-amylase from the crude preparation Sorghum (Table 1) and Millet (Table 2) was carried out using the following steps - heat treatment, ammonium sulphate precipitation and gel filtration using sephadex G150-120.

To test the degree of purification of the enzyme after each step, the total volume, protein and enzyme activity of the various enzyme fractions were determined as shown in Tables 1 and 2.

Heat Treatment

The optimum conditions (Temperature and pH) for preferential thermal denaturation of β-amylase of crude extracts from sorghum (figure 1) and millet (figure 2) were found to be 70°C and pH 6.0 and in the presence of 5% and 4% activated decolourising charcoal respectively.

Ammonium Sulphate Precipitate

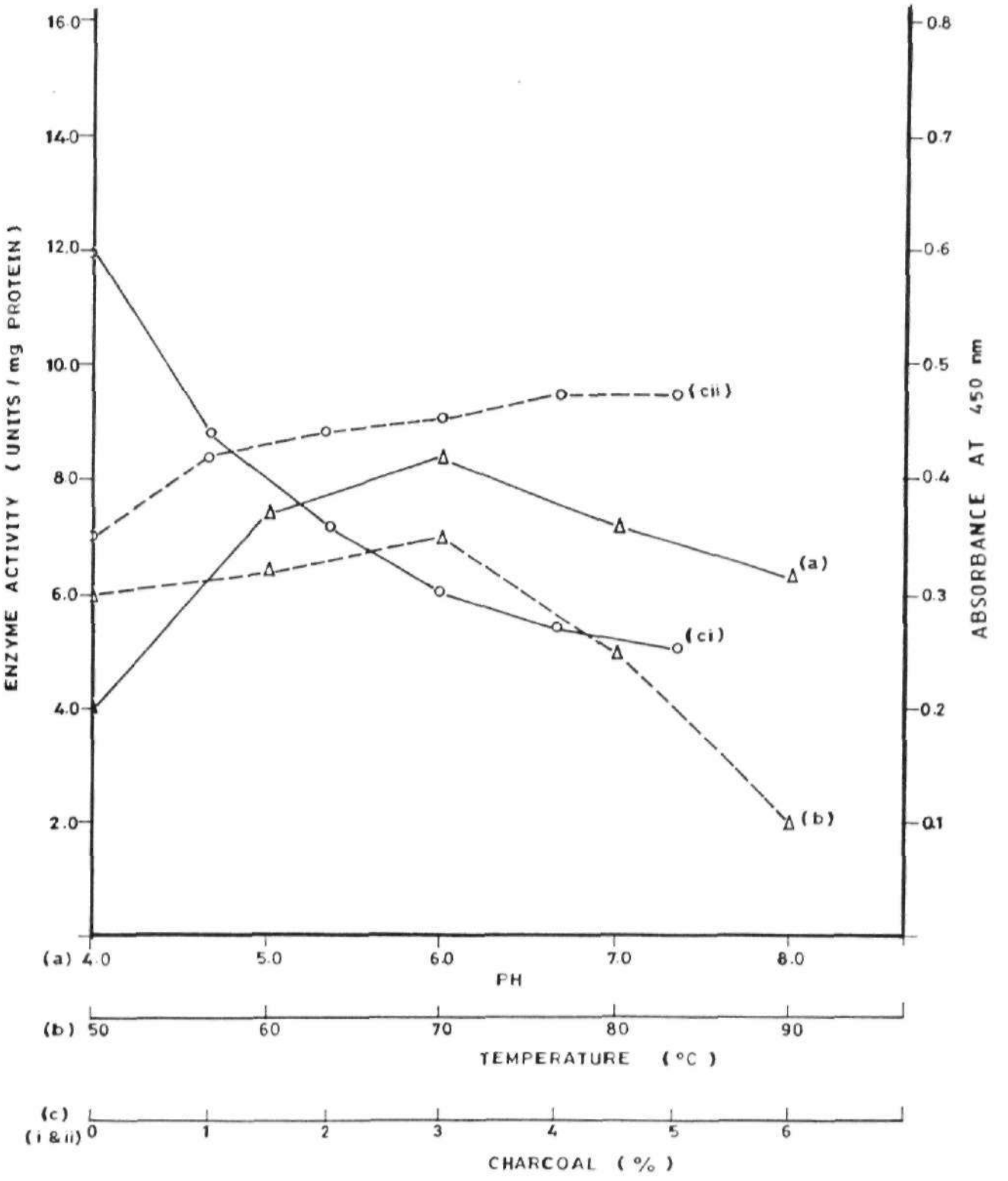
A pilot run carried out to establish the appropriate percentage of ammonium sulphate saturation that is required to precipitate the enzyme after heat treatment is shown in Table 3.

TABLE 1. PURIFICATION PROFILE OF MALTED SORGHUM α-AMYLASE

PURIFICATION STEP	TOTAL PROTEIN CONCENTRATION (mg)	TOTAL α-AMYLASE ACTIVITY (Units)	SPECIFIC ACTIVITY unit/mg	RECOVERY %	PURIFICATION FOLD
Crude extract	2345	1306.5	0.56	100	1
Heated at 70° with 5% charcoal	1254	1034.4	0.82	53.5	1.5
Ammonium sulphate Precipatation	72	196.56	2.73	3.07	4.88
Sephadex G150-120 chromatography	44.6	301.1	6.75	1.92	12.05

TABLE 2: PURIFICATION PROFILE OF MALTED MILLET α-AMYLASE

PURIFICATION STEP	TOTAL PROTEIN CONCENTRATION (mg)	TOTAL α-AMYLASE ACTIVITY (Units)	SPECIFIC ACTIVITY Units/mg	RECOVERY (%)	PURIFICATION FOLD
Crude extract	3330	3738	1.12	100	1
Heated at 70°C with 4% charcoal	2914	3705	1.28	87.9	1.10
Ammonium sulphate Precipitation	96	256.4	2.62	2.94	2.34
Sephadex G150-120 chromatography	75	1140	15.2	2.7	13.57


ENZYME ACTIVITY (UNITS / mg PROTEIN)
ABSORBANCE AT 450 nm
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
(a)
(b)
(ci)
(cii)
(a) 4.0
5.0
6.0
7.0
8.0
PH
(b) 50
60
70
80
90
TEMPERATURE (°C)
(c) (i & ii) 0
1
2
3
4
5
6
CHARCOAL (%)

49

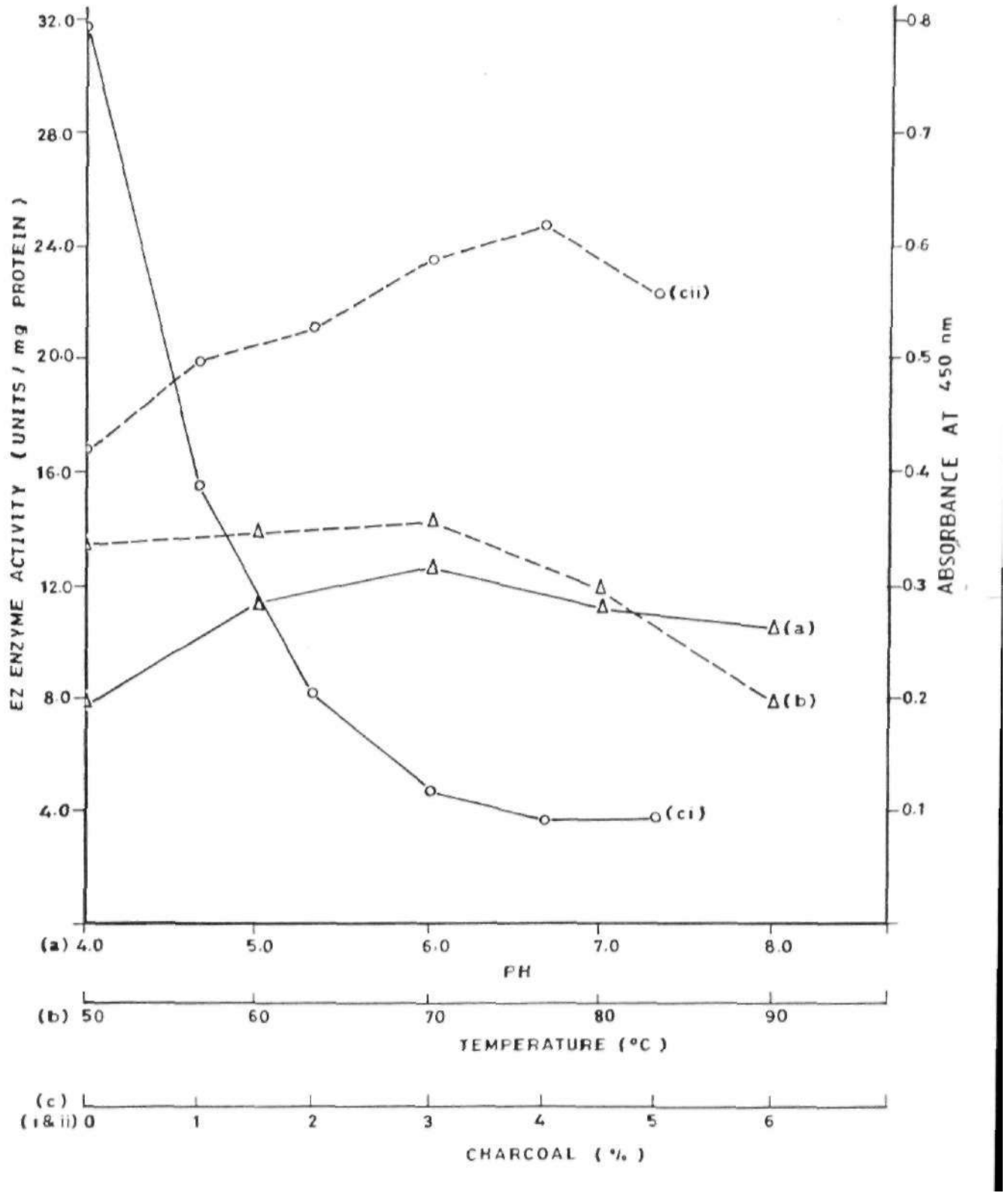

EZ ENZYME ACTIVITY (UNITS / mg PROTEIN)
32.0
28.0
24.0
20.0
16.0
12.0
8.0
4.0
ABSORBANCE AT 450 nm
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
(cii)
Δ(a)
Δ(b)
(ci)
(a) 4.0
5.0
6.0
7.0
8.0
PH
(b) 50
60
70
80
90
TEMPERATURE (°C)
(c)
(i & ii) 0
1
2
3
4
5
6
CHARCOAL (%)

TABLE 3: PILOT RUN FOR AMMONIUM SULPHATE PRECIPITATION

PERCENTAGE $(NH_4)_2SO_4$ SATURATION	α-AMYLASE ACTIVITY (UNITS) MALTED MILLET	MALTED SORGHUM
0-20	0.70	0.00
20-30	1.05	0.35
30-40	1.17	0.50
40-50	0.68	0.82
50-60	0.35	1.28
60-70	0.09	0.88
70-80	-	0.44
80-90	-	0.00

Much of the α-amylase of the sorghum malt was precipitated between 30-60% ammonium sulphate saturation (Table 3) whereas, millet malt enzyme was mainly precipitated at 50% saturation (Table 3). Although ammonium sulphate precipitation produced an increase in the purification fold of both α-amylases, much of the enzyme protein is lost especially in the purification of the sorghum enzyme (Tables 1 and 2).

Gel Filtration chromatography on sephadex G150-120

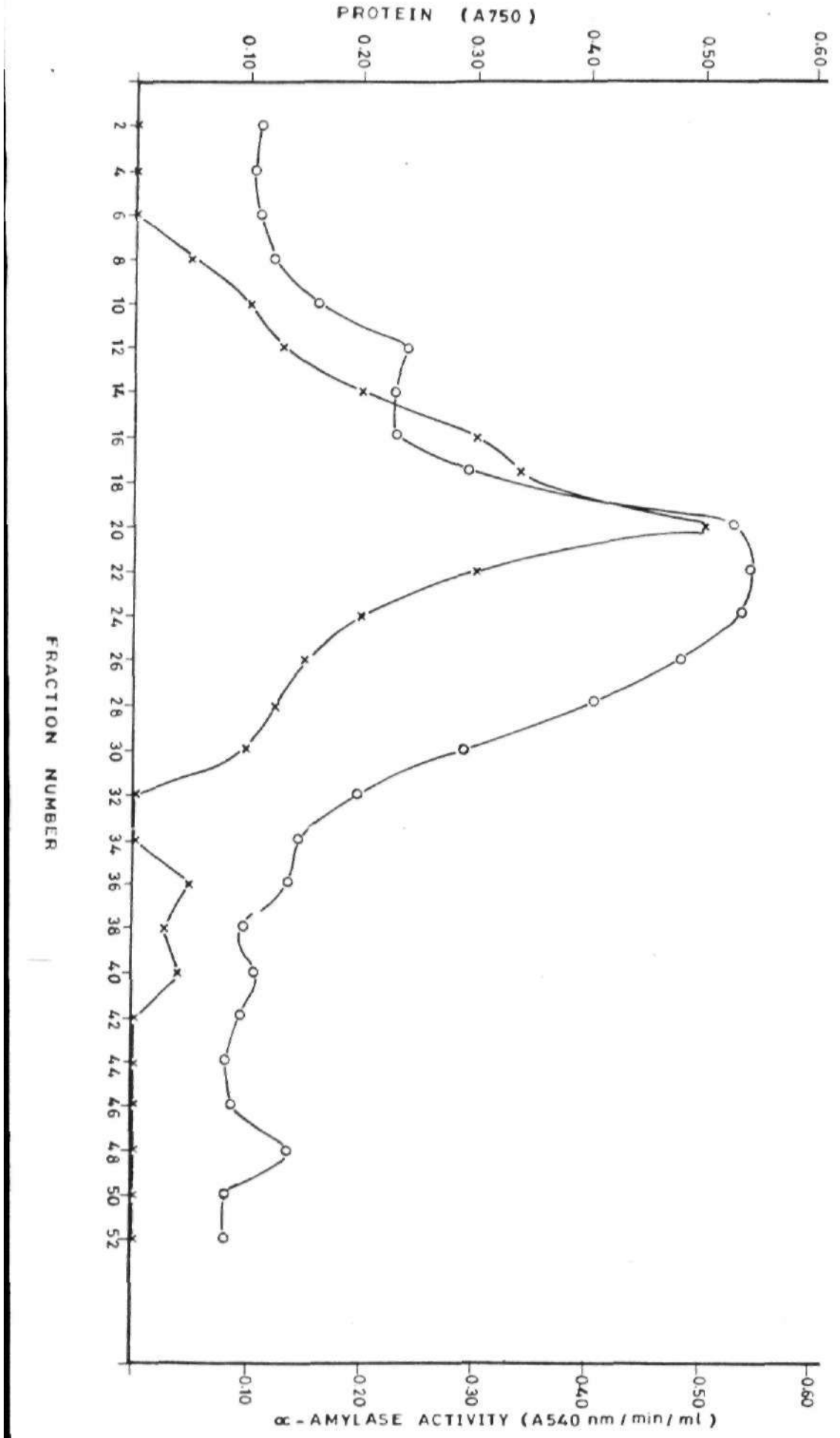
The elution profile of α-amylase from sephadex G150-120 column showed a fairly broad peak (figures 3 and 4). Although the peaks were not very discrete, they were however single. Gel filtration of the ammonium sulphate precipitate on sephadex G150-120 yielded a 12- and 14-fold purification over the crude enzyme from sorghum (Table 1) and millet (Table 2) malts respectively.

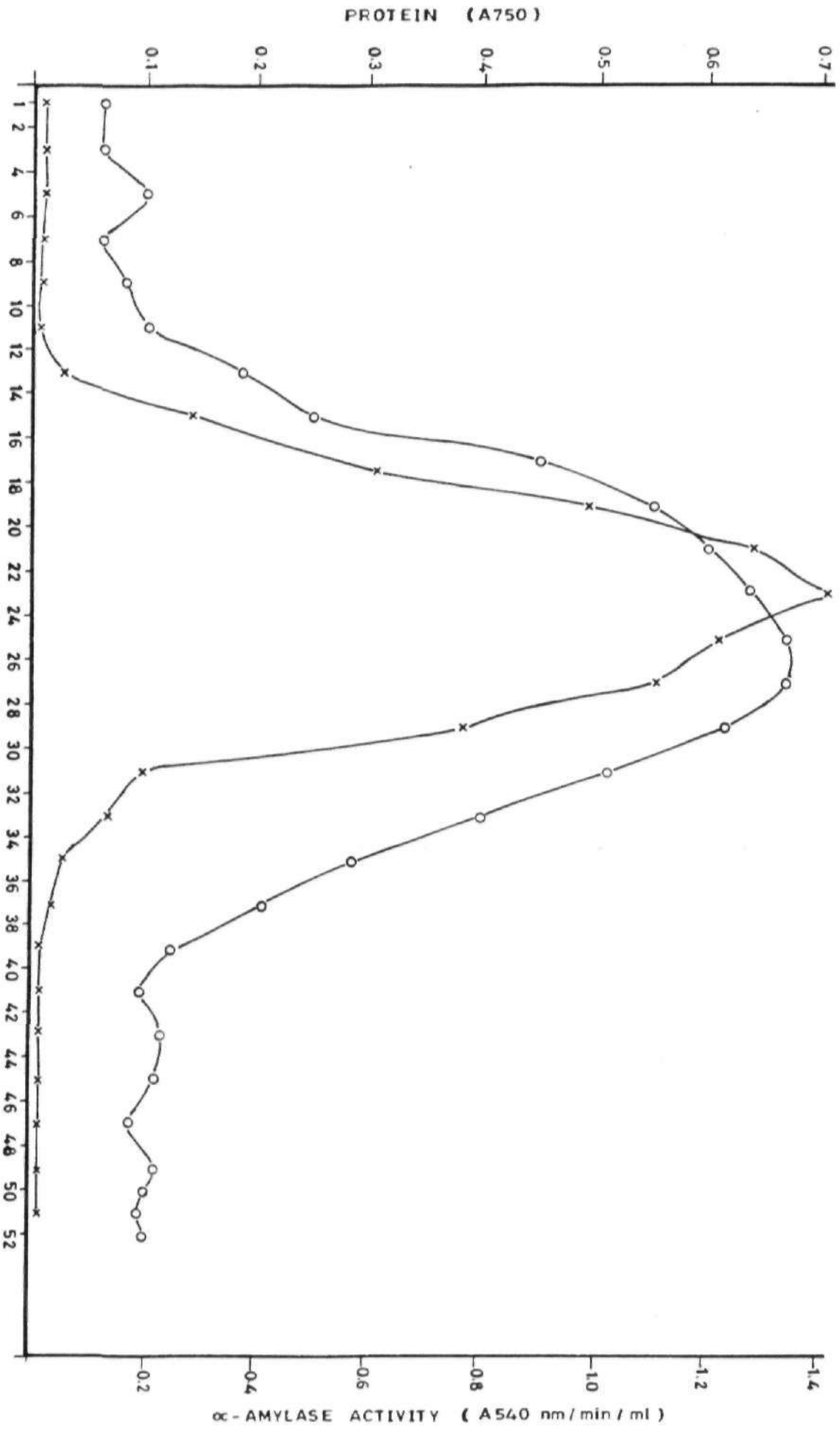
Polyacrylamide gel electrophoresis

Single protein bands were obtained from the electrophoresis of α-amylase from sorghum (Plate 1) and millet (Plate 2) pooled after sephadex G150-120 gel chromatography.

Effect of pH on Enzyme Activity

The effects of pH on the activity of partially purified α-amylase from malted sorghum and millet as well as on purified bacterial α-amylase (Rohalase AT) and fungal α-amylase (Rohalase MS) were studied.


PROTEIN (A750)
0.10
0.20
0.30
0.40
0.50
0.60
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46 48 50 52
FRACTION NUMBER
0.10
0.20
0.30
0.40
0.50
0.60
α - AMYLASE ACTIVITY (A540 nm / min / ml)


PROTEIN (A750)
0.1
0.2
0.3
0.4
0.5
0.6
0.7
1 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46 48 50 52
FRACTION NUMBER
0.2
0.4
0.6
0.8
1.0
1.2
1.4
α - AMYLASE ACTIVITY (A540 nm / min / ml)

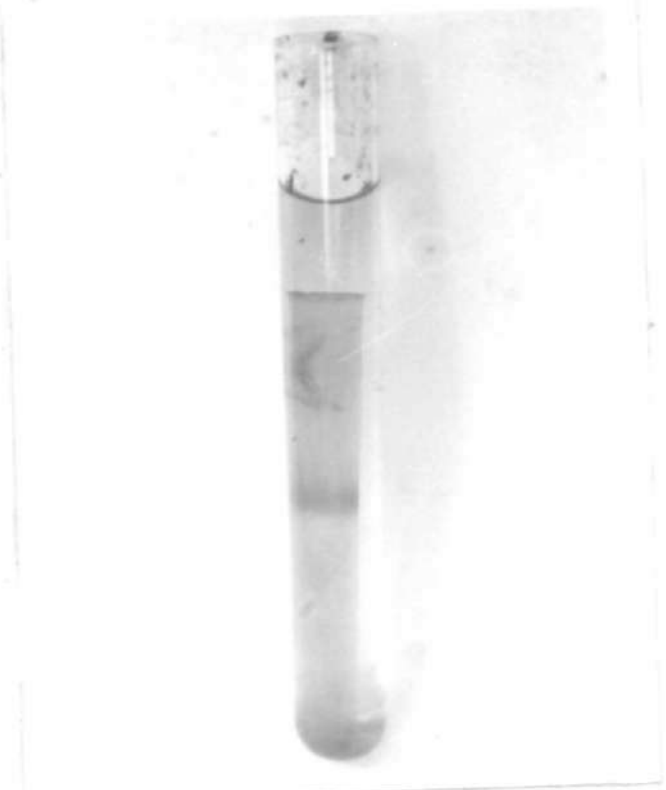
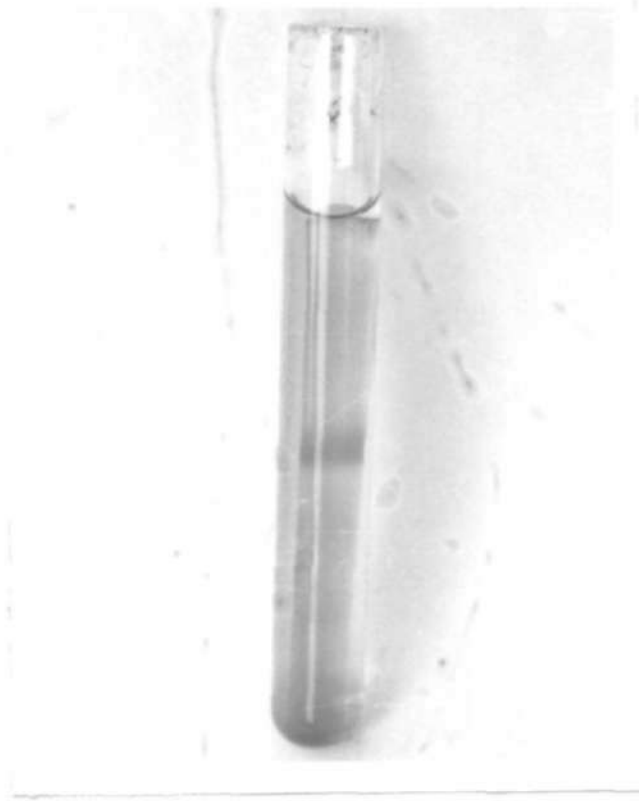


Plate 1 Polyacrylamide gel electrophoresis of partially purified Sorghum α-amylase



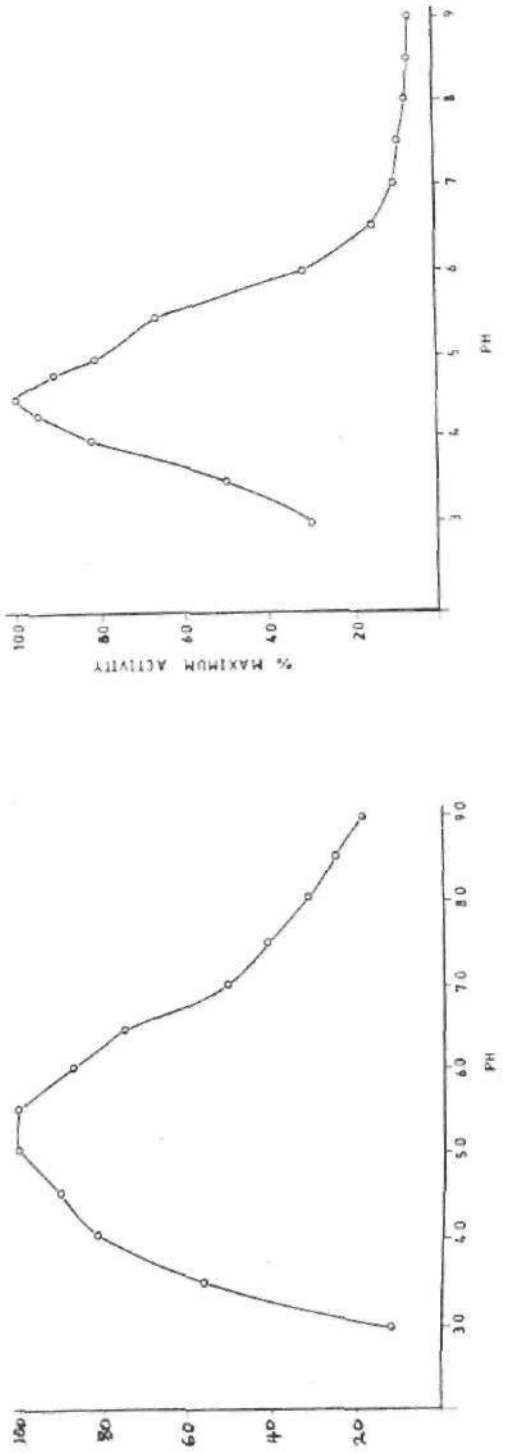
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Ahmadu Bello University
Zaria : Nigeria.

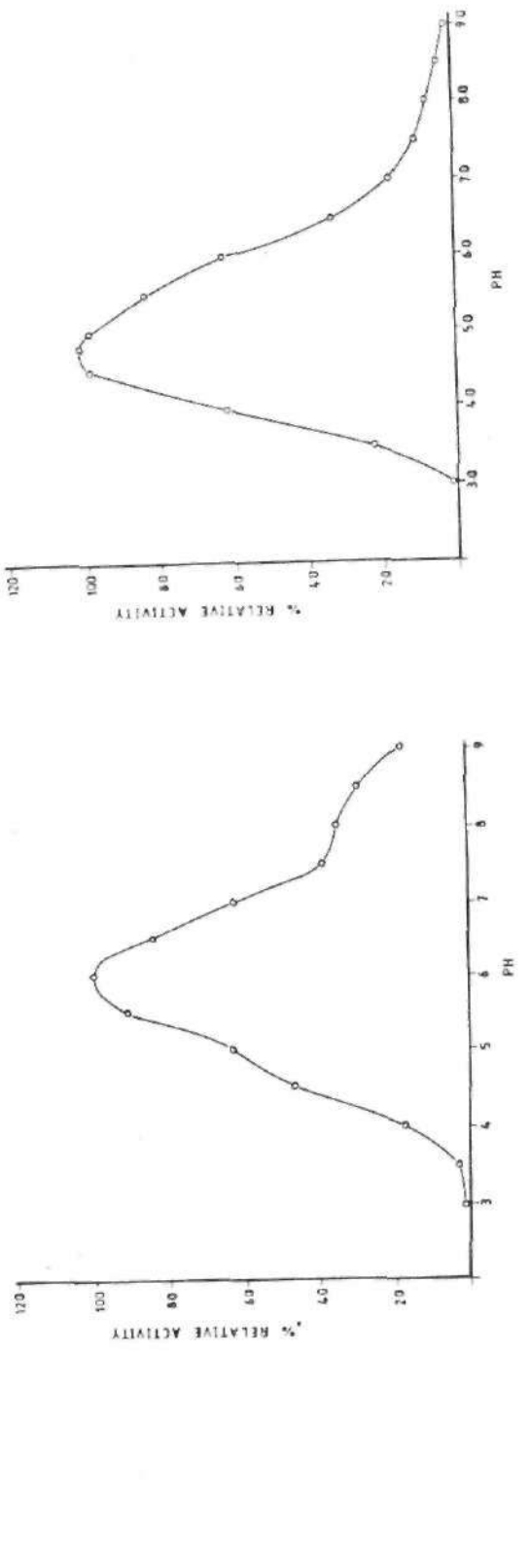
Plate 2 Polyacrylamide gel electrophoresis of partially purified Millet α-amylase

Figures 5, 6, 7, and 8 show that maximum activity (optimum pH of activity) for malted sorghum, and millet, and for bacterial and fungal α-amylase occured at pH values 5.0-5.5, 4.5, 6.0 and 4.6-4.8 respectively. The pH activity curve shows very low enzyme activity in alkaline pH as well as acidic pH values below pH 3.0, except for the millet malt enzyme which exhibited 30% of its maximum activity at pH 3.0 (figure 6).

Effect of Temperature

The effect of heat on the stability as well as on the activity of the α-amylases is shown in figures 9, 10, 11 and 12. Exposure of the enzymes to temperatures up to (50°C) for thirty minutes did not result in denaturation of the enzyme. However, heating the enzymes above this temperature resulted in a decrease in enzyme activity due to heat inactivation or denaturation. The bacterial enzyme, Rohalase AT retained full activity at 60°C and starts denaturing above this temperature (figure 11). The process of heat inactivation of the cereal α-amylases appears to be biphasic, the initial phase (between 50° and 70°C) where the inactivation was almost linear and a region of rapid denaturation (70-80°) (figures 9 and 10). Bacterial α-amylase was rapidly inactivated between 60° and 80°C (figure 12). The fungal amylase, exhibited almost the same pattern of heat denaturation as the bacterial amylase especially between 50° and 60°C (figure 11).


% MAXIMUM ACTIVITY
100
80
60
40
20
3
4
5
6
7
8
9
PH


% RELATIVE ACTIVITY
120
100
80
60
40
20
30
40
50
60
70
80
90
PH

100
80
60
40
20
30
40
50
60
70
80
90
PH

% RELATIVE ACTIVITY
120
100
80
60
40
20
3
4
5
6
7
8
9
PH

When the results of the effect of temperature on the rate of hydrolysis examined over the temperature range of 20°-50°C were plotted in the conventional Arrhenius manner, they exhibited linearity (figures 13, 14, 15 and 16). The average energy of activation was calculated to be 10.05 kcal, 7.48kcal, 6.57kcal and 5.39 kcal for the sorghum malt, millet malt, bacterial and fungal α-amylases respectively (Appendix 5).

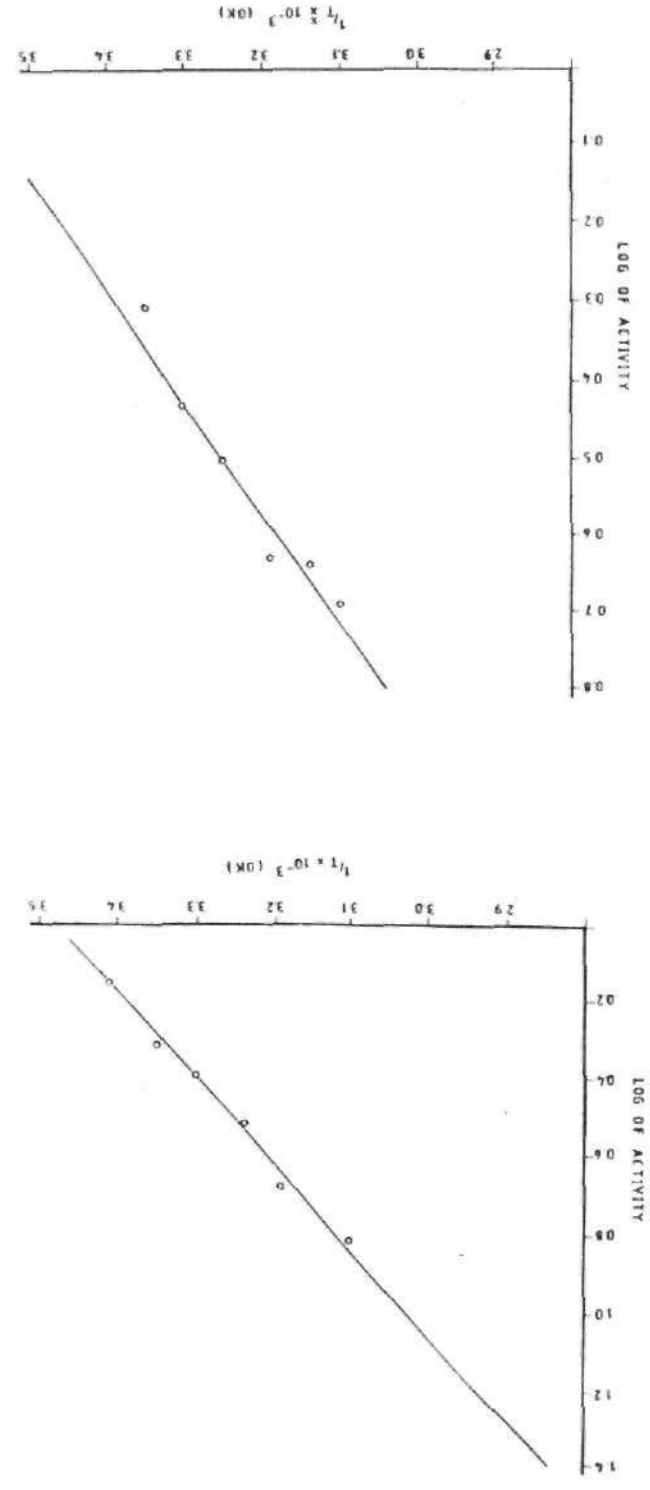
Effect of substrate concentration

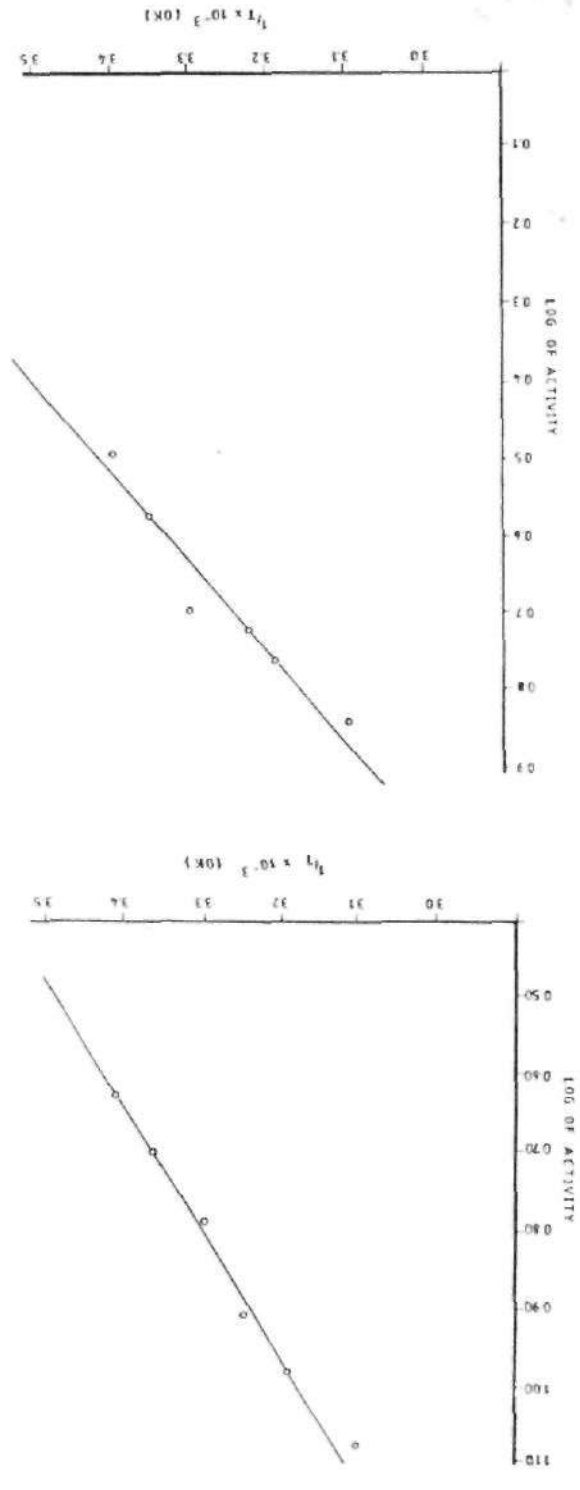
The Michaelis constant (Km) for the enzyme-substrate complex and the maximum velocity (Vmax) of α-amylases from sorghum malt, millet malt, bacteria and fungi as determined from the double reciprocal plots of Lineweaver and Burk, (1934) are shown in figures 17, 18, 19 and 20.

The Km values of 0.156mg starch/ml, 1.56mg starch/ml, 2.10mg starch/ml and 3.55mg starch/ml were calculated for sorghum malt, millet malt, bacterial and fungal α-amylase respectively (Appendix 6). The maximum velocity of this hydrolysis was calculated to be 0.0656mg maltose/min/ml, 0.164mg maltose/min/ml, 0.25mg maltose/min/ml, and 0.345mg/min/ml for these enzymes respectively (figures 17, 18, 19, 20).

Storage stability of α-Amylase

The stability of the enzyme preparations under storage was determined by monitoring the change in activity for a period of

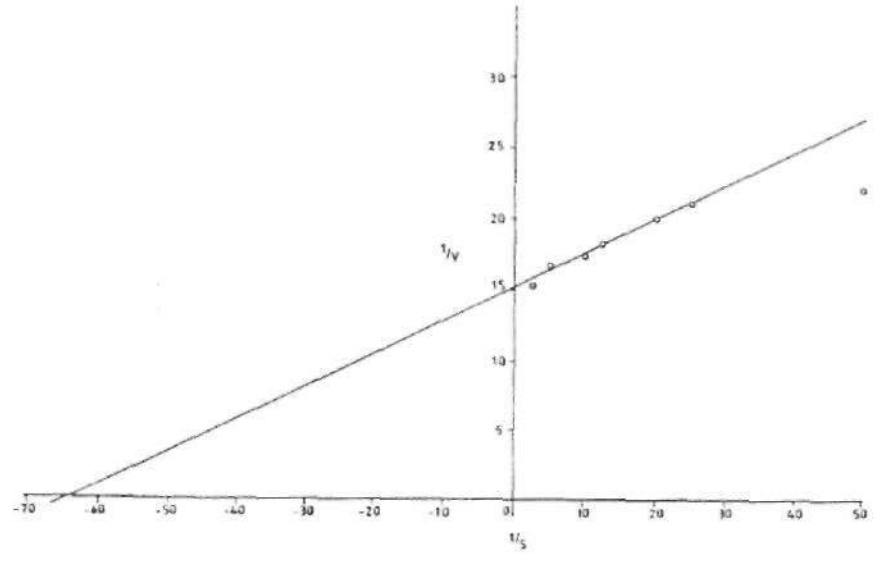

LOG OF ACTIVITY
$^1/_T \times 10^{-3}$ (0K)


LOG OF ACTIVITY
$^1/_T \times 10^{-3}$ (0K)

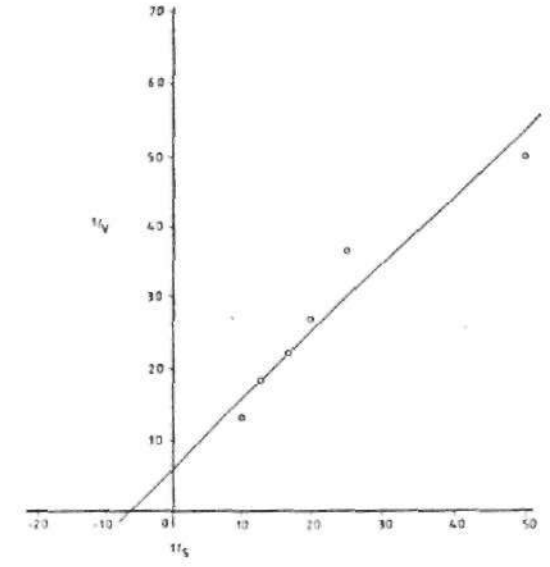
LOG OF ACTIVITY
$^1/_T \times 10^{-3}$ (0K)

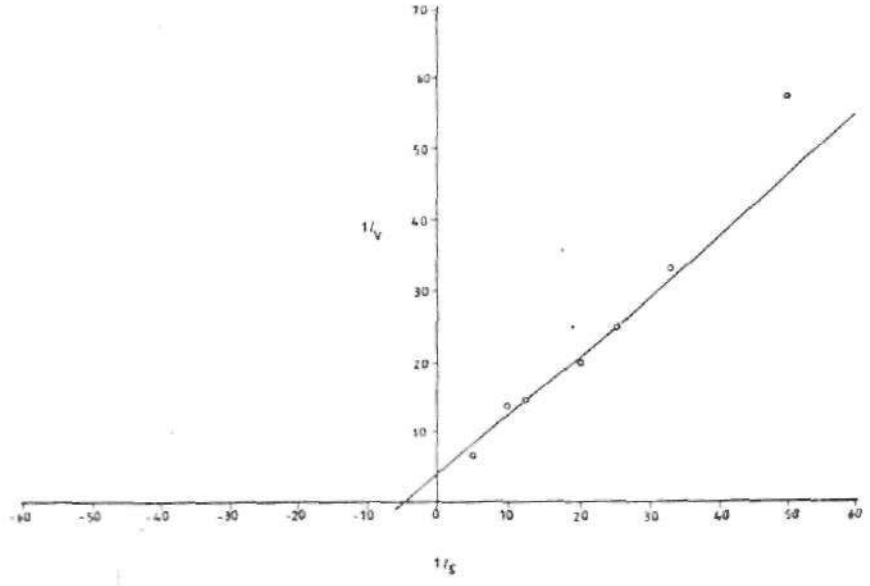
LOG OF ACTIVITY
$^1/_T \times 10^{-3}$ (0K)

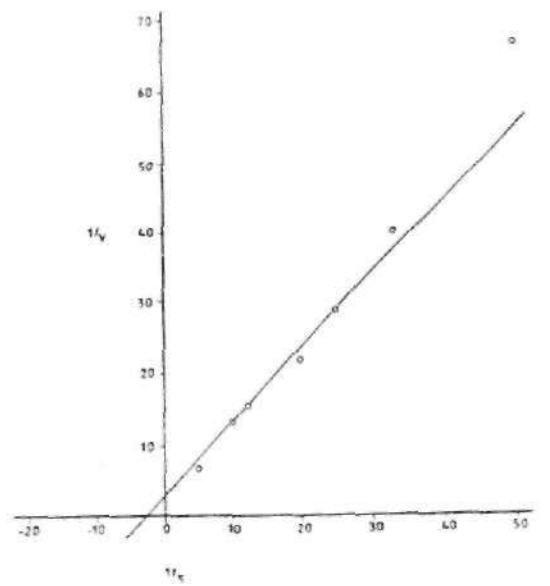
G1



G2







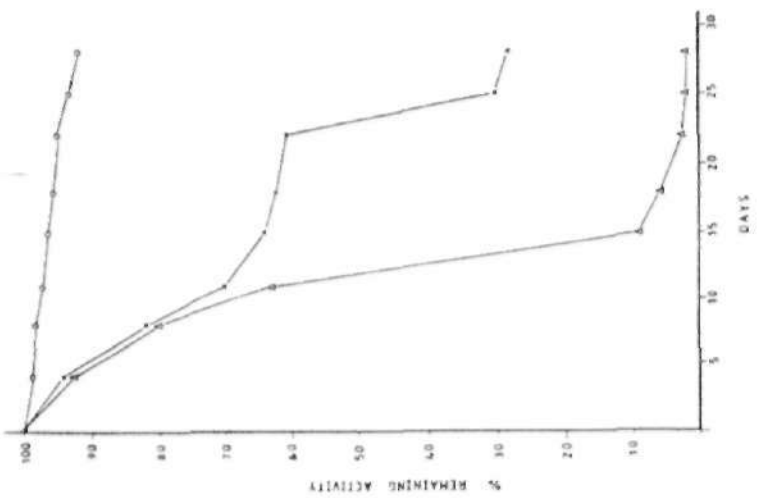
28 days at room temperature, refrigerator temperature, and in frozen state. This has been presented as a plot of remaining activity against time (days) (Figs. 21, 22, 23 and 24).

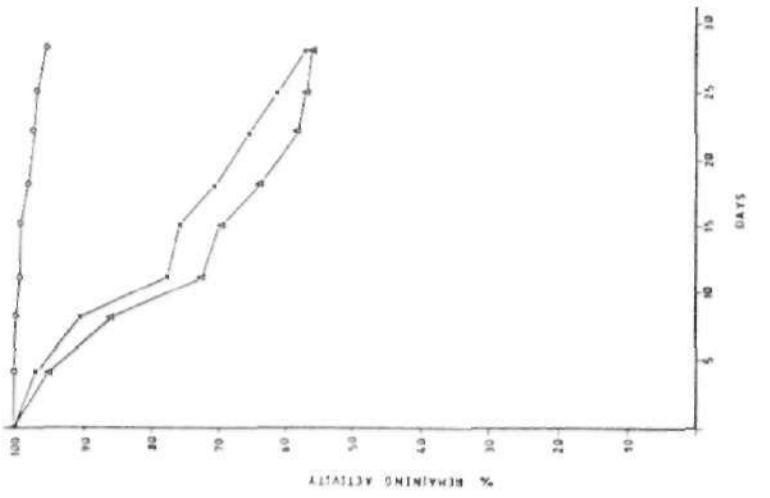
The enzymes stored at frozen state retained over 90% of their initial activity after 28 days of storage (figures 21, 22, 23 and 24). While sorghum malt and fungal α-amylase retained over 50% of their original activity when stored in the refrigerator (figures 21 and 24), the bacterial enzyme completely lost its activity, and retained only 5% on the 14th day of storage (figure 23). Millet α-amylase retained over 60% of its initial activity after 22 days of storage in the refrigerator, then there was rapid loss of activity (figure 22).

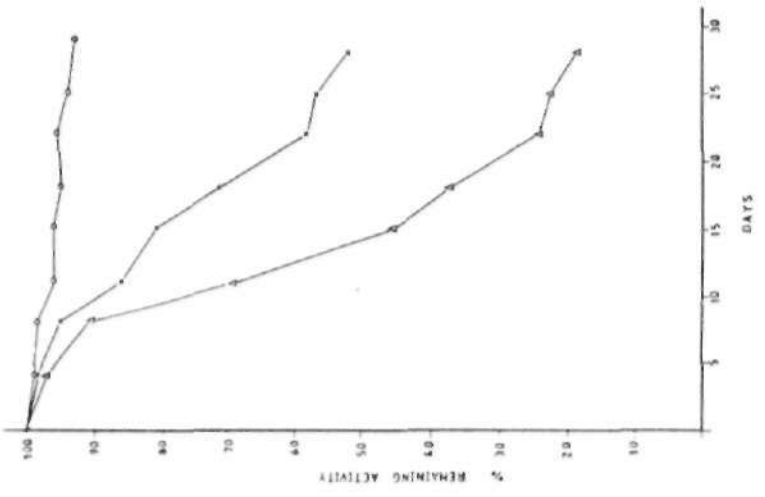
The loss of enzyme activity at room temperature was more rapid than at the other states (figures 21, 22, 23), except for the fungal α-amylase which retained 54% of its original activity after 28 days of storage (figure 24).

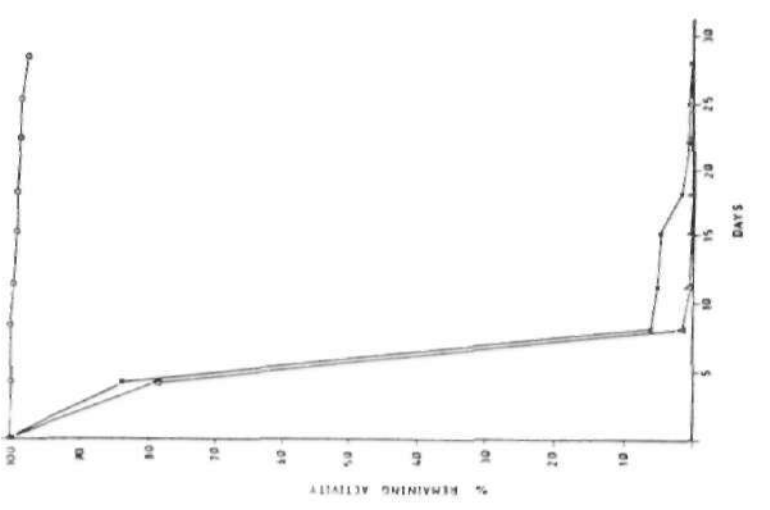
Determination of Molecular Weight

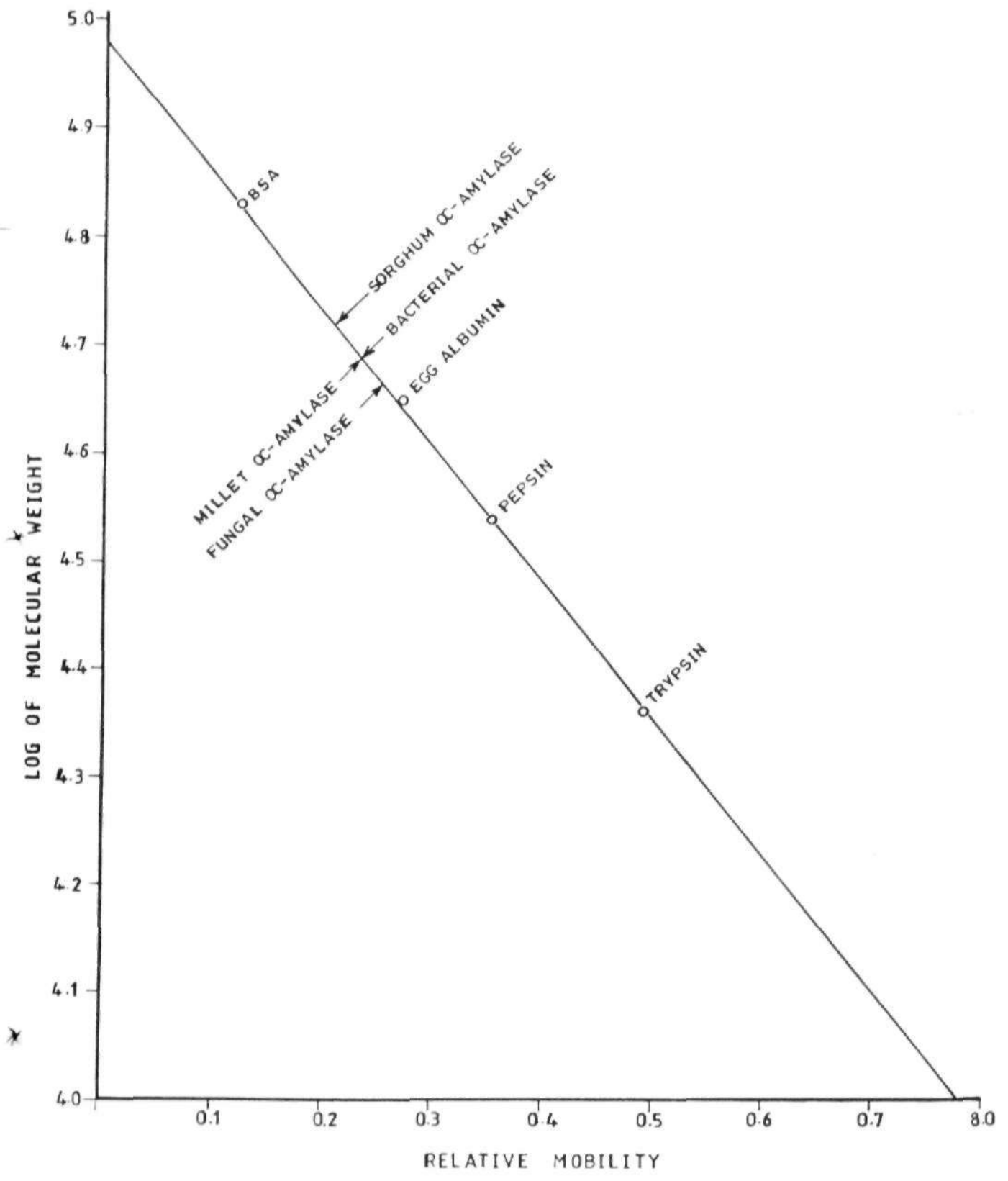
The average molecular weights of the α-amylases determined from two different runs of SDS-polyacrylamide gel electrophoresis were 52, 668 ± 1114, 48, 698 ± 397, 48, 991 ± 1,595 and 47, 140 ± 1022 daltons for sorghum malt, millet malt, bacterial and fungal enzymes respectively (figure 25).


% REMAINING ACTIVITY
DAYS


% REMAINING ACTIVITY
DAYS


% REMAINING ACTIVITY
DAYS


% REMAINING ACTIVITY
DAYS


5.0
4.9
4.8
4.7
4.6
4.5
4.4
4.3
4.2
4.1
4.0
LOG OF MOLECULAR WEIGHT
0.1
0.2
0.3
0.4
0.5
0.6
0.7
8.0
RELATIVE MOBILITY
BSA
SORGHUM α-AMYLASE
BACTERIAL α-AMYLASE
EGG ALBUMIN
MILLET α-AMYLASE
FUNGAL α-AMYLASE
PEPSIN
TRYPSIN

Effect of ions on α-Amylase activity

The result of the effect of copper II (Cu^{2+}), iron III (Fe^{3+}), Magnesium (Mg^{2+}), Aluminium (Al^{3+}), Zinc (Zn^{2+}) and Chloride (Cl^-) ions on the amylolytic activity of α-amylase is shown on table 4.

Copper (Cu^{2+}), Aluminium (Al^{3+}) and Iron (III) Fe^{3+} were observed to inhibit the enzymes especially at 2.5mM concentration (Table 4). Cu^{2+} completely inhibited the cereal α-amylase and reduced the activity of the microbial enzymes by over 75% (Table 4). Chloride ions (Cl^-) had no effect on the cereal α-amylases, however, 2.5mM Cl^- increased the activity of the bacterial enzyme by over two-fold, while it caused a 21% reduction in the activity of the fungal α-amylase. Zn^{2+} and Mg^{2+} were mildly inhibitory to sorghum α-amylase while Mg^{2+} had no effect on millet α-amylase activity. Both ions had no effect on the activity of the microbial enzymes (Table 4). Generally, the activity of the bacterial enzyme was least affected by the presence of the metallic ions than the amylases from other sources.

TABLE 4: EFFECT OF SOME IONS ON α-AMYLASE ACTIVITY

Mode of Effect	Sorghum α-Amylase	Millet α-Amylase	Bacterial α-Amylase	Fungal α-Amylase
1. INHIBITION				
A. Potent	Cu^{2+}	Cu^{2+}(b)		Al^{3+}(b)
				Fe^{3+}(b)
				Cu^{2+}(b)
B. Mild	Fe^{3+} Mg^{2+}	Cu^{2+}(a) Fe^{3+}	Fe^{3+}(b)	Fe^{3+}(a)
	Al^{3+} Zn^{2+}	Al^{3+} Zn^{2+}	Al^{3+}(b) Cu^{2+}	Cu^{2+}(a)
				Al^{3+}(a)
2. NO EFFECT	Cl^-	Cl^- Mg^{2+}	Fe^{3+}(a) Mg^{2+}	Mg^{2+} Zn^{2+}
			Al^{3+}(a) Zn^{2+}	Cl^-
3. ACTIVATION:	-	-	Cl^-	-

Note: (a) ion concentration at 1.0mM only

(b) ion concentration at 2.5mM only.

Determination of Ionizing groups in α-Amylase active Site

From the studies of the effect of pH variation of enzymic activity, the ionization constants of groups believed to be at the active centres of these α-amylases were calculated as described by Alberty and Massey (1954) (Appendix 7) and shown in Table 5.

TABLE 5: IONIZATION CONSTANTS OF α-AMYLASES

Source of α-Amylase	pK_a	pK_b
Sorghum malt	3.45	7.05
Millet malt	3.64	5.54
Bacterial (Rohalase AT)	4.67	7.33
Fungal (Rohalase MS)	3.84	5.56

N-Bromosuccinimide cause almost complete inhibition of amylolytic activity (Table 5). P-chloromercuribenzoate had little or no effect on the enzyme activity as compared to the control (Table 5). The effect of acetylation was generally unspecific (Table 5) since the activity of the enzyme was reduced by only one-half.

TABLE 6: EFFECT OF SITE MODIFYING AGENT ON AMYLOLYTIC ACTIVITY

Reagent	Percentage remaining activity			
	Sorghum malt α-amylase	Millet Malt α-Amylase	Bacterial α-amylase	Fungal α-amylase
N-Bromosuccinimide	6.8	5.7	7.5	8.0
p-chloromercuri-benzoate	94.9	92.4	96.0	92.7
Acetic anhydride	54	60	62	58

DISCUSSION

The α-amylases from sorghum and millet malts were extracted with acetate buffer (pH 4.8) which has been reported (Baisia and Varriano-Marston, 1981) to be very efficient especially when it contains 0.2% calcium acetate or 0.01M calcium chloride. The high activity observed in the crude acetate extract compared to the heat treated fraction occurs because, the assay method measures the activity of both α- and β-amylase (Bernfeld, 1955; Thoma et al, 1971).

Purification of the crude extract was achieved by a combination of several methods (Tables 1 and 2). The preferential thermal inactivation of β-amylase in the presence of the α-component has been reported (Bates et al, 1967) to be characteristic of cereal amylases and has been of wide application in the isolation of α-amylase from these sources. Though Bobe and Nordin, (1961) were unable to demonstrate this preferential thermal β-amylase inactivation with malted sorghum enzymes. Bates et al (1967) showed that when crude malt extract was heated at 70°C for 20 minutes in the presence of 0.2% calcium acetate, β-amylase activity was destroyed while α-amylase remained. The preferential thermal inactivation of β-amylases in the malted sorghum and millet extract in this study conforms with the observation of Bates et al, (1967).

In the determination of the optimum conditions for the heat treatment step, the effect of pH and temperature for thermal denaturation of ß-amylase activity was established (figures 1 and 2) as these parameters especially pH are very crucial in obtaining an active α-amylase preparation (Baleja and Varriano-Marston, 1981). In both enzyme preparations the optimum pH and temperature for inactivation of ß-amylase were pH 6.0 and 70°C respectively (figures 1 and 2). This observation conforms with the suggestion of Bates et al. (1967) that the optimum temperature, pH and time of heat inactivation of cereal ß-amylase are 70°C, 6.0 and 15 minutes respectively, although the time of heating at pH 6.0 has little effect on the specific activity of the α-amylase after 20 minutes of heating (Bates et al. 1967).

The enzyme recovery of millet and sorghum extract was 88% and 55% respectively (Tables 1 and 2). This variation in enzyme recovery could have been influenced by the amount of protein solubilised during the extraction procedure, indicating that high protein concentration in the enzyme extract during heat treatment is critical for removing ß-amylase and recovering α-amylase as suggested by Baleja and Varriano-Marston (1981).

The colouring materials (tannins) found in sorghum and millet extract were removed by means of decolourising charcoal. This treatment also appreciably increased the enzyme activity (figures 1 and 2). This increase in activity is attributed to increase in the stability of the enzyme preparation as a result

of the removal of polyphennis which are known to complex with and inhibit activity of α-amylases (Daiber, 1975).

The determination of α-amylase activity in the crude extract before and after heat treatment showed that about 7% and 22% of the amylase activity was lost during heat treatment of millet and sorghum malt extract (Table 1 and 2). This small drop in activity confirms the report (Aisien, 1989) that sorghum and presumably millet, contains a low level of β-amylase activity. It is possible that millet contains a lower level of β-amylase hence the smaller drop in activity (Table 1 and 2). Subsequent steps in the purification of both enzymes increased the specific activity several times over the heat treated extract. Ammonium sulphate precipitation though it increased the specific activity by almost three-fold in both enzymes (Tables 1 and 2), it was accompanied by low protein recovery. The sphadex G150-120 chromatography increased the specific activity of the millet and sorghum malt enzymes by almost 14- and 12-fold over that of the crude extract (Table 1 and 2). The elution profile of both enzymes showed single peaks (figures 3 and 4) suggesting the presence of one protein in the eluate. These values conform with the 14-fold and 17-fold purification reported by Botes et al. (1967) and Abdul-Hussain and Varriano-Marston (1982) for sorghum malt and millet malt α-amylases respectively. However, Belsia and Varriano-Marston (1981) reported 21-fold and 60-fold purification for millet malt α-amylase purified by starch- and glycogen-complex formation respectively. This variation is not

unexpected since the methods of purification used by these investigators are different from those used in this study.

Electrophoresis on polyacrylamide gel at pH 7.0, gave a single protein band for the purified enzyme preparations of both malted sorghum and millet (Plate 1 and 2). It has been reported (MacGregor, 1977) that many α-amylases including those of cereals origin, are not homogenous. Although, Bates et al. (1967) and Baleja and Varriano-Marston (1981) showed the occurence of isoenzyme forms in sorghum and millet α-amylase respectively, results from this work conform with those of Abdul-Hussain and Varriano-Marston (1982) who observed a single protein band after electrophoresis.

The occurence of a single band of enzyme protein on electrophoresis does not necessarily imply the non-existence of isoenzyme forms as observed by Bates et al (1967) for sorghum α-amylase, since the properties of these isoenzymes may be so close as to evade detection but it essentially establishes the state of purity of the enzyme preparation (Bates et al, 1967).

The α-amylases from malted sorghum and from fungi, showed broad pH optimum of 5.0-5.5 and 4.6-4.8 respectively (figures 5 and 8) while the optimum pH of the α-amylase from millet malt and bacteria were distinct - pH 4.5 and 6.0 respectively (figures 6 and 7). This optimum pH of sorghum malt is slightly higher than 4.8 reported by Acharya (1934), 4.6 by Dube and Nordin (1961) and

4.5-5.0 by Bates et al. (1967). The optimum pH of 4.5 obtained for millet α-amylase compares well with 4.4 and 4.4-4.8 reported by Balain and Varriano-Marston (1981) and 4.0-4.5 obtained by Abdul-Hussain and Varriano-Marston (1982). Although the pH optimum of sorghum malt enzyme was slightly higher than other reported data, it agrees with the pH optimum of 5.0 reported for barley malt enzyme by Acharya (1984), and 5.5 by MacGregor et al. (1974). Thachuk and Kruger (1974) and MacGregor (1978b) for immature barley, wheat and barley malt α-amylases respectively. It also conforms with 4.4-5.4 reported (Fuda et al. 1975a) for barley malt α-amylase. The pH optimum of 4.5 obtained for the mold α-amylase, Rohalase M3 was in good agreement with 4.5 obtained for acid-stable α-amylase from Aspergillus aureus (A. aureus) (Tanaka et al. 1976) and slightly at variance with 4.8-5.2 reported by Ghai and Chahal (1980) for Aspergillus Flavus (F-14) (A. Flavus (F-14)) and 4.0 for Aspergillus Niger (A. Niger) acid-stable α-amylase (Thoma et al. 1970). However, this pH value was lower than 6.3 obtained for schwanniomyces alluvius (S. alluvius) (Ingledew and Wilson, 1982), 6.6 and 7.1 for two α-amylase fractions of lentinus edodes (L. edodes) (El-Zalaki and Hamza, 1979).

The bacterial α-amylase exhibited an optimum pH of 6.0 (figure 7). This agrees with the 5.8-6.8 and 6.0 reported respectively for Bacillus subtilis (B. subtilis) α-amylase by Fischer and de Montmollin (1957) and Menzi et al (1957). This optimum pH also conforms with pH optimum reported for α-amylase

produced by other strains of bacteria especially those from thermophilic organisms. Markouritz et al (1956) reported an optimal pH range of 5.25-5.75 for Pseudomonas saccharophila (P. saccharophila) α-amylase; Manning and Campbell (1961), 4.6-5.1 for Bacillus stearothermophilus (B. stearothermophilus; Chiang et al, (1970), 6.0-7.0 for Bacillus licheniformis (B. licheniformis; Madda and Chandra (1960), 7.5-8.5 and 9.5 for new strains of B. coagulans and B. licheniformis) repectively; Srivastava (1987), 6.9 for B. stearothermophilus and Stukalin et al(1968) 5.5-7.0 for B. subtilis 1621 strain.

Generally, α-amylases from cereals and microorganisms exhibited maximal activity in a slight to moderate acidic environment having their pH optima mostly in the region of 4.5-7.0 (figures, 5, 6, 7, and 8). The acid tolerance capacity is in the order of Millet malt > sorghum malt > fungal > bacteria (figures 5, 6, 7 and 8). Millet and sorghum malt α-amylases, like the enzyme from immature barley, barley malt and wheat (Tkachuk and Kruger, 1974; MacGregor et al, 1974; MacGregor, 1978b), possessed an unsually high activity and stability in the pH region of 4-5, where most α-amylases from higher plants undergo rapid loss of activity (Dube and Nordin, 1961).

α-Amylases from malted millet and from fungi retain very little activity at pH values greater than or equal to neutrality (10% and 17% respectively at pH 7.0) (figures 6 and 8). On the other hand, the α-amylases from bacteria and sorghum malt exhibit fairly high (61% and 50% respectively) activities at neutral pH

(figures 5 and 7). The acidic preference of the fungal enzyme was attributed to the preponderance of acidic amino acids in these enzymes (Stein et al. 1960). It is also suggested (Abdul-Hussain and Varriano-Marston, 1982) that the acid tolerance of the millet enzyme may be due to its poor basic amino acid content. One feature which is not a common characteristic of these enzymes was the occurence of double pH optima as reported by Acharya (1934) for sorghum α-amylase.

The temperatures of maximum activity of sorghum malt, millet malt, bacterial and fungal α-amylase as determined with 0.1% acetate buffer pH 4.6 containing 0.2% calcium chloride were 60°, 64-65°, 70-75° and 50°C respectively (figures 9, 10, 11 and 12). This optima temperature for sorghum malt enzyme was slightly higher than the 50° reported by Acharya (1934). Also the value for millet malt observed in this study was slightly higher than 55° and 45-55oC reported respectively by Belein and Varriano-Marston, (1981) and Abdul-Hussain and Varriano-Marston, (1982). The temperature optimum for sorghum and millet malt α-amylases also show slight variation from the 55°C reported for both immature barley (MacGregor et al. 1974) and barley malt (MacGregor, 1976b) α-amylase. However, they agree with 55°-60°C reported (Foda et al. 1975b) for barley malt enzyme.

The temperature optimum obtained for the bacterial enzyme conforms with the values reported by other investigators; 60°-70°C for B. coagulans and B. stearothermophilus (Campbell, 1955); 55°-70°C for B. stearothermophilus (Manning and Campbell, 1961);

85°C for B. licheniformis (Chiang et al. 1979); 85°C and 91°C for B. coagulans and B. licheniformis (Mehta and Chandra, 1988); 80°C for B. stearothermophilus (Srivastava, 1987) and 60°-75°C for the strain B. subtilis 1621 (Stukalin et al. 1988). Similarly, the optimal temperature obtained for the fungal enzyme was in conformity with those reported for α-amylases produced by a number of molds, viz. 55°C for P. saccharophila (Markovitz et al. 1956); 60°C for L. edodes (El-Zalaki and Hamza, 1979), A. flavus (F-14) (Ghai and Chahal, 1980) and S. olivaceus (Ingledew and Wilson, 1982) α-amylases. Although, these purified enzymes exhibited slight variations in their temperatures of maximum activity compared to reported values (figures 9, 10, 11 and 12), these differences may however, not be significant as these enzyme preparations are of different purity states. Therefore the differences may be due to variation in purity rather than any inherent differences in the chemical and physical properties of the enzymes.

The α-amylases from sorghum, millet and bacteria are unstable at their optimal temperatures, retaining only 84%, 76% and 76% respectively of their initial activity at these temperatures (figures 9, 10 and 11) while the fungal enzyme retained 100% activity at its optimal temperature (figure 12). Heat inactivation of these α-amylases and presumably many other enzymes is dependent on the state of purity of the enzyme, therefore the stability of the enzyme decreases as the state of purity increases (Tkachuk and Kruger, 1976). This explains the

high stability of the malt α-amylase in the crude extraction at 70°C when compared to the purified enzyme. Similarly, the differences in the temperature of maximal stability and that of activity could be accounted for by the protection of the enzymes by the starch substrate (Tkachuk and Kruger, 1976). This protective effect of starch on α-amylase implies that, the formation of enzyme-substrate complex results in a tightening of the configuration of the α-amylase molecule, which in turn strenghtens the binding of the calcium ion (Vallee et al, 1959).

The temperature optimum of sorghum and millet α-amylases is of industrial interest as one of the major problems militating against the complete and successful replacement of barley malt by sorghum is the high gelatinisation temperature (69°-75°C) of sorghum grain starch (Aisien, 1989; Arri, 1989). Therefore the lower gelatinisation temperature of the millet starch (55°-70°C) (Wankhede et al, 1979; Belaia et al, 1980) vis-a-vis the higher optimum temperature and stability of its α-amylases portrays millet as a better alternative to barley than sorghum.

Arrhenius plots of the results of the effect of temperature on the enzyme activity gave activation energies of 10.05 kcal, 7.43kcal, 6.57kcal and 5.39kcal per mole per degree over the temperature range of 20°-50°C for sorghum, millet, bacterial and fungal α-amylases respectively (figures 13, 14, 15 and 16). The energy of activation for the sorghum enzyme varies slightly from the 12kcal/mole/degree reported by Botes et al (1967) but it is considerably and slightly higher than the 7.05kcal/mole/degree

(Fischer and de Montmollin, 1951); and 9.7kcal/mole/degree (MacGregor, 1978b) reported for barley malt enzyme and 9.7kcal/mole/degree for immature barley and malted wheat α-amylase (MacGregor et al, 1974; Tkachuk and Kruger, 1974) respectively. The value for millet enzyme agrees with that for barley (Fischer and deMontmollin, 1951). With some exceptions, most reports for plant α-amylase activation energy are in the range of 7.1 to 8.6kcal/mole/degree (Tkachuk and Kruger, 1974).

There is a wide variation in the energy of activation obtained for the microbial enzymes in comparism with the cereal α-amylases as shown by the reports of many investigators. Fischer and de Montmollin (1951) reported 10.50kcal/mole/degree for A. oryzae α-amylase while 14.0kcal/mole/degree was calculated for P. saccharophila enzyme (Markovitz et al, 1956). Menzi et al. (1957) reported 15.0kcal (0-12°) and 11.0kcal (> 12°) for B.subtilis enzyme. DePinto and Campbell, (1968) reported 12.5kcal/mole/degree for B. Macerans α-amylase. The most satisfactory explanation for this variation in energy of activation is that most of these determinations were carried out at widely varying temperature ranges.

The Michaelis constant (Km) values calculated from the effect of substrate concentration on α-amylase activities plotted according to Lineweaver and Burk (1934) (figures 17, 18, 19 and 20) were 0.156mg starch/ml, 1.56mg starch/ml, 2.10mg starch/ml and 3.55mg starch/ml respectively for surghum, millet, bacterial and fungal α-amylases. Though the reported Km for α-amylases of

animal, plant and microbial origin ranged from 0.18 to 8.65mg starch/ml, the data obtained from this study as well as those of other investigators (Markovitz et al, 1956; Bates et al, 1967; DePinto and Campbell, 1968; Marchylo et al, 1974; Ghai and Chahal, 1980; Ingledew and Wilson, 1982) vary considerably from this range. One striking feature from the Km data of these enzymes is the lower Km values of the plant α-amylases. This strong affinity of the plant α-amylase for the starch substrate is however not surprising since this substrate is from a plant source.

The data on figures 21, 22, 23, 24 show that all the enzyme preparations store excellently in the frozen state since all of them retained over 90% of their initial activity. The small drop in the activity of these enzymes might be due to thawing. When kept in the refrigerator for 4 weeks, the sorghum, millet, bacterial and fungal enzyme preparations lost 48, 91.5, 100 and 43% of the initial activity respectively (figure 21, 22, 23 and 24). Storage of these enzymes at room temperature led generally to very high loss of enzyme activity. The fungal α-amylase which retained over 50% of its initial activity after a 28-day storage was the only exception. The complete loss of activity of the bacterial α-amylase when stored at both refrigerator and room temperatures implies that this enzyme preparation is highly unstable at these temperatures and probably at temperatures above 0°C. This observation is of industrial importance since presently, brewing with sorghum malt involves the use of exogen-

ous thermostable α-amylase (Aisien 1989; Arri, 1989) most of which are of bacterial origin.

The effect of calcium ions on α-amylase activity was extensively studied by Stein and his colleagues (1964) and they concluded that calcium ions play a vital role in the stability of all α-amylases. In addition calcium ions were found to be essential for activity of cereal α-amylases (Thoma et al. 1971). In this study, copper ion was observed to be a potent inhibitor of the cereal α-amylase (Table 4), while iron and aluminium ions in addition to copper ion potently inhibited the fungal α-amylase (Table 4). Iron, Magnesium, Aluminium and Zinc ions were generally mildly inhibitory (Table 4). Whereas chloride ion had no effect on the activity of the cereal and fungal α-amylase, it increased the activity of the bacterial enzyme (Table 4). This activating effect of chloride ions on the bacterial α-amylase conforms with the report of Fischer and de Montmollin (1951) and Fischer and Stein (1960). The observation that the bacterial enzyme is not potently inhibited by any ion coupled with the activating effect of chloride ion might also be of industrial interest.

The reduction in activity of the enzymes in the presence of copper, iron and aluminium ion in this study is consistent with previous findings (Mous et al. 1956; El-Farra et al. 1976; Tanaka et al. 1976; Srivastava, 1987). Mous et al. (1956) concluded that the heavy metals typified by lead, silver and copper shorten the half-life of α-amylases much quicker than

neutral salts. Although the mechanism of this "heavy metal" inactivation is not well understood (Greenwood and Milne, 1968b), Feeney et al. (1956) earlier proposed that this inactivation could be due to complex formation or to heavy metal catalysed hydrolysis of peptide or disulfide linkages or to breakage of hydrogen bonds.

The estimated molecular weight (M.W) of purified α-amylases from malted sorghum, and millet and from bacterial and fungal origin were 52, 668 ± 1114, 48, 698 ± 397, 48, 991 ± 1595 and 47,1400 ± 1022 daltons respectively. The molecular weight for millet α-amylase agrees very well with 48,000 reported by Abdul-Hussain and Varriano-Marston (1982) and between 31,000 and 53,000 by Belais and Varriano-Marston (1981). Also the 52,668 obtained for sorghum α-amylase conforms with 47,700-53,000 reported by Botes et al. (1967). Although the molecular weights obtained here for the malt enzyme vary slightly from the 42,000-46,000 reported by Greenwood and Milne (1968a) for most cereal α-amylases, they compare well with the molecular weight of 41,500-42,500 reported for malted wheat, and barley (Thachuk and Kruger, 1974; Rudaway, 1978), and 52,000-54,000 reported for immature wheat and malted barley (Marchylo et al. 1976; MacGregor, 1978b) α-amylases. However, these values are at variance with the 59,500 daltons previously reported (Schwimmer and Balls, 1949) for the barley malt enzyme.

The molecular weight of 48,991 obtained for the bacterial α-amylase is also in good agreement with 47,000-48,000 reported for

B. subtilis and B. stearothermophilus α-amylases (Junge et al. 1959; Thoma et al. 1971; Srivastava, 1987). The 47,050 daltons obtained for the fungal α-amylase, though differing from 58,000; 60,000 and 61,000 daltons (Tanaka et al. 1976; Kvesitadze et al. 1978) reported for A. Aureus α-amylase and an acid-stable α-amylase from A. Niger respectively, it agrees with 50,000 and 51,000 reported (Stein et al. 1960; Sirisinha and Allen, 1965a; Kvesitadze et al. 1978; Takagi, 1981) for A. Flavus and A. oryzae α-amylases. Although a multiplicity of molecular weights has been reported (Bates et al. 1967; MacGregor, 1977; Belaia and Yarriano-Marston, 1981) for cereal α-amylases, such occurance is not compatible with the result of this study and those of Abdni-Hussain and Yarriano-Marston, (1982).

The nature of the ionizing groups in the enzyme active site concerned with the breakdown of the enzyme substrate complex was infered from the pK values determined from the pH profiles. The group with pKa of 3.45, 3.64, 4.67 and 3.84 for sorghum malt, millet malt, bacterial and fungal α-amylases respectively (Table 5) is likely to be a carboxyl group (as in aspartic and glumatic acid) while the group with pK_a of 7.05, 5.54, 7.33 and 5.56 for sorghum, millet, bacterial and fungal α-amylases respectively (Table 5) is most probably an imidazolium ring (as in histidine).

To confirm the presence of histidine in the active centre of these α-amylases, the enzyme preparations were assayed, after being treated with 3.0×10^{-5}M p-chloromercuric benzoate, 5×10^{-3}M N-Bromosuccinimide and acetic anhydride. Almost complete

inhibition of the α-amylolytic activity by N-Bromosuccinimide suggests an involvement of phenolic, imidazolium or sulfyhydryl group in α-amylase catalysis (Table 6). However, the lack of appreciable effect of the p-chloromercuribenzoate (Table 6) implies that sulfyhydryl group is not essential for α-amylolysis. The lowering of activity by acetic anhydride (Table 6) indicates that ε-amino group may play some role in the catalytic action of α-amylase. However, since this reagent is only fairly specific for ε-amino group, coupled with the fact that acetylation induces minor but significant changes in the physical and biologic properties of proteins (Thoma et al, 1971), the most probable explanation for the reduction in enzyme activity on acetylation is therefore the change in protein conformation and the non-specificity of the process. Thus imidazolium or phenol group or both form part of the active centre. The specific involvement of phenolic group in the catalytic process of enzymes has not been confirmed (Zeffren and Hall, 1973). This is due to the inability of group specific reagents to cause complete loss of activity when one or two tyrosine residues are modified. This suggests that though phenolic groups may not be involved in catalysis, a reactive phenolic groups in the vicinity of the active site may be involved in the modification. Therefore the catalytic function of these amylases could be assigned to carboxyl and imidazolium groups. This is , moreso considering that the pK of tyrosine phenolic group ranges from 9.8 to 10.4 as suggested by Thoma et al, (1971).

CHAPTER 5

CONCLUSION AND RECOMMENDATION

From the results obtained in this study, there is a strong indication that it is possible to substitute barley malt with those of our locally grown cereals - millet and sorghum for brewing.

Biochemical profiles show that there exist close similarities in the optimum temperature and pH of α-amylases from malted sorghum, millet and barley and the fungal α-amylase. Also similar trends were observed in the thermal stabilities of these enzymes. The bacterial α-amylases were found to possess higher optimum pH and temperature and thermal stability values.

It was observed that all the enzymes were very stable when stored in the frozen state, stability decreasing with increase in storage temperature. The effects of ions indicate that copper is a potent inhibitor of the cereal α-amylases while Zinc, Aluminium, Iron and Magnesium are mild inhibitors. Similarly, Copper in addition to Aluminium and Iron are strong inhibitors of the fungal α-amylase whereas they mildly inhibit the bacterial enzyme. However, chloride ion was found to have no effect on the α-amylases except the bacterial enzyme which it activated. Investigation of the functional groups involved in catalysis implicates to carboxyl and imidazole groups as being involved in

the amylolysis by these enzymes. From the results of this research, it could be concluded that although much emphasis has been placed on sorghum as the substitute to barley in brewing, millet α-amylase appears to have better brewing properties.

In line with the above findings, it is suggested that intensive malting studies of millet grains be embarked upon, alongside studies on sorghum, since the major problem negating large-scale millet malting is the size of the millet grain. Also results obtained from this research need further investigation. The α-amylase from other local sorghum and millet varieties should also be studied.

APPENDIX 1

Definition

One unit of enzyme activity is equivalent to that amount of enzyme that will liberate from soluble starch, one micromole of reducing groups (calculated as maltose) per minute at 50°C and pH 4.8 under the specified conditions.

Activity (units) = micromoles of maltose liberated

$$= \frac{\text{µg maltose/min/ml} \times \text{dil}}{342}$$

$$\text{Specific Activity units/mg protein} = \frac{\text{µg maltose/min/ml} \times \text{dil}}{342 \times \text{mg enzyme protein}}$$

APPENDIX 2

Preparation of Reagents:

Preparation of Dinitrosalicyclic Acid (DNS) colour Reagent.

Dinitrosalicylic acid (1.0g) was dissolved in 20ml of 2N sodium hydroxide (NaOH) solution. This mixture was warmed and then 30g of sodium potassium tartarate added slowly. The volume was then made up to 100ml with distilled water.

Preparation of 1% starch solution.

Soluble starch (1.0g) was dissolved in 100ml 0.1M acetate buffer pH 4.8. This was boiled gently and cooled.

APPENDIX 3

Standard Maltose curve for determination of α-amylase activity.

APPENDIX 4

Lowry's Protein Determination (Lowry et al. 1951) as described by Cooper (1977).

Reagents:

A 10% Na_2CO_3 in 0.5% NaOH

A 1% $CuSO_4.5H_2O$ solution

C 2% Potassium tartarate

D 10% Folin-Ciocalteau reagent

Bovine serum albumin (BSA) stock solution (300μg/ml).

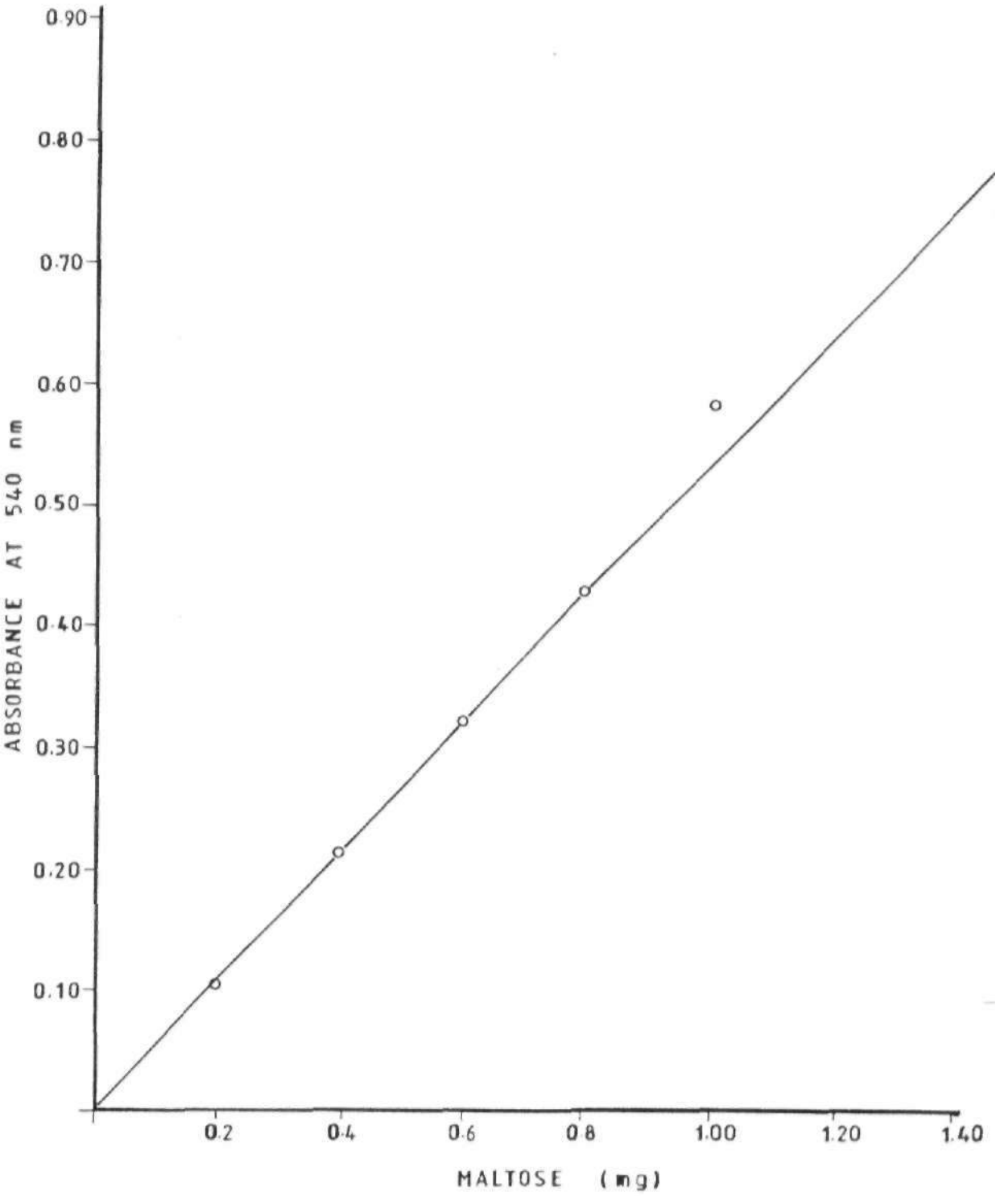
Procedure

1. Standard BSA solutions of concentration range 0-300μg/ml were prepared in different test tubes.

2. To 15ml of reagent A, 0.75ml reagent B and 0.75ml of reagent C were added.

3. 1.0ml of the solution of step 2 was added to each of the tubes in step 1, mixed thoroughly and allowed to stand for 15 minutes.

4. At the end of incubation, 3.0ml of the 10% Folin-Ciocalteau reagent immediately prepared was added, mixed vigorously and allowed to stand for 45 minutes.


0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
ABSORBANCE AT 540 nm
0.2
0.4
0.6
0.8
1.00
1.20
1.40
MALTOSE (mg)

Purification Profile of sorghum and millet α-amylases

Sorghum or Millet meal (200g)

Acetate buffer (0.1M) pH 4.8, 0.01M $CaCl_2$

↓

Extract 3 hr.

Centrifuge 15 mins. at 8,000g

↓

↓	↓
Precipitate	Supernatant
(Discard)	↓
	Filter (Whatman No 1)
	↓
	Adjust to pH 6.0, 0.2% Calcium chloride. 5% or 4% charcoal. Heat at 70°C for 15mins. Centrifuge 9,000g for 15 mins.
	↓
Precipitate	Supernatant, Make 55% or 45% saturated with $(NH_4)_2SO_4$. Centrifuge, 10,000rpm, for 10mins. for 4°C.
(Discard)	↓
↓	↓
Supernatant	Precipitate Redissolve in 0.1M Tris Buffer pH 7.0 containing 0.2% $CaCl_2$
(Discard)	↓
	Sephadex G150-120 Column chromatography
	↓
	Elute with 0.1M Tris buffer pH 7.0
	↓
	Purified α-Amylase

5. The absorbance of each tube was read at 750nm against a blank which contained no BSA.

6. The data were plotted as shown in figure 27. Test samples were treated in the same way and the corresponding protein contents read off from the clibration curve.

APPENDIX 5

Determination of Kinetic Constants of α-Amylase

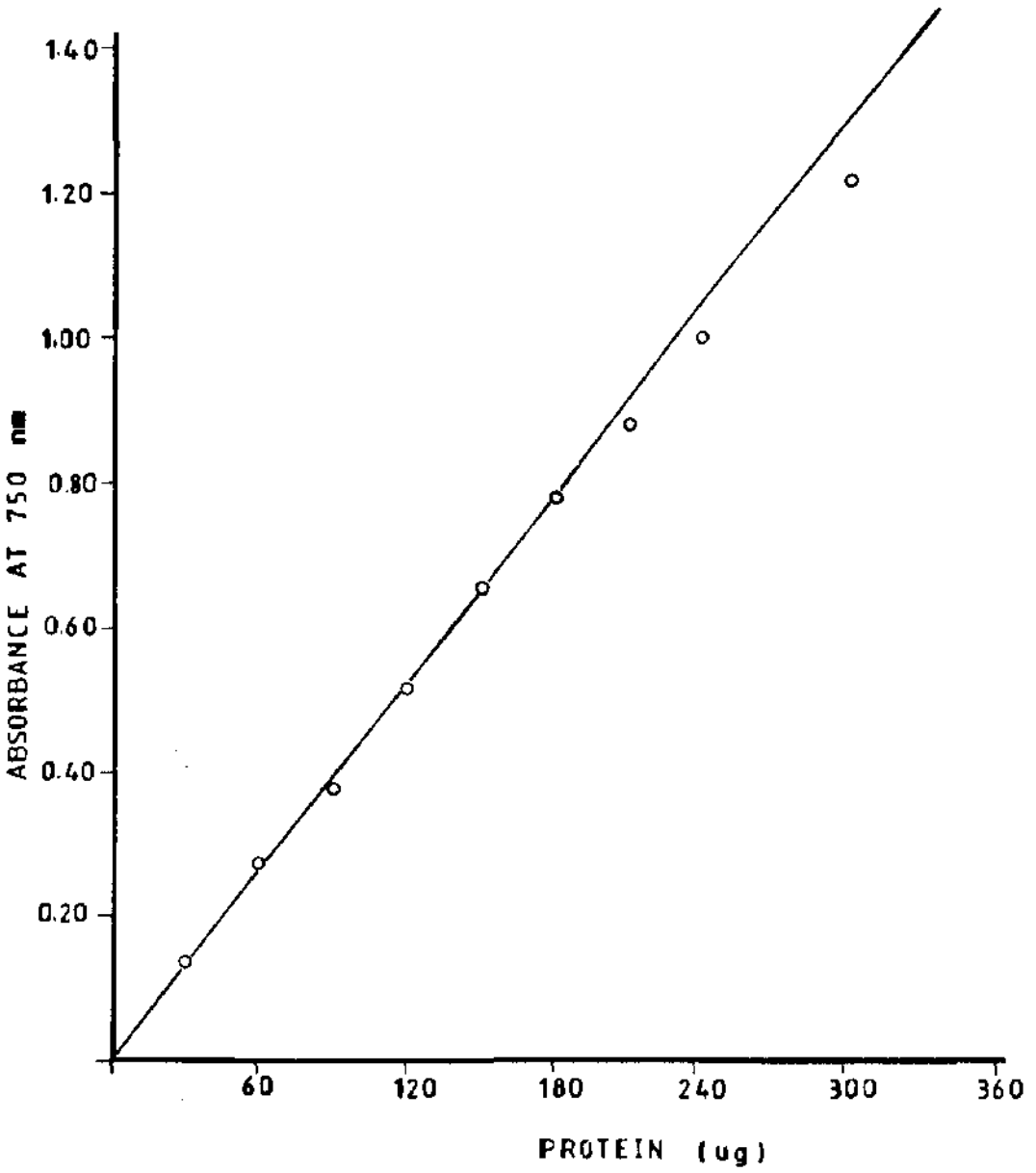
Substrate Conc.		Sorghum		Millet		Bacterial		Fungal	
S (%)	1/S	V mg maltose	1/V	V mg maltose	1/V	V mg maltose	1/V	V mg maltose	1/V
0.02	50	0.045	22.22	0.02	50	0.0775	57.14	0.015	66.67
0.04	25	0.0475	21.05	0.0275	36.3	0.04	25.00	0.035	28.57
0.05	20	0.050	20.00	0.0375	26.6	0.05	20.00	0.045	22.22
0.06	16.67	0.055	18.18	0.045	22.2	0.055	18.18	0.050	20.00
0.08	12.5	0.0575	17.39	0.055	18.8	0.070	14.28	0.065	15.40
0.10	10	0.060	16.67	0.075	13.3	0.075	13.33	0.075	13.33

Calculation of Km

Sorghum α-amylase

$$\text{Slope} = \frac{Km}{Vmax} = \frac{20 - 15.5}{20 - 0} = \frac{4.75}{20} = 0.2375$$

$$1/Vmax = 15.25$$


1.40
1.20
1.00
0.80
0.60
0.40
0.20
ABSORBANCE AT 750 nm
60
120
180
240
300
360
PROTEIN (ug)

$\therefore$ V_{max} = 0.0656mg maltose/min/ml

K_m = slope x V_{max}

= 0.2375 x 0.0656

= 1.56×10^{-2}g starch/100ml

= 0.156mg starch/ml

Bacterial α-amylase

$$\text{Slope} = \frac{K_m}{V_{max}} = \frac{30 - 4}{31 - 0} = \frac{26}{31} = 0.84$$

$1/V_{max}$ = 4.0

$\therefore$ V_{max} = 0.25mg maltose/min/ml

K_m = slope x V_{max} = 0.84 x 0.25

= 0.210g starch/100ml

= 2.10mg starch/ml

Fungal α-amylase

$$\text{Slope} = \frac{K_m}{V_{max}} = \frac{44 - 10}{40 - 7} = \frac{34}{33} = 1.03$$

$1/V_{max}$ = 2.9

$\therefore$ V_{max} = 0.345mg maltose/min/ml

K_m = 0.345 x 1.03

= 0.33g starch/100ml

= 3.3mg starch/ml

Millet α-Amylase

$$\text{Slope} = \frac{K_m}{V_{max}} = \frac{30 - 6}{25 - 0} = \frac{24}{25} = 0.96$$

1/Vmax = 6.1

∴ Vmax = 0.164mg maltose/min/ml

Km = 0.96 x 0.164

= 0.157g starch/100ml

= 1.57mg starch/ml

APPENDIX 6

Determination of Energy of Activation of α-Amylases

Temperature °C	1/Tx10⁻³ °A	Sorghum Val µm/min	Sorghum log₁₀V	Millet Val µm/min	Millet log₁₀V	Bacterial Val µm/min	Bacterial log₁₀V	Fungal Val µm/min	Fungal log₁₀V
20	293	3.41	1.46	0.16	4.24	0.63	1.37	0.14	2.51	0.40
25	298	3.35	2.09	0.32	4.97	0.70	2.0	0.30	3.74	0.57
30	303	3.30	2.50	0.40	6.14	0.79	2.65	0.42	4.98	0.70
35	308	3.24	3.31	0.52	8.07	0.91	3.57	0.55	5.32	0.73
40	313	3.19	4.57	0.66	9.59	0.98	4.33	0.64	5.74	0.76
50	323	3.10	6.61	0.82	12.18	1.08	4.91	0.69	6.9	0.84

Calculation of Energy of activation (Ea)

Slope of Arrhenius plot $\frac{Ea}{2.303R}$ (Sigal, 1976)

Ea = 2.303 x R x slope

Sorghum α-Amylase

$$\text{Slope of figure 13} = \frac{1.06 - 0.4}{3.3 - 3.0 \times 10^{-3}}$$

$$= 2.2 \times 10^{3}$$

$$R = 1.987 \text{ cal/degree/mole}$$

$$\therefore Ea = 2.2 \times 2.303 \times 1.987 \times 10^{3}$$

$$Ea = 10.05 \text{ Kcal/degree/mole}$$

Millet α-Amylase

$$\text{Slope of figure 14} = \frac{Ea}{2.303R} = \frac{1.16 - 0.64}{3.4 - 3.08 \times 10^{-3}}$$

$$= 1.625 \times 10^{3}$$

$$\therefore Ea = 1.625 \times 2.303 \times 1.987 \times 10^{3}$$

$$Ea = 7.43 \text{Kcal/degree/mole}$$

Bacterial α-Amylase

$$\text{Slope of figure 15} = \frac{Ea}{2.303R} = \frac{0.86 - 0.4}{3.32 - 3.0 \times 10^{-3}}$$

$$= 1.4375 \times 10^{3}$$

$$Ea = 1.4375 \times 10^{3} \times 2.303 \times 1.987$$

$$Ea = 6.57 \text{ kcal/degree/mole}$$

Fungal α-Amylase

$$\text{Slope of figure 16} = \frac{Ea}{2.303R} = \frac{0.87 - 0.4}{3.5 - 3.1 \times 10^{-3}}$$

$$= 1.16 \times 10^{3}$$

$$Ea = 1.16 \times 2.303 \times 1.987 \times 10^{3}$$

$$Ea = 5.39 \text{ kcal/degree/mole}$$

APPENDIX 7

Calculation of pK values from pH effect profile

$\Delta n = (H^+)a + (H^+)b - 4(H^+)opt.$ and $(pH)_{opt} = \frac{1}{2}(pKa + pKb)$

(Alberty and Massey, 1954)

$(H^+)_a$ = hydrogen ion concentration at 50% activity on the acid side of the pH profile.

$(H^+)_b$ = hydrogen ion concentration at 50% activity on the basic side of the pH profile

$(H^+)_{opt}$ = hydrogen ion concentration at maximum activity of pH profile.

Sorghum α-Amylase

$(pH)_a = 3.42$

$= \log 2.36 + \log 10^{3}$

$= \log 0.263 \times 10^{4}$

$$= \log \frac{1}{3.84 \times 10^{-4}}$$

$(H^+)_a = 3.84 \times 10^{-4} M$

$(pH)_b = 7.0$

$= \log 1 + \log 10^{7}$

$= \log 0.1 \times 10^{8}$

$$= \log \frac{1}{10 \times 10^{-8}}$$

$(H^+)_b = 10 \times 10^{-8} M$

$(pH)_{opt} = 5.25$

$= \log 1.778 + \log 10^{6}$

$= \log 0.178 \times 10^{6}$

$$= \log \frac{1}{5.623 \times 10^{-6}}$$

$(H^+)_{opt} = 5.623 \times 10^{-6} M$

$Ka = (H^+)_a + (H^+)_b - 4(H^+)_{opt}$

$= 3.80 \times 10^{-4} + 10 \times 10^{-8} - 4(5.623 \times 10^{-6})$

$= 3.801 \times 10^{-4} - 2.249 \times 10^{-5}$

$Ka = 3.576 \times 10^{-4}$

$pKa = 3.45$

$(pH)_{opt} = \frac{1}{2}(pKa + pKb)$

$\therefore pKb = 2 \times (pH)_{opt} - pKa$

$pKb = 2 \times 5.25 - 3.45$

$\underline{pKb = 7.05}$

Millet α-amylase

$(pH)_a = 3.45$

$= \log 2.82 + \log 10^{3}$

$= \log 0.28 \times 10^{4}$

$$= \log \frac{1}{3.55 \times 10^{-4}}$$

$(H^+)_a = 3.55 \times 10^{-4}M$

$(pH^+)b = 5.74$

$= \log 5.50 + \log 10^{5}$

$= \log 0.55 \times 10^{6}$

$$= \log \frac{1}{1.82 \times 10^{-6}}$$

$(H^+)b = 1.82 \times 10^{-6}M$

$(pH)_{opt} = 4.5$

$= \log 3.16 \times 10^{4}$

$= \log 0.316 \times 10^{5}$

$$= \log \frac{1}{3.16 \times 10^{-5}}$$

$(H^+)_{opt} = 3.16 \times 10^{-5}M$

$Ka = 3.55 \times 10^{-4} + 1.82 \times 10^{-6} - 4(3.16 \times 10^{-5})$

$= 2.30 \times 10^{-4}$

<u>pKa = 3.64</u>

$pKb = 2 \times (pH)_{opt} - pKa$

$= 2 \times 4.5 - 3.64$

<u>pKb = 5.54</u>

<u>Bacterial α-amylase</u>

$(pH)_a = 4.6$

$= \log 3.98 + \log 10^{4}$

$= \log 0.398 \times 10^{5}$

$= \log \frac{1}{2.512 \times 10^{-5}}$

$(H^+)_a = 2.51 \times 10^{-5}M$

$(pH)_b = 7.6$

$= \log 3.98 + \log 10^{7}$

$= \log 0.398 \times 10^{8}$

$= \log \frac{1}{2.572 \times 10^{-8}}$

$(H^+)b = 2.51 \times 10^{-8}M$

$(pH)_{opt} = 6.0$

$= \log 1 + \log 10^{6}$

$= \log 0.1 \times 10^{7}$

$$= \text{ing} \quad \frac{1}{10 \times 10^{-7}}$$

$(H^+)_{opt} = 10 \times 10^{-7}M$

$Ka = (H^+)_a + (H^+)_b - 4(H^+)_{opt}$

$= 2.57 \times 10^{-5} + 2.51 \times 10^{-5} - (4 \times 10 \times 10^{-7})$

$Ka = 2.115 \times 10^{-5}$

$\underline{pKa = 4.67}$

$(pH)_{opt} = \frac{1}{2}(pKa + pkb)$

$pKb = 2 \times (pH)_{opt} - pKa$

$= 2 \times 6.0 - 4.67$

$\underline{pKb = 7.33}$

Fungal α-amylase

$(pH)a = 3.65$

$= \text{ing } 4.47 + \text{ing } 10^{3}$

$= \text{ing } 0.447 \times 10^{4}$

$$= \text{ing} \quad \frac{1}{2.24 \times 10^{-4}}$$

$(H^+)a = 2.24 \times 10^{-4}M$

$(pH)_b$ = 6.3

= lng 1.995 + lng 10^6

= lng 0.1995 x 10^7

$$= \text{lng } \frac{1}{5.012 \times 10^{-7}}$$

$(H^+)_b$ = 5.01 x 10^{-7}M

$(pH)_{opt}$ = 4.7

= lng 5.012 + lng 10^4

= lng 0.5012 x 10^5

$$= \text{lng } \frac{1}{1.995 \times 10^{-5}}$$

$(H^+)_{opt}$ = 1.995 x 10^{-5}M

Ka = 2.24 x 10^{-4} + 5.01 x 10^{-7} - 4 (1.995 x 10^{-5})

= 1.447 x 10^{-4}

pKa = 3.84

$(pH)_{opt}$ = ½(pKa + pKb)

pKb = 2 x $(pH)_{opt}$ - pKa

= 2 x 4.7 - 3.84

pKb = 5.56

APPENDIX B

Determination of Molecular Weight by SDS-gel eletrophoresis

The following values were obtained from two runs.

Run	Sorghum	Millet	Bacterial	Fungal
1st	51,880	48,978	47,863	47,863
2nd	53,456	48,417	50,119	46,417
Mean ($\bar{x}$)	52,668	48,698	48,991	47,140

Calculation of standard deviation

Sorghum α-amylase

Molecular weight (x)	Deviation from the mean ($x - \bar{x}$)	$(x - \bar{x})^2$
51,880	- 788	620944
53,456	788	620944

$$\Sigma (x - \bar{x})^2 = 1241888$$

$$\text{standard deviation} = \frac{\Sigma (x - \bar{x})^2}{n - 1}$$

$$= 1241888$$

$$= 1114$$

Molecular weight = 52,668 ± 1114 daltons

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Millet α-amylase

Molecular weight (x)	Deviation from the mean $(x - \bar{x})$	$(x - \bar{x})^2$
48978	280	78400
48417	− 281	78961

$\Sigma (x - \bar{x})^2 = 157361$

Standard deviation = 157361

= 397

Molecular Weight = 48.698 ± 397 daltons

Bacterial α-amylase

Molecular weight (x)	Deviation from the Mean $(x - \bar{x})$	$(x - \bar{x})^2$
47,863	− 1128	1272384
50,119	1128	1272384

$\Sigma (x - \bar{x}) = 2544768$

Standard deviation = 2544768

= 1595

Molecular weight = 48,991 ± 1595 daltons

Fungal α-amylase

Molecular weight (x)	Deviation from the mean $(x - \bar{x})$	$(x - \bar{x})^2$
47.663	723	522729
46.417	- 723	522729

$\Sigma(x - \bar{x})^2 = 1045458$

Standard deviation = $\sqrt{1045458}$

= 1022

Molecular weight = 47.140 ± 1022

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