STRUCTURE, FUNCTION AND STABILITY OF α-AMYLASE FROM JOWAR (*Sorghum bicolor*)

A THESIS SUBMITTED TO THE UNIVERSITY OF MYSORE FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN BIOCHEMISTRY

BY

R. SIVA SAI KUMAR

Department of Protein Chemistry and Technology

Central Food Technological Research Institute

Mysore - 570020, Karnataka, India

AUGUST 2008

R. SIVA SAI KUMAR
Senior Research Fellow
Department of Protein Chemistry and Technology
Central Food Technological Research Institute
Mysore - 570020, Karnataka, India

DECLARATION

I hereby declare that the thesis entitled **"Structure, Function and Stability of α-Amylase from Jowar (*Sorghum bicolor*)"** submitted to the University of Mysore for the award of degree of DOCTOR OF PHILOSOPHY in BIOCHEMISTRY is the result of the research work carried out by me in the Department of Protein Chemistry and Technology, Central Food Technological Research Institute, Mysore, India under the guidance of **Dr. A. G. Appu Rao** during the period of 2003 – 2008.

I further declare that the research work embodied in this thesis has not been submitted for the award of any other degree.

Signature of the Doctoral candidate
Date:

Signature of Guide
Date: Counter signed by

Signature of Chairperson/Head of Department/
Institution with name and official seal

Dr. A.G. APPU RAO
Head, Department of Protein Chemistry and Technology

August 2008

CERTIFICATE

I hereby certify that the thesis entitled **"Structure, Function and Stability of α-Amylase from Jowar (*Sorghum bicolor*)"** submitted to the University of Mysore for the award of the degree of DOCTOR OF PHILOSOPHY in BIOCHEMISTRY by **Mr. R. Siva Sai Kumar** is the result of the research work carried out by him in the Department of Protein Chemistry and Technology, Central Food Technological Research Institute, Mysore under my guidance and supervision during the period of 2003 – 2008. This has not been submitted either partially or fully to any degree or fellowship earlier.

A.G.APPU RAO

(Guide)

STRUCTURE, FUNCTION AND STABILITY OF α-AMYLASE FROM JOWAR (*Sorghum bicolor*)

ABSTRACT

The focus of this investigation has been to screen cereals for thermostable α-amylases, which retain activity when hot water is added to supplementary foods to reconstitute them. The isolation, characterization and stability parameters of the α-amylase from sorghum are reported for the first time.

The thermostability of α-amylase from malted sorghum is higher compared to α-amylases from barley (*Hordeum jubatum*) and ragi (*Eleusine coracana*). The digestion of gelatinized starch, by this enzyme, results in maltose, glucose and maltotriose, which increase nutrient density by decreasing its water absorption capacity. The major amylase from malted jowar, a 47 kDa α-amylase, is rich in β structure (~60%) like other cereal amylases.

α-Amylase from *Sorghum bicolor* is reversibly unfolded, by GuHCl and urea at pH 7.0 in 50 mM Hepes containing 13.6 mM calcium and 15 mM DTT. The isothermal equilibrium unfolding at 27°C is characterized by two-state transition with $\Delta G(H_2O)$ of 16.5 kJ mol^{-1} and 22 kJ mol^{-1} for GuHCl at pH 4.8 and 7.0, respectively and $\Delta G(H_2O)$ of 25.2 kJ mol^{-1} for urea at pH 4.8. The pH dependent stability described by $\Delta G(H_2O)$ and the effect of salt on urea induced unfolding confirm the dominant role of electrostatic interactions in enzyme stability. Homology modeling studies of sorghum α-amylase, using barley

AMY1 as a template, have shown a structure similar to that expected from the high sequence identity.

The reactivity of the conserved cysteine residues, during unfolding, suggests that unfolding starts from the 'B' domain of the enzyme. The conserved cysteine residues are essential for enzyme activity but not for the secondary and tertiary fold acquired during refolding of the denatured enzyme.

T_m, the midpoint of thermal inactivation, is found to be 69.6 ± 0.3 °C. Thermal inactivation of α-amylase follows first-order kinetics at pH 4.8, the pH optimum of the. NaCl is a destabilizer, while sucrose is a stabilizer against thermal inactivation.

Calcium content of sorghum α-amylase is determined to be 0.8 mole of Ca^{2+}/ mole of protein. Removal of calcium leads to decreased thermo stability and enzymatic activity, and increased susceptibility to proteolytic degradation. The loss of sorghum amylase activity upon removal of calcium suggests that Ca^{2+} is important for maintenance of the configuration of the active site. Activity recovery of the EDTA inactivated sorghum α-amylase in presence of barium is similar to that of calcium.

These studies on structure function and stability of sorghum α-amylase may lead to the development of enzymes with improved stability and in help in understanding the folding and refolding of a multi – domain protein.

ACKNOWLEDGEMENTS

I would like to express my profound gratitude and sincere thanks to….

Dr. A.G. Appu Rao, Head, Department of Protein Chemistry and Technology, CFTRI, Mysore, India for suggesting the research problem, inspiring guidance, and constant encouragement through out the course of investigation .

Dr. V. Prakash, Director, CFTRI, Mysore for providing the necessary facilities to carry out the research work in the institute and permitting me to submit the results in the form of a thesis.

Dr. Sridevi Annapurna Singh, for CD experiments, data analysis and also for her constant support during the course of investigation.

Dr. Lalitha R. Gowda**,** for helping me with amino acid analysis and amino-terminus sequencing.

Mr. P. S. Kulasekhar for rendering help to make improvements to the presentation of the material in the thesis.

Staff and students of Protein Chemistry & Technology, Library and of Central Instrumentation Facility & Services for their help/technical assistance rendered during the entire course of investigation.

My parents, wife and friends who constantly supported and eagerly waited for this moment of rejoice.

CSIR**,** New Delhi**,** for the financial support in the form of research fellowship.

R.SIVA SAI KUMAR

CONTENTS

ABBREVIATIONS

ΔC_p	Excess heat capacity
ΔH_g	unfolding enthalpy
AHA	*Alteromonas haloplanctis* α-amylase
AMY1	Barley α-amylase isozyme 1
AMY2	Barley α-amylase isozyme 2
BAA	*Bacillus amyloliquefaciens* α-amylase
BLA	*Bacillus licheniformis* α-amylase
BstA	*Bacillus stearothermophilus* α-amylase
CAPS	3-[cyclohexylamine]-1-propane sulfonic acid
CD	Circular dichroism
DNS	3,5-dinitrosalicylic acid
DP	Degree of polymerization
DTNB	5,5′ –dithiobis-(2-nitrobenzoic acid)
DTT	Dithiothreitol
$E^{1\%}$	Absorption coefficient of 1% protein solution
Ea	Activation energy
EDTA	Diaminoethanetetraacetic acid
GuHCl	Guanidine hydrochloride
HCl	Hydrochloric acid
Hepes	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HPLC	High performance liquid chromatography
K	Kelvin
kcal	Kilo calories
kDa	kilo Daltons
K_m	Michaelis constant
LEM	Linear extrapolation model
MIANS	2-(4′-maleimidylanilino)naphthalene-6-sulfonicacid
min	Minutes
mrw	Mean residue weight
NaCl	Sodium chloride
PAGE	Polyacrylamide gel electrophoresis
PEG	Poly(ethyleneglycol)
PPA	Porcine pancreatic α-amylase
PVDF	Polyvinyl difluoride
SDS	Sodium dodecyl sulfate
TEMED	*N*,*N*,*N*′,*N*′-tetramethylethylenediamine
T_M	Transition temperature
UV	Ultraviolet
V_{max}	Maximum velocity

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

INTRODUCTION

The glycolytic pathway and the citric acid cycle play a pivotal role in metabolism. Variations in the above pathways persist in a few organisms; yet, the orchestra of enzymes as well as their function, in a definite reaction, remains conserved (Huynen et al., 1999; Dandekar et al., 1999). Glycolysis is the pathway, which breaks down sugars, primarily glucose, into readily available energy forms and the conservation of this pathway suggests that glucose is a major source of energy for many organisms (Nielsen and Borchert et al., 2000). Glucose, in nature, occurs in such polymeric states as glycogen in animals and cellulose as well as starch in plants (Vihinen et al., 1989). Glucose molecules are readily available, in abundance, to many organisms which can hydrolyze glucose polymers. The variance observed among the glucose-polymer hydrolyzing enzymes emphatically signifies the necessity of glucose polymers for life.

Human beings can digest starch and glycogen which consist of α-(1-4)-glycosidic linkages in glucose polymers. These are hydrolyzed by such enzymes as α- and β-amylases. Lack of enzymes that can hydrolyze polymers such as cellulose, with β-(1-4)-glycosidic linkages, renders these polymers nearly impossible to digest by the humans (Ramasubbu et al., 1996; Brayer et al., 1995). α-Amylases are of immense importance among all the starch-hydrolyzing enzymes, due to their involvement in solubilisation of starch. Starch consists of two glucose polymers: amylose (10-20%), which is exclusively α-(1-4) linked

(Figure 1) and amylopectin (80-90%) containing, in addition to the α-(1-4) linkages found in amylose, many α-(1-6) branch points (Vihinen et al., 1989; Guzman-Maldonado et al., 1995**)** (Figure 2). α-Amylases actively catalyze random hydrolysis of internal α-(1-4)-glycosidic linkages of amylose, amylopectin and other related polysaccharides, giving rise to low molecular weight oligosaccharides and glucose. The exact ratio of these saccharides depends on the source and the nature of the α-amylase used (Brosnan et al., 1992). These enzymes, found in both eubacteria and eukaryota, have a wide variety of substrate specificities as well as a huge variation in both temperature and pH optima (Vihinen et al., 1989).

History

Originally named as diastase, amylase is the first enzyme to be found and isolated from malt solution by Anselme Payen in 1833. A diastase (from Greek, meaning "separation") is any one of a group of enzymes which catalyses the breakdown of starch into maltose. Today, diastase means any α-, β-, or γ-amylase (all hydrolases) that can break down carbohydrates. The commonly used -ase suffix for naming enzymes is derived from the name diastase. The advent of commercialization of α-amylases from fungal and bacterial sources is reported to have begun in the late 19th and early 20th centuries respectively (Muralikrishna and Nirmala, 2005).

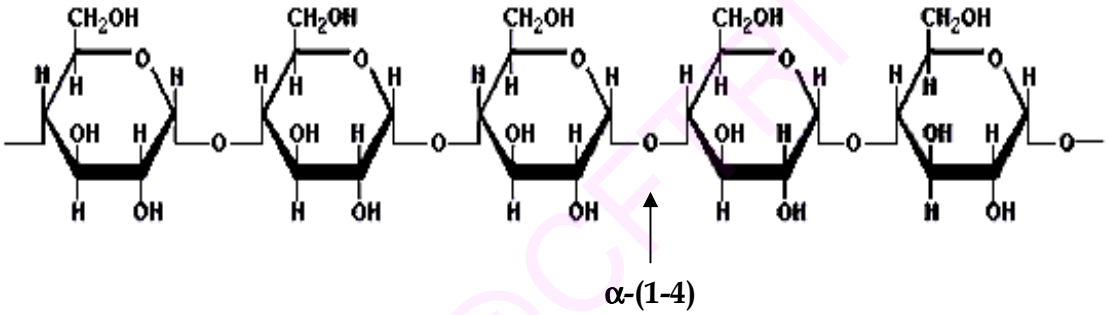


Figure 1. Representative partial structure of amylose

Amylose is a planar polymer of glucose linked mainly by α-(1-4)-glycosidic bonds. The number of repeated glucose subunits runs up to thousands (usually in the range of 300 to 3000).

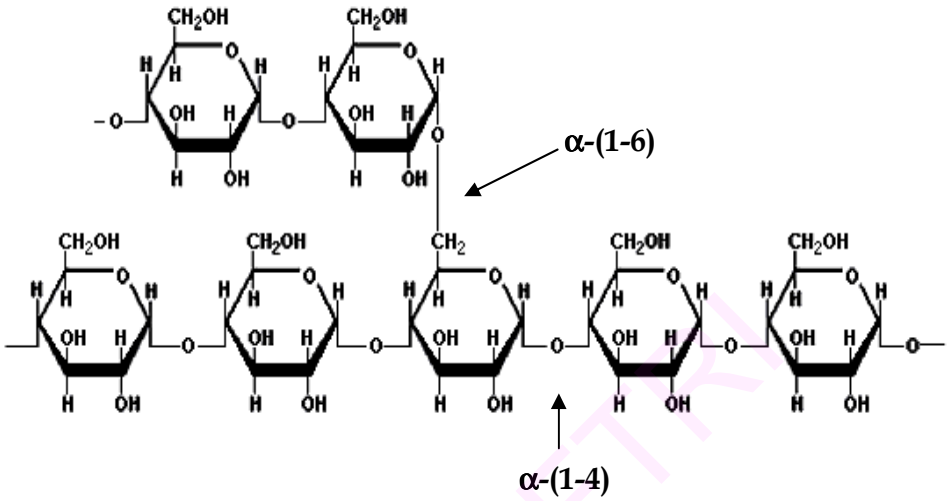


Figure 2. Representative partial structure of amylopectin

Amylopectin is a polysaccharide composed of linearly linked α-(1-4) glucose units with occasional α-(1-6)- glycosidic bonds which provide branching points. Each amylopectin molecule may contain 100,000-200,000 glucose units with each branch about 20 or 30 glucose units in length. Glycogen ("animal starch"), found in the liver and muscles, is effectively similar in structure to amylopectin, but it has shorter branches: 8-12 glucose units.

Classification

Enzymes which can degrade starch and related poly- and oligosaccharides can be classified based on the following distinctions in their behavior : (1) endo- versus exo-mode of attack, (2) inversion versus retention of the anomeric configuration of the substrate, (3) preference for poly-, oligo- or di-saccharides, (4) α-(l-4), α-(1-6), or dual bond-type specificity, and (5) hydrolytic versus glucosyl-transfer activity. Amylases are classified as α, β or γ-amylases. Characteristic features of amylases are shown in Table 1. Specificity of these enzymes on amylose and amylopectin are shown in Figure 3 A and B.

α-Amylase

α-Amylases (EC 3.2.1.1) (Alternate name: 1,4-α-D-glucan glucanohydrolase; glycogenase), also known as "liquefying" enzymes or endoamylases, are calcium metalloenzymes. α-Amylase breaks down long-chain carbohydrates, to ultimately yield either maltotriose and maltose from amylose, or maltose, glucose and "limit dextrin" from amylopectin. The α-configuration of the C_1 of the reducing glucose unit obtained after the hydrolysis of the oligosaccharides, renders the name α-amylases to this class. Since it can act anywhere on the substrate, α-amylase tends to act faster than β-amylase. It is a major digestive enzyme in animals with an optimum pH range of 6.7-7.0. In human physiology, both the salivary as well as pancreatic amylases are α-amylases. They are also found in plants, fungi and bacteria.

β-Amylase

β-amylase (EC 3.2.1.2) (Alternative names: 1,4-α-D-glucan maltohydrolase; glycogenase; saccharogen amylase), also known as "saccharifying" or exoamylases, cleave α-(1-4)- glucosidic bonds in amylose, amylopectin and glycogen from non-reducing end, leading to the removal of successive maltose/glucose units. β-amylase is also synthesized by bacteria, fungi and plants. Unlike α-amylases, β-amylases release products with β-configuration at the C1 glucose unit due to the inversion. β-Amylase is present prior to germination, whereas α-amylase appears once germination has begun**.** Animal tissues do not contain β-amylase, although it may be present in microrganisms within the digestive tract.

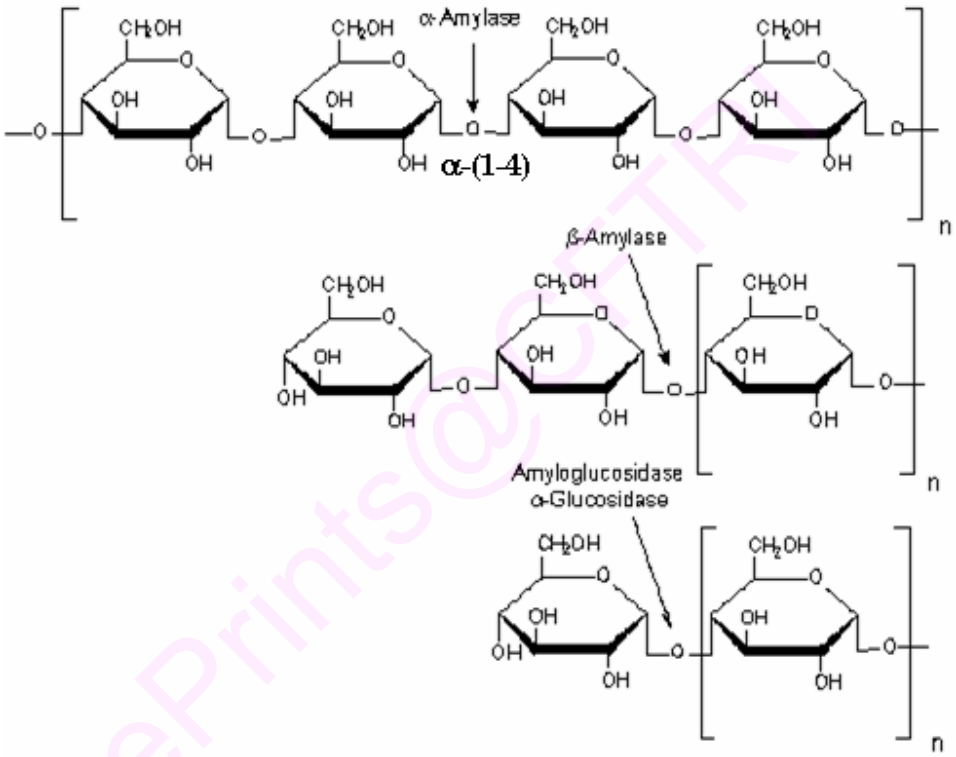
γ-Amylase

γ-amylase (EC 3.2.1.3) (Alternative names: Glucan 1,4-α-glucosidase; amyloglucosidase; Exo-1,4-α-glucosidase; glucoamylase; lysosomal α-glucosidase; 1,4-α-D-glucan glucohydrolase), cleaves α-(1-6)- glycosidic linkages, in addition to cleaving the terminal α-(1-4)glycosidic linkages at the nonreducing end of amylose and amylopectin, yielding glucose. Unlike the other forms of amylase, γ-amylase, which is the most efficient in acidic environments, has an optimum pH of 3.

Table 1 Characteristic features of amylases

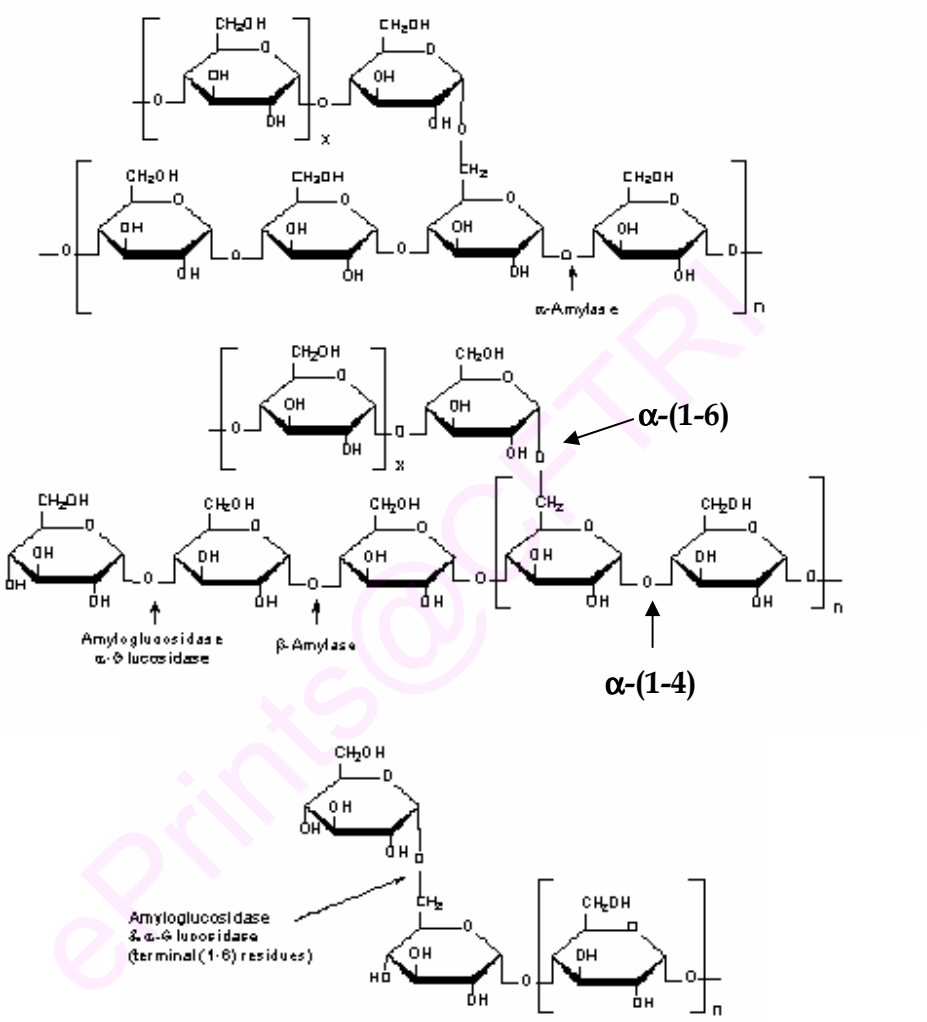
Characteristic	α-Amylase	β-Amylase	γ-Amylase
Cleavage point	α, 1→4 Glucosidic bonds Cleaves C_1-O_4 bond	α, 1→4 Glucosidic bonds Cleaves C_1-O_4 bond	α, 1→4 and α, 1→6 Glucosidic bonds Cleaves C_1-O_4 and C_1-O_6 bond
Configuration of new reducing unit	α	β	β
Mechanism	Endo-attack	Exo-attack	Exo-attack
End products	Oligosaccharide mixture	Maltose	Glucose
Decrease in Viscosity and Iodine staining	Rapid	Slow	Slow
Action at branch point	Can bypass	Cannot bypass	Cannot bypass

(*Maldonado and Lopez, 1995; http://en.wikipedia.org/wiki/Amylase*)



Amylases specificity on amylose

Figure 3 A



Amylases specificity on amylopectin

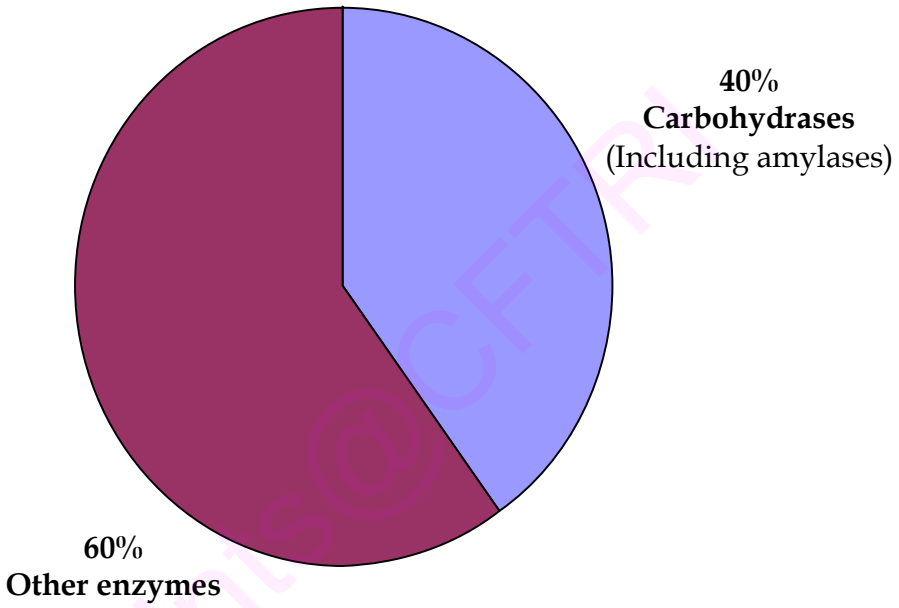
Figure 3 B

Applications

α-Amylase finds a number of important commercial applications in the sugar, brewing, alcohol and textile industries for hydrolysis of starch (Nielsen and Borchert, 2000). Currently, these enzymes of α-amylase family constitute about ~ 40% of the world's enzyme production (Sivaramakrishnan et al., 2006) (Figure 4). Industrial production of dextrose powder and dextrose crystals from starch using α-amylase and glucoamylase has had a beginning in 1959 (Aiyer, 2005). Since then, amylases are being used for various purposes. Conversion of starch into sugar, syrups and dextrins forms the major activity of the starch processing industry. Amylases, especially alkaline amylases, are used in detergents. Amylases are also used, to some extent, as digestive aids (Aiyer, 2005) to supplement the diastatic activity of flour and to improve digestibility of some of the animal feed ingredients.

α-Amylases find application in supplementary foods too. The enhanced bulk acquired by supplementary foods on reconstitution with hot water due to absorption of large quantity of water is attributable to their pregelatinized starch content. Malted cereals being a rich source of hydrolytic enzymes (α-amylases) can be an integral part of supplementary foods. α-Amylases in the supplementary food, predigest starch, leading to a decrease in water absorption capacity and an increase in nutrient density. Thermal stability of α-amylase is a desirable feature as the supplementary food is reconstituted with hot water/milk before consumption. An understanding of the basis of amylase activity in

correlation with its structure can lead to the development of more efficient and varied applications. Purification of α-amylases is a prerequisite for studying its structure-function relationships and biochemical properties.



(*Sivaramakrishnan et al., 2006*)

Figure 4. The contribution of carbohydrases (including amylases) to the total sale of enzymes is indicated.

α-Amylases

α-Amylases (EC 3.2.1.1) (Alternate name: 1,4-α-D-glucan glucanohydrolase; glycogenase), are also known as "liquefying" enzymes or endoamylases. These are the most important amongst all amylases commercially. α-Amylases are ubiquitous in nature. Bacterial α-amylases are amongst the most thermostable enzymes known and are widely used. α-Amylases are dependent on calcium for their activity.

Purification

Conventional and classical methods have been employed to purify several amylases (Brena et al., 1996). These methods are based on differences in: (i) solubility, (ii) non-specific adsorption-desorption processes on inorganic supports. (iii) electrostatic interactions as a function of pH (ion exchange chromatography, preparative electrophoresis) and (iv) molecular sieving (gel filtration chromatography). Dependence of the solubility of proteins on solvent composition, mostly, forms the starting point for purification of a protein following which, it is subjected to chromatography, usually affinity, ion exchange and/or gel filtration.

The need for cost-effective method for the purification of proteins, on a large scale, has lead to the development of several techniques, including adsorption of enzymes on their substrates and affinity precipitation, which help obtain the enzyme of desired purity in fewer processing steps (Sommers et al., 1989; Sadana and Beelaram, 1994). Affinity precipitation is based on the use of a

heterobifunctional ligand that interacts in free solution with the target molecule by affinity interaction. The affinity complex is subsequently separated from the reaction mixture by exploiting the property of the second functionality of the heterobifunctional ligand (Aksoy etal., 1998). Various studies have been carried out on constraining the enzyme on to a solid support thereby immobilizing it and increasing its stability towards temperature (Emne'us and Gorton, 1990; Linne 1992). Immobilized amylases have several advantages, such as easy recovery, simplification of purification procedure, besides affording scale up (James and Simpson, 1996).

Most of the wide variety of supports employed for immobilization of amylases, modify the enzyme chemically, hampering its performance. Alginate, a naturally occurring copolymer comprising mannuronic acid and guluronic acid, is a polymer of choice for entrapment of cells and enzymes due to its cost-effectiveness. Alginates, apart from offering a relatively inert aqueous environment within the matrix, afford high gel porosity which allows high diffusion rates of macromolecules. Affinity precipitation has been used for the purification or enrichment of carbohydrate-splitting enzymes, namely α-amylase, glucoamylase, polygalacturonase and β-amylase (Sardar and Gupta, 1998; Sharma et al., 1998; Teotia and Gupta., 2001; Teotia, Khare et al., 2001). Glucoamylase from *Bacillus amyloliquefaciens* has also been purified to homogeneity by affinity precipitation (Teotia, Lata et al., 2001).

Assay methods

A variety of analytical techniques are available for the determination of α-amylase activity. The quantitative procedure, widely used, entails the measurement of new reducing groups formed upon the amylolytic hydrolysis of starch or the decrease in the iodine color of the treated substrate or the decrease in viscosity of the starch suspension. One unit of enzyme activity is represented as the micromoles of products formed or substrate transformed per minute under defined conditions (Greenwood and MacGregor, 1965).

Several methods have been developed based on the reactivity of reducing sugars, released as products of enzyme reaction. The most commonly used among them is DNS method because of its reliability and simplicity (Bernfield, 1955; Robyt and Whelan, 1968).

A semi-quantitative determination of starch hydrolysis by α-amylase involves the measurement of decrease in the blue color produced by starch when complexed with iodine solution. This procedure, which shows the endocleavage of starch, can be used, routinely to assay α-amylases. Decrease in the viscosity of the starch solution can also be a measure of α-amylase activity (Greenwood, MacGregor and Milne, 1965). Since this procedure measures only endoactivity, it can be used to detect α-amylase.

Chromogenic substrates have been developed for α-amylase assays, especially for clinical samples. The dye is covalently linked to starch or one of its constituents (amylose or amylopectin) to give an insoluble material (azure

derivative) (Rinderkneet et al., 1967). When these substrates are acted on by α-amylase, fragments containing dye get solubilized. The remaining insoluble substrate is removed by centrifugation. Absorbance of the supernatant is taken to be a measure of amylase activity. *p*-Nitrophenyl derivative of oligosaccharide has also been used to detect both α- and β-amylase activities. The amount of *p*-nitrophenol released correlates with the amylase activity (Jansen and Wydeveld, 1958). This method is very appropriate and accurate to differentiate between α- and β-amylase activities; yet, it is not used for routine analysis due to the high cost factor. Table 2 gives the various assay methods used for amylases.

Table 2. Assay methods for amylases

Method	**Reagent**	**Reaction analyzed**	**Wave length at reaction measured (nm)**	**Reference**
DNS	Alkaline 3,5-dinitro salicylate	Increase in reducing sugars	540,550	Bernfeld et al., 1962; Robyt and Whelan, 1968
Starch-iodine	Iodine solution	Decrease in the color intensity	550-700	Xiao et al., 2006
Viscosity reduction	-	Decrease in viscocity	-	Greenwood et al., 1965
Degradation of color-complexed substrate	Dyed (Reactone Red 2B) amylopectin	Derivatives of dyed amylopectin	540 nm	Babson et al., 1970

Structural features common to α-amylases

Starch hydrolases and related enzymes have been sorted into more than fifty families based on the common features in their amino acid sequences brought out by the hydrophobic cluster analysis method (Gaboriaud et al., 1987; Lemesle-Varloot et al., 1990; Henrissat, 1991; Henrissat and Bairoch, 1993). β-Amylase and glucoamylase comes under families 14 and 15, respectively, while the rest of starch hydrolases get grouped into the family 13, glycosyl hydrolases. α-amylase family presently covers around 20 different enzyme specificities (Table 3).

The common feature is the presence of a parallel $(\alpha/\beta)_8$ -barrel domain A, which, invariantly inserts a long loop between the third β-strand and the third α-helix (the so-called domain B). Much of the specificity of each enzyme is, mostly defined by amino acid residues of domain A and B (Ann MacGregor et al., 2001).

Structure

Primary structure

α-Amylases are single chain polypeptides with molecular weights in the range 20-55 kDa. The structure of AMY2 and AMY1 of barley contain 403 and 407 amino acid residues, respectively. AMY2, with 80% of its sequence being identical with that of AMY1, is the major isozyme constituting about 80–98% of the total α-amylase (Buckow et al., 2007).

Table 3. The members of the α-amylase family

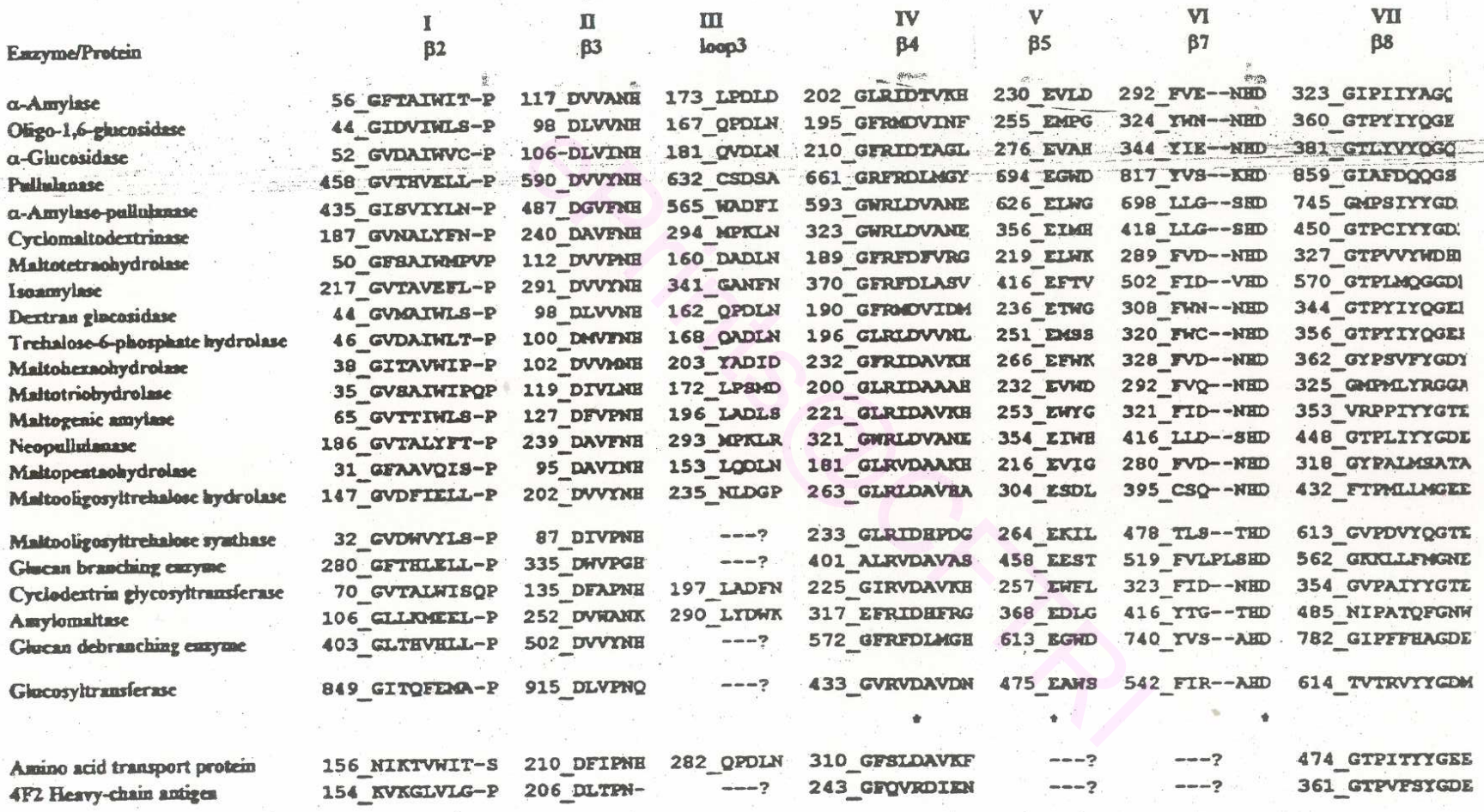
Enzyme code number (EC*)	Enzyme/protein
3.2. 1. 1	α-Amylase
3.2. 1. 10	Oligo- 1,6-glucosidase
3.2. 1 .60	Maltotetraohydrolase
2.4.1.19	Cyclodextrin glycosyltransferase
3.2.1.20	α-Glucosidase
3.2.1.41	Pullulanase
3.2.1.1/41	Amylopullulanase
3.2.1.54	Cyclomaltodextrinase
3.2.1.68	Isoamylase
3.2.1.70	Dextran glucosidase
3.2.1.93	Trehalose-6-phosphate hydrolase
3.2.1.98	Maltohexaohydrolase
3.2.1.116	Maltotriohydrolase
3.2.1.133	Maltogenic amylase
3.2.1.135	Neopullulanase
	Maltopentaohydrolase
	Maltopentaohydrolase
	Maltooligosyltrehalose hydrolase
2.4.1.18	Glucan branching enzyme
2.4.1.25	Amylomaltase
2.4.1.25/3.2.1.33	Glucan debranching enzyme
	Maltooligosyltrehalose synthase
	Glucosyltransferase
2.4.1.5	Glucosyltransferase
	Amino acid transport-related
	4F2 Heavy-chain cell surface antigen

*EC numbers are given if known (*Janecek et al., 1997*).

The isozymes exhibit several differences, which include: isoelectric point, calcium ion affinity and stability at acidic pH and elevated temperature (Bush et al., 1989).

α-Amylases from various sources (microorganism, plants and animals) exhibit only 10% sequence similarity whereas α-amylases from the same group of sources exhibit greater similarity in sequence (Nakajima et al., 1986). Three regions of similarity have been identified between sequences of PPA and TAA (regions II, IV & VI in Figure 5), which, along with the catalytic and substrate binding sites, have been shown in bacterial (Friedberg, 1983) and plant α-amylases (Rogers, 1985). Thereafter, the fourth conserved region (region V in Figure 5) has been well established due to its resemblance in all enzyme specificities (Nakajima et al., 1986). Asp-206, Glu-230, and Asp-297 (in TAA) and their equivalents play, role in catalysis by acting at active site, determined either experimentally by X- ray crystallography and site directed mutagenesis or theoretically by the similarity of known sequences (Holm et al., 1990; Vihinen et al., 1990; Takase et al., 1994). Undoubtedly, ten of the invariant residues, seven of which are involved in the active site (Asp-117, His-122, Arg-204, His-296, Gly-56, Pro-64 and Gly-323), establish low sequence similarity among alpha amylase family (Figure 5).

Figure5. The conserved sequence regions of the members of the α-amylase family. The best conserved parts of the α-amylase-type $(\alpha/\beta)_8$-barrel comprise the strands β2, β3, β4, β5, β7 and β8. There is also a short conserved sequence located near the C-terminus of the longest loop 3. The three proposed catalytic residues (Asp, Glu and Asp in the strands β4, β5 and β7, respectively) are marked by an asterisk (Janecek ., 1997)



Enzyme/Protein	I β2	II β3	III loop3	IV β4	V β5	VI β7	VII β8
α-Amylase	56_GFTAIWIT-P	117_DVVANH	173_LPDLD	202_GLRIDTVKH	230_EVLD	292_FVE--NHD	323_GIPIIYAGQ
Oligo-1,6-glucosidase	44_GIDVIWLS-P	98_DLVVNH	167_QPDLN	195_GFRMDVINF	255_EMPG	324_YWN--NHD	360_GTPYIYQGE
α-Glucosidase	52_GVDAIWVC-P	106-DLVINH	181_QVDLN	210_GFRIDTAGL	276_EVAH	344_YIE--NHD	381_GTLYVYQGQ
Pullulanase	458_GVTHVELL-P	590_DVVYNH	632_CSDSA	661_GRFRDLMGY	694_EGWD	817_YVS--KHD	859_GIAFDQQGS
α-Amylase-pullulanase	435_GISVIYLN-P	487_DGVFNH	565_WADFI	593_GWRLDVANE	626_ELWG	698_LLG--SHD	745_GMPSIYYGD
Cyclomaltodextrinase	187_GVNALYFN-P	240_DAVFNH	294_MPKLN	323_GWRLDVANE	356_EIMH	418_LLG--SHD	450_GTPCIYYGD
Maltotetraohydrolase	50_GFSAIWMPVP	112_DVVPNH	160_DADLN	189_GFRFDFVRG	219_ELWK	289_FVD--NHD	327_GTPVVYWDH
Isoamylase	217_GVTAVEFL-P	291_DVVYNH	341_GANFN	370_GFRFDLASV	416_EFTV	502_FID--VHD	570_GTPLMQGGD
Dextran glucosidase	44_GVMAIWLS-P	98_DLVVNH	162_QPDLN	190_GFRMDVIDM	236_ETWG	308_FWN--NHD	344_GTPYIYQGE
Trehalose-6-phosphate hydrolase	46_GVDAIWLT-P	100_DMVFNH	168_QADLN	196_GLRLDVVNL	251_EMSS	320_FWC--NHD	356_GTPYIYQGE
Maltohexaohydrolase	38_GITAVWIP-P	102_DVVMNH	203_YADID	232_GFRIDAVKH	266_EFWK	328_FVD--NHD	362_GYPSVFYGD
Maltotriohydrolase	35_GVSAIWIPQP	119_DIVLNH	172_LPSMD	200_GLRIDAAAH	232_EVWD	292_FVQ--NHD	325_GMPMLYRGG
Maltogenic amylase	65_GVTTIWLS-P	127_DFVPNH	196_LADLS	221_GLRIDAVKH	253_EWYG	321_FID--NHD	353_VRPPIYYGT
Neopullulanase	186_GVTALYFT-P	239_DAVFNH	293_MPKLR	321_GWRLDVANE	354_EIWH	416_LLD--SHD	448_GTPLIYYGD
Maltopentaohydrolase	31_GFAAVQIS-P	95_DAVINH	153_LQDLN	181_GLRVDAAKH	216_EVIG	280_FVD--NHD	318_GYPALMSAT
Maltooligosyltrehalose hydrolase	147_GVDFIELL-P	202_DVVYNH	235_NLDGP	263_GLRLDAVHA	304_ESDL	395_CSQ--NHD	432_FTPMLLMGE
Maltooligosyltrehalose synthase	32_GVDWVYLS-P	87_DIVPNH	---?	233_GLRIDHPDG	264_EKIL	478_TLS--THD	613_GVPDVYQGT
Glucan branching enzyme	280_GFTHLELL-P	335_DWVPGH	---?	401_ALRVDAVAS	458_EEST	519_FVLPLSHD	562_GKKLLFMGN
Cyclodextrin glycosyltransferase	70_GVTALWISQP	135_DFAPNH	197_LADFN	225_GIRVDAVKH	257_EWFL	323_FID--NHD	354_GVPAIYYGT
Amylomaltase	106_GLLKMEEL-P	252_DVWANK	290_LYDWK	317_EFRIDHFRG	368_EDLG	416_YTG--THD	485_NIPATQFGN
Glucan debranching enzyme	403_GLTHVHLL-P	502_DVVYNH	---?	572_GFRFDLMGH	613_EGWD	740_YVS--AHD	782_GIPFFHAGD
Glucosyltransferase	849_GITQFEMA-P	915_DLVPNQ	---?	433_GVRVDAVDN	475_EAWS	542_FIR--AHD	614_TVTRVYYGD
				*	*	*	
Amino acid transport protein	156_NIKTVWIT-S	210_DFIPNH	282_QPDLN	310_GFSLDAVKF	---?	---?	474_GTPITYYGE
4F2 Heavy-chain antigen	154_KVKGLVLG-P	206_DLTPN-	---?	243_GFQVRDIEN	---?	---?	361_GTPVFSYGD

(*Janecek et al., 1997*)

See previous page for legend for Figure 5

Secondary structure

Structures of α-amylases, reported till date, are characterized by three distinct domains: a catalytic A domain, containing $(\beta/\alpha)_8$ barrel, which forms the core of the molecule and contains the three active site residues (Nielsen and Borchert, 2000).

A large loop protruding between the third β-strand and third helix of the barrel, is known as the B domain. This domain has irregular structure and is the least similar domain in α-amylases. The irregular structure of domain B in barley amylase is stabilized by three calcium ions. This domain from two bacillus α-amylases, one from sacharifying enzyme produced by *Bacillus subtilis* (Yang et al., 1983) and the other from the liquefying enzyme produced by *Bacillus licheniformis* (Yuuki et al., 1985), differs in its length (MacGregor, 1988; Machius et al.,1995). The amino acid sequence of these two domains are hardly alignable (Janecek et al., 1997).

The domain C in the C-terminus, is made up of β-strands, is assumed to stabilize the catalytic domain by shielding hydrophobic residues of domain A from the solvent. The possibility of domain C helping in substrate binding has also been understood (Dauter et al., 1999; Lawson et al., 1994).

Tertiary structure

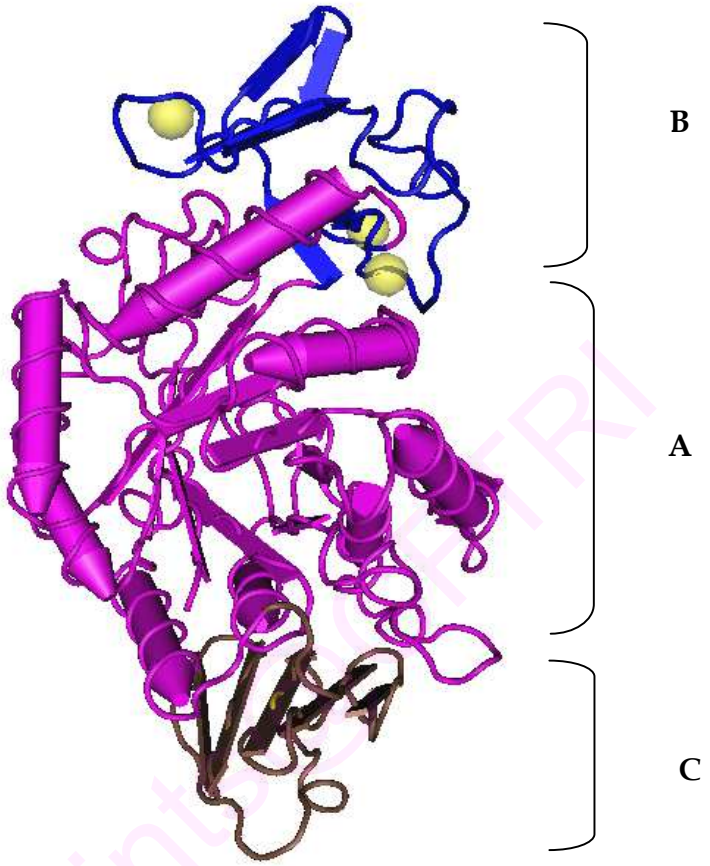
Three-dimensional structures solved by X-ray crystallography are known for: (1) α-amylases from *Bacillus licheniformis* (Machius et al., 1995; Song et al., 1996),

Aspergillus oryzae (TAA; Matsuura et al., 1984; Swift et al., 1991), *Aspergillus niger* (Brady et al.,1991), barley (Kadziola et al., 1994), pig pancreas (PPA; Qian et al., 1993), human pancreas (Brayer et al., 1995), and human saliva (Ramasubbu et al., 1996).

There are many structures solved as complexes of the enzymes with their substrates (Kadziola et al., 1998) or inhibitors (Vallee et al., 1998). The three dimensional structure of barley AMY1 is given in Figure 6. The backbone structures of AMY1 and AMY2 are almost identical, but for only small, local changes (Robert et al., 2002).

Active site

The active site is located in a 3 nm long cleft, between the carboxylic end of the A domain and the B domain. The variation in specificity of α-amylases associated with the hydrolysis of a polysaccharide, such as amylose, has been explained in terms of number of subsites at the active site. A region of the active site that interacts with one glucose ring is known as a subsite. The active site of different α-amylases are made up of 5-11 subsites (A-K) (Figure 7). The reducing end of the α-glucose chain is located towards the K subsite. It is found that the α-amylases from *Aspergillus oryzae*, barley, hog pancreas, *Bacillus amyloliquefaciens*, and *Bacillus subtilis* (saccharifying) contain subsites C-K (Knodo et al., 1980), A-I (MacGregor and MacGregor, 1985), D-H (Robyt and French, 1970; Seigner et al., 1987), A-J (Torgerson et al., 1979), and D-H (Hiromi et al., 1983), respectively.



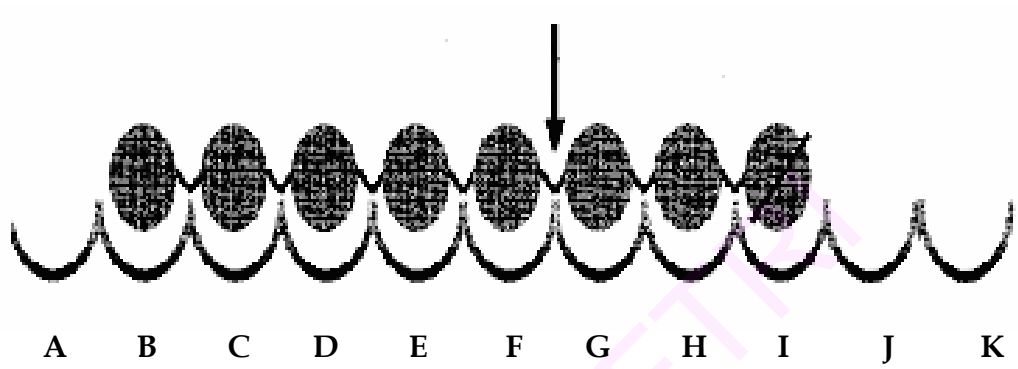
(*Robert et al., 2003*)

Figure 6. Crystal structure of barley α- amylase isozyme 1 at 1.5A° resolution. Three calcium atoms stabilizing B domain is shown in yellow color.

PDB ID: 1HT6

In general, all α-amylases contain subsites D-H, and vary with respect to their containing subsites A-C and I-K. Barley α-amylase and BAA containing subsite A, differ from those of mammalian α-amylases (MacGregor, 1988).

Catalytic site, situated between subsites F and G, is occupied by two Asp and one Glu residues (Asp-179, Asp-289, Glu-204 in barley AMY2, Asp-206, Asp-297, Glu-230 in TAKA and Asp-197, Asp-300, Glu-233 in PPA) (Kadizola et al., 1994). Conservation of the His in all α-amylases at the position equivalent to 122 of *Aspergillus oryzae* suggests an important role for this residue in the interaction with the C-6 primary hydroxyl group of a glucose ring (Matsuura et al., 1984). The three invariant histidine residues, His-122, His-210 and His-296, present in the active site of mammalian pancreatic α-amylases play a role in substrate recognition (Ishikawa et al., 1993) and in the transition state stabilization, with no direct involvement in the catalysis of barley α-amylase (Sogaard et al., 1993). The active site of AMY2, in the crystal structure of a complex between the enzyme and acarbose (Kadziola et al., 1998), appears to be a large V-shaped depression on one side of the enzyme, formed by parts of domain A and domain B (Figure 8). Only two AMY2 residues involved in substrate binding and catalysis differ in AMY1: Lys182/Arg183 and Ser208/Asn209 (AMY2/AMY1 numbering)



(*Adopted from Ann MacGregor., 2001*)

Figure 7. Schematic representation of subsite arrangement

Oligosaccharide occupying subsites B to I. Cleavage occurs between subsites F and G as indicated by the arrow. The reducing end group is bound at subsite I.

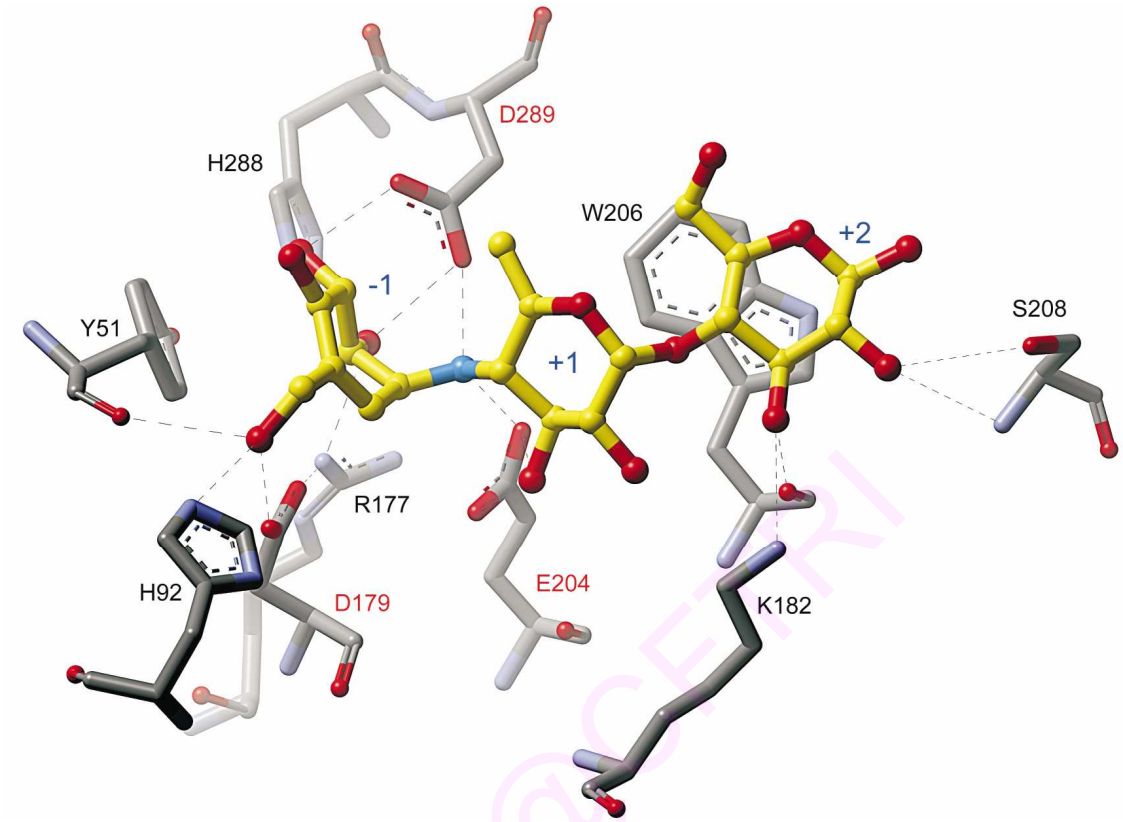


Figure 8. Crystal structure of active site of barley α-amylase 2 (*Robert et al., 2002*) Active site of AMY2 in complex with the acarbose inhibitor (Kadizola et al., 1998) showing catalytic residues (labeled in red) along with the remaining substrate-binding residues. Direct hydrogenbond interactions are shown by dot-and-dash lines. The three sugar rings of acarbose seen in the structure (colored in yellow) are located in subsites −1, +1 and +2 corresponding to F, G and H in Figure 7.

Catalytic Mechanism

Most of the amylases and their related enzymes, undoubtedly, exhibit structural similarity. Reportedly, the similarity in the secondary structure is common among amylases and their related enzymes. Apart from structural similarity, common catalytic mechanism relates these enzymes with one family (Kuriki and Imanaka, 1989; Svensson, 1994).

The anomeric configuration is preserved when the substrate is converted to product. In the other words, enzymes act on α-linkages in glucans or glucosides to yield α-linked products. The reaction is expected to follow by a double displacement mechanism (Figure 9). During the first displacement, an acid group on the enzyme protonates the glycosidic oxygen, bringing about scission of the C1-O bond and transient formation of an oxocarbenium ion-like transition state (Davies et al., 1998; Ly and Withers, 1999; Uitdehaag et al., 1999). The attack of a nucleophilic acid group at the sugar anomeric centre to give a β-glycosyl enzyme intermediate as well as the aglycone leaving the substrate, occur simultaneously. In hydrolysis, during the second displacement, the attack at the anomeric centre by a water molecule, activated by the carboxylate form of the former proton donor, is perfectly reversed (Figure 8). The second stage of the reaction proceeds via an ion-like transition state, as before, to yield a product with α-anomeric configuration, apart from reprotonation of the original acid group.

Three acidic residues, one glutamic and two aspartic acids, have been found at the centre of the active site (Matsuura et al., 1984). Subsequent mutational studies have confirmed the role of these residues in catalysis (Janecek, 1997; Svensson, 1994). The glutamic acid residue is now believed to be the proton donor, while the first of the two conserved aspartic acids appearing in the amino acid sequence of an α-amylase family member is assumed to act as the nucleophile. The role of the second aspartic acid is less certain, it is found to be involved in stabilizing the oxocarbenium ion-like transition state, in addition to maintaining the glutamic acid in the correct state of protonation for activity (Uitdehaag et al., 1999).

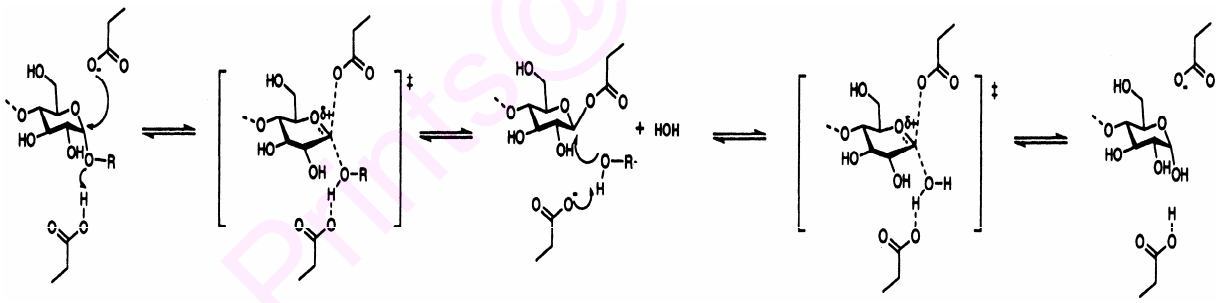


Figure 9. Catalytic mechanism of α-amylase (*Ann MacGregor., 2001*).

The proton donor protonates the glycosidic oxygen and the catalytic nucleophile attacks at C1 leading to formation of the first transition state. The catalytic base promotes the attack of the incoming molecule ROH (water in hydrolysis) on the formation of the covalent intermediate resulting in a second transition state, leading to hydrolysis or transglycosilation product.

Sulfhydryl groups

The number of cysteine residues in α amylase is source specific. Barley α-amylases, AMY1 and AMY2 are found to contain four and three cysteine residues, respectively, among which three are conserved (Kadizola et al., 1994). It is reported that PPA contains two (Pommier et al., 1974) and TAKA amylase A contains one cysteine residue (Hiroko Toda et al., 1968), in masked state. It has been noticed that after denaturation with urea/GuHCl or EDTA, these cysteine residues become accessible to DTNB. Complete blocking of sulfhydryl groups results in total loss of enzyme activity. Although sulfhydryl groups are not part of the active site, they are found to play a crucial role in maintaining the active configuration by chelating with the essential calcium atom (Hiroko Toda et al., 1968).

The major cause of thermal inactivation of α-amylase is the oxidation of sulfhydryl groups. Cys-362 has been identified to be the oxidation prone residue in BStA (Tomazic and Klibanov, 1988; Brosnan et al., 1992). It is reported that methionine residues are also prone to oxidation at higher temperatures. Met-197, located near the active site, has been shown to be responsible for inactivation of BLA (Borchert et al., 1995). The replacement of sulphur groups with any non-sulphur containing amino acid at position 197, considerably reduces the oxidation sensitivity of BLA (Borchert et al., 1995).

α-Amylase from *Pyrococcus furiosus* is more thermostable than its bacterial and archaeal homologues (e.g., *Bacillus licheniformis* TAKA-term and *Pyrococcus*

kodakaraensis KOD1 α-amylases, respectively). *P. furiosus* enzyme contains five cysteines (C152, C153, C165, C387, and C430) of which Cys165 is involved in Zn^{2+} binding and is required for the stability of *P.furiosus* α-amylase at very high temperatures (Savchenko et al., 2002). α-Amylases are strongly destabilized by ethylenediaminetetraacetic acid, suggesting that metal binding may be involved in this enzyme's thermostability (Bush et al., 1989).

α- Amylases as metalloenzymes

Calcium ions are known to play a pivotal role in the stabilization of α-amylases. Loss of α-amylase activity on dialysis or incubation with metal-binding agents, such as fluoride, oxalate, citrate, polyphosphate or EDTA, emphasizes the fact that α-amylase might contain calcium. α-amylase can bind more than one calcium ion (Janecek, 1997). If the enzyme is exposed to Ca^{2+} ions before dialyzing thoroughly against distilled water, the protein molecule will retain as many as ten and seven atoms of calcium in the case of mold amylase and bacterial amylase, respectively (Vallee et al., 1959).

α- Amylases, from various sources, are known to contain a conserved calcium ion, at the interface between domain A and B (Machius et al., 1995; Machius et al., 1998; Boel et al., 1990), essential for the stability and activity of the enzyme. It is found that the role of conserved calcium ion is structural (Larson et al., 1994., Buisson et al., 1987), as it is too far away from the active site to participate directly in catalysis.

Calcium is required to maintain the structural integrity of α-amylases. Removal of calcium leads to decreased thermostability and/or enzymatic activity and increased susceptibility to proteolytic degradation (Saboury et al., 2000). Addition of calcium is the most common approach to stabilize the enzyme and favor its crystallization. So far, it has not been possible to obtain crystals of α-amylase from BAA with bound calcium (Machius et al., 1995). Five crystalline α-amylases originating from different biological sources (human, porcine, bacterial, fungal and cereal), purified according to five different procedures, have each been found to contain at least one gram atom of calcium per mole (Vallee et al., 1959).

The role of calcium in the catalytic activity of the α-amylase was unknown for a few decades due to three misleading facts (Fischer et al., 1960): first, calcium is bound so firmly that ordinary dialysis against metal-binding agents could not remove it; second, under drastic conditions, irreversible denaturation occurred and addition of calcium failed to reactivate the enzyme; lastly, irrespective of the strategies for reversible removal of calcium employed, the loss of enzyme activity could not be detected, because of the very high affinity of most α-amylases for calcium ubiquity of this metal. Traces of calcium might be included in the assay system by glassware, buffers and predominantly by starch, which comprises 0.01 to 0.1% calcium that becomes a thousand fold molar excess of metal with respect to the microgram amount of enzyme used in the assay system. This reactivated the enzyme during incubation with the substrate (Vihinen et al., 1989).

The sufficiency of a single gram-atom of calcium per mole of protein for the restoration of full enzymatic activity of amylase from calcium free sources (bacterial, porcine, human and fungal) establishes the role of calcium in the catalytic activity. It is clearly understood that calcium plays a crucial role, not only in the proper configuration for biological activity but also in stabilizing the secondary and tertiary structure, thereby conferring a compact architecture to the amylase molecule (Bush et al., 1989).

α-Amylase of malted barley binds calcium rather loosely compared to microbial or mammalian amylases. *Aspergillus niger* has primary and secondary binding sites. The primary site, which plays a role in folding around the active site, contains a tightly bound Ca^{2+} whereas the secondary site found at the bottom of the substrate binding cleft involves in the catalytic site residues (Asp-206 and Glu-230) demonstrating the inhibitory effect of calcium, at higher concentrations (Boel et al., 1990). The conserved primary calcium binding site is present in the structure of TAA (Matsuura et al., 1984; Swift et al., 1991), barley (Kadizola et al., 1994) and mammalian alpha amylases (Qian et al., 1993., Larson et al., 1994). The most conserved residue is Asp 175 coming from C-terminal end of domain B (Janecek, 1992).

It is reported that calcium ion is necessary for the correct refolding of the reduced Taka-amylase A enzyme (Takagi and Isemura, 1965) and BLA (Nazmi et al., 2006). There are also reports about other cations such as Sr^{2+}, Mg^{2+}, Ba^{2+} and Na^{+} playing a role in the stability of the enzyme (Bush et al., 1960).

Cereal α-Amylases

α-Amylases, ubiquitous in nature, have been isolated, purified and characterized from a number of animal, plant, fungal and bacterial sources (Hagenimana et al., 1992). Although α-amylases have been isolated from a wide variety of sources, cereal α-amylases gain importance due to their suitability for biotechnological applications in supplementary foods, breweries and starch saccharification. Sources of the commonly used cereal α-amylases include, sorghum (Botes et al., 1967), barley (MacGregor et al., 1978), rye (Baker et al., 1921), wheat (Tkachuk et al., 1974) and ragi (Nirmala and Muralikrishna, 2003). Malted sorghum is an important constituent of supplementary foods.

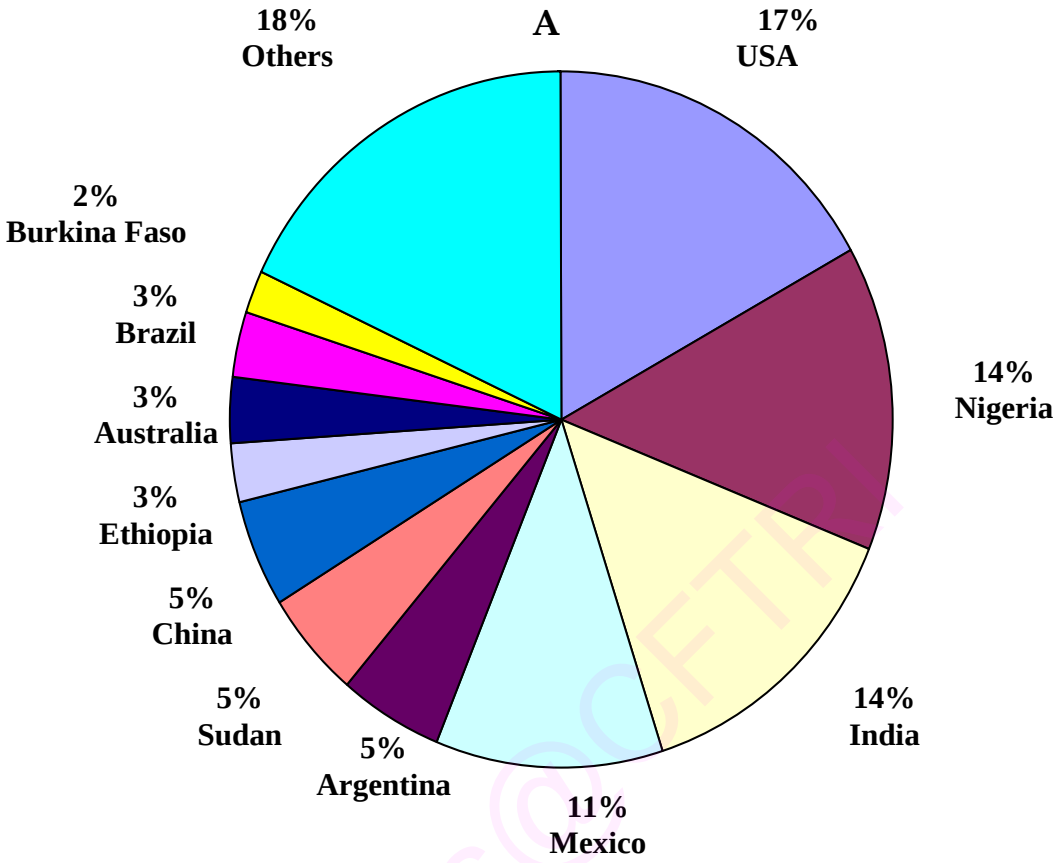
Sorghum bicolor

Sorghum is popularly known as sorghum, great millet, guinea corn, kafir corn, aura, mtama, jowar, cholam, kaoliang, milo, milo-maize. Sorghum belongs to the order of *Cyperales* and family *Poaceae* of the plant kingdom. The top sorghum producing countries (see Figure 10 A) are the United States (17% of the world production), Nigeria and India (each with 14%) and Mexico (11%) (http://www.gramene.org/species/sorghum/sorghum_maps_and_stats.html).

Sorghum bicolor (Figure 10 B and 10 C) is the primary *Sorghum* species cultivated for grain for human consumption and animal feed. Most of the species being drought and heat tolerant are of special important in the arid regions. They form an important component of pastures in many tropical regions. *Sorghum*

species, an important food crop in Africa, Central America and South Asia, is the "fifth most important cereal crop grown in the world" (Adebiyi, A. O et al., 2005). Its ability to grow in areas that are low in moisture and fertility, coupled with photosynthetic efficiency, makes it an attractive alternative to other cereals (Mutisya et al., 2003). Seed weight and chemical composition of sorghum is shown in Table 4. α-Amylase is present in trace amounts in ungerminated jowar but increases, largely, during germination.

One of the major obstacles to the use of cereal enzymes is their inactivation at higher temperatures (Tomazic and Klibanov, 1988). Physical and chemical instabilities of proteins are the most daunting and challenging tasks in the development of a suitable protein formulation. Formerly, only a few α-amylases have been commercially available for meeting the very diverse application conditions. A single α-amylase, obviously, cannot be sufficient to meet the particular demands of every industrial process; optimization of α-amylases for each individual application is, therefore, desirable. In spite of long history of these enzymes, the structure-function and structure-stability relationships have been elucidated only for a few of the α-amylases. Since α-amylases are widely used in industrial process for starch degradation and often at higher temperatures, a clear understanding of the molecular mechanisms of inactivation and structure-function-stability of these enzymes are likely to be very useful for designing stable enzymes.



Source: http://www.gramene.org/species/sorghum/sorghum_maps_and_stats.html

B

C

Figure 10. ***Sorghum bicolor***

A. Global sorghum production; B. Sorghum plant; C. Sorghum seeds

Table 4. Chemical composition of sorghum seed

Component	Content (%)
Protein	14.1
Starch	70.8
Soluble sugars	1.3
Crude fibre	1.9
Fat	3.3
Ash	2.1

(*Jambunathan, R. et al., 1984*)

Stability

Stability is a prerequisite for enzymes to be functional at extreme conditions. Studies on protein stability explore the sequence-structure-stability relationship. Sequence defines structure, whose interactions with each of the domains/subunits stabilize the protein (Razvi and Scholtz, 2006). The stability of a protein can be determined by studying the effect of temperature and denaturants on its structure. A precise understanding of thermodynamic and conformational stability contributes to the prediction of enzyme stability.

Conformational stability measurements assume that the molecule may belong only to two thermodynamic states, the folded state (typically denoted N or F) and the unfolded state (typically denoted U). This "all-or-none" model of protein folding, first proposed by Tim Anson (1945), is believed to hold only for small, single structural domains of proteins (50-200 amino acid residues). As the length of the polypeptide chain increases, it becomes energetically unfavorable to form one large domain. Thus, globular proteins with molecular weights over 30 kDa tend to form multidomain structures with different degrees of interdomain interactions (Griko et al., 2001). Barley AMY2 and PPA α-amylases, reportedly, have intimate association between the three structural domains (Sogaard et al., 1993).

Some proteins regain their native and functional structure upon removal of the denaturant. This kind of unfolding, also called thermodynamically reversible unfolding, comes handy in the determination of the thermodynamic

parameters. It is generally accepted that the driving force for the refolding of a protein resides in its amino acid sequence (Strucksberg et al., 2007). Refolding of small globular proteins, with amino acid residues 100-200, is feasible. One of the major problems in the refolding of multi-domain proteins is related to aggregation of non-native states, which often inherently reduce the efficiency of the refolding process. The proper refolding of these proteins, *in vivo*, takes place under the influence of molecular chaperons, which serve to prevent aggregation of proteins.

The following limitations in the experimental approach of folding have been identified to minimize aggregation and to optimize the renaturation yield of large proteins.

1. Low protein concentrations, which aid folding over aggregation.
2. To carry out folding under strong native conditions.
3. Proper physical and solvent conditions. The yields and rates of renaturation of large proteins considerably vary with pH, temperature, ionic strength, higher salts, presence of folding assisting agents like detergents, residual denaturants etc.

In other cases, the refolding of a protein cannot be achieved due to covalent modifications during unfolding, such as thiol modification, deamidation, cleavage of labile peptide bonds, removal of prosthetic group, etc. (Sudharshan and Rao, 1999)

In this work, we have studied reversibility of unfolding transition for a medium sized, multidomain protein, namely, sorghum α-amylase. Effect of different additives such as cyclodextrins or glycerol or polyethylene glycol or DTT has been added to the dilution buffer in order to study aggregation and inactivation of enzyme during the refolding process.

Structural basis for thermal stability

Thermal inactivation of enzymes occurs in two steps.

$$N \leftrightarrow U \rightarrow I,$$

where N is the native enzyme, U is the reversibly unfolded enzyme and I is the irreversibly inactivated enzyme. The first reversible step is partial unfolding of protein molecules. The second step is irreversible due to conformational and covalent processes. The major conformational processes include aggregation due to enhanced hydrophobic interactions and formation of incorrect structures. The covalent processes are hydrolysis of Asp-X peptide bonds, destruction of disulphide bonds and deamidation (Tomazic and Klibanov, 1988).

For multidomain proteins, such as α-amylases, the unfolding transitions are accompanied by an irreversible step, often related to aggregation. As a consequence the analysis of thermostabilities in terms of equilibrium dynamics is not applicable. α-Amylases, in nearly all cases, unfold irreversibly. AHA is the largest multidomain (~ 50 kDa) that has been shown to unfold/refold reversibly by a cooperative two-state mechanism. It is supposed that chemical

modifications, such as deamidation, cysteine oxidation or peptide bond hydrolysis, take place once the protein is unfolded. One approach to reduce protein aggregation of unfolded α-amylases is to add co-solvents such as non-denaturant sulfobetaine or cyclodextrins (Fitter et al., 2005).

Many of the industrial amylases generally require their reactions to be conducted under harsh conditions, such as high temperatures and pressures, to improve productivity and reduce microbial contamination (Gupta, 1991). The stability of these enzymes, usually the factor that limits their utility, has long been a practical concern. The thermal stability of α-amylases can be changed intrinsically by alteration of amino acids or extrinsically by addition of suitable stabilizing effectors (e.g., cations, polyhydric alcohols, substrates, etc.). Extensive literature is available on the studies of the thermal stability of bacterial amylases. However, very less information is available on the thermal stability studies of cereal amylases.

α-Amylases from *Bacillus* species *licheniformis*, *amyloliquifaciens* and *stearothermophilus* are among the most studied amylases. These α-amylases are homologues with respect to primary and tertiary structure (Declerck et al., 1997). Sequence similarly between α-amylase from: BLA and BAA is 80% and BLA and BstA is 65% (Yuuki et al., 1985). This is evident from the crystal structures of these enzymes. Despite the structural similarities between the α-amylases, they exhibit significant differences in their stability. Studies of proteins differing

slightly in structure should allow us to gain a good understanding of the forces that determine the conformations of proteins and to optimize their stabilities.

Bacterial α-amylases, *Bacillus stubtilis*, *Bacillus amyloliquifacients* and *Bacillus Stearothermophilus* exhibit similar trend (Janecek, 1997). Their thermostabilities are directly proportional to the hydrophobicity of the interior and hydrophilicity of the exterior of their protein molecules (Janecek, 1993b). It has been proposed that the mutation of His-133 and Ala- 209 by hydrophobic amino acid residues stabilizes *Bacillus licheniformis* amylase (Declerck et al., 1990; Joyet et al., 1992). The established structural model of *Bacillus licheniformis* α-amylase has demonstrated that the gain in protein stability can be ascribed in the case of His-133 to the β-sheet forming potential of the inserted leucine and in the case of Ala-209 to the cavity filling effect of the inserted valine (Declerck et al., 1995).

Site-directed mutagenesis experiments (SDM) and comparisons of structure and stability of α-amylases revealed some important factors that contribute to the remarkable stability of α-amylases. SDM of the BAA revealed that deletion of Arg-176 and Gly-177, substitutions of Ala for Lys-269 and Asp for Asn-266 are responsible for the enhancement in thermal stability (Suzuki et al., 1989). The extra stability of BLA compared to its counterparts BAA and BStA appeared to be mainly due to the additional salt bridges involving a few specific lysine residues (Lys-385 and Lys-88 and/or Lys- 253) (Tomazic and Klibanov, 1988).

Proline's pivotal role for thermostabilization of proteins has been well studied (Matthews et al., 1987). Replacement of Arg-124 (corresponding to Pro-122 of BLA) to proline in an alkalophilic *Bacillus* species was found to stabilize the enzyme (Nielsen and Borchert, 2000). Similar results have been observed for the α-amylase from *Bacillus* species KSM-1378 (Igarashi et al., 1999)

Interestingly, after hundreds of α-amylase mutants have been made and characterized, the following observations have emerged:

1. Thermostability determinants of bacterial α-amylases are concentrated in domain B and in the nearby region of the central A domain (Figure 11).
2. Single mutations in this region can have large destabilizing (upto −22°C in T_{50}) and considerable stabilizing (+ 5°C range) effects.
3. Combining mutations in this region yielded a hyperstable enzyme showing a 23 ◦C increase in T_{50} to 106°C (Declerck et al., 2003; Machius et al., 2003).
4. All stabilizing mutations affect the surface of the protein.

The conformational stability measurements of sorghum α-amylase have been followed by unfolding, induced by pH, denaturants and temperature. The role of conserved cysteine residues, in structure and activity, has been followed by covalent fluorescence labeling. These studies have provided clues to the conformational stability of the molecule and the role of cysteine residues and electrostatic interactions in structure and activity.

Figure 11. Indication of key mutations in *Bacillus licheniformis* α-amylase (*Vincent G.H. Eijsink et al., 2004*)

Domain B (residues 101-205) is shown in dark green. Green balls specify the seven residues (positions 133, 156, 181, 190, 209, 264, 265) that were mutated in a hyperstable enzyme (Declerck et al., 2003). Mutations at positions 204, 237 (dark blue balls) are responsible for remarkable destabilization. Light blue and orange balls indicate calcium and sodium ions, respectively.

AIM AND SCOPE OF PRESENT INVESTIGATION

The starch-converting enzymes have been exploited by the starch processing industry as a replacement for acid hydrolysis in the production of starch hydrolysates and other applications. Presently, these enzymes comprise about 30 % of the world's enzyme production (Khajeh et al., 2006). α-Amylases are among the most important starch-converting enzymes.

Each application of α-amylase requires unique properties with respect to specificity, pH dependence, temperature and stability. Screening of various sources with higher alpha-amylase activities could therefore, facilitate the discovery of novel amylases suitable to new industrial applications. In the past two decades amylases from various sources have been isolated and characterized with respect to their stabilities.

Supplementary foods absorb large quantity of water on reconstitution with hot water due to their pregelatinized starch content. Malted cereals being a rich source of hydrolytic enzymes (α-amylases) can be an integral part of supplementary foods. α-Amylases, in the supplementary food, predigest starch, leading to a decrease in water absorption capacity and an increase in nutrient density. Thermal stability of α-amylase is a desirable feature as the supplementary food is reconstituted with hot water/milk before consumption.

Thermostable alpha-amylases have had extensive commercial applications in starch processing, brewing and sugar production. Attempts are being made to

stabilize novel enzymes against extreme environmental conditions by genetic and protein-engineering techniques by targeting random sites in the gene or protein molecule (Brosnan et al., 1992; Knowles, 1987). However, to successfully achieve this it is necessary to understand the structure – function and stability relationships of these enzymes under different conditions.

The main objective of the present investigation has been to screen cereals for thermostable α-amylases, which retain activity when hot water is added to supplementary foods to reconstitute them and to study the structure-function and structure-stability relationships as well as the folding and refolding behavior of α-amylase which can lead to the development of more efficient and diverse applications.

With the above objectives the following studies were undertaken.

1. To compare the action of malted cereal amylases in supplementary foods; and
2. To understand the structure and stability of α-amylase from sorghum.

With the above studies an attempt has been made to understand the interactions that contribute to the stability of α-amylase. These studies have been carried out by employing different spectroscopic and activity measurements.

The salient findings of the present investigation are

1. Isolation, purification and characterization of α-amylase from malted sorghum and comparison with other cereal amylases with respect to the products of starch degradation
2. Entrapment of α-amylase in alginate beads: single step protocol for purification and thermal stabilization
3. Conformational stability measurements of α-amylase –effect of pH, salts, ionic strength, denaturants and temperature
4. Mechanism of inactivation of α-amylase: Role of divalent cations (Ca^{2+}) in stabilization

These studies have provided clues to the conformational stability of the molecule and the role of non-covalent and covalent interactions in structure and activity.

MATERIALS AND METHODS

MATERIALS AND METHODS

Materials

DEAE-Cellulose, Sephadex G-75, Acrylamide, Bis-acrylamide, TEMED, Ammonium per sulphate, Protein molecular weight markers (SDS), 3,5-dinitrosalicylic acid, Maltose and maltooligosaccharides, Calcium chloride, Tris base, Hepes, CAPS, Urea and GuHCl, DTT, Iodoacetamide, α-Cyclodextrin, MIANS, PEG, Glycerol, DTNB, Chelex sodium, EDTA, Trypsin , Sodium alginate (A-2158, low viscosity alginate) were from Sigma Chemical Co. (St. Louis, MO, USA); Termamyl1® 120 LS from *Bacillus licheniformis* and Fungamyl1® 2500 SG from *Aspergillus oryzae* were commercial preparations (M/s Novozymes, Denmark). All other chemicals used were of analytical/HPLC grade.

Malted cereals

Barley (*Hordeum jubatum*, commercial variety), Ragi (*Eleusine coracana*, commercial variety) and Jowar (*Sorghum bicolor* variety M-35-1) were purchased from local market, Mysore, Karnataka, India. The seeds, cleaned and soaked in water for 12 hours, were allowed to germinate for 60 hours. After germination, the seeds were air-dried and vegetative growth portions were removed by gentle, manual brushing. Seeds were powdered to a particle size of 355 μm. The powder was stored in an airtight container at 4°C after being defatted using hexane.

Purification of sorghum malt α-amylase

α–Amylase was purified at 4°C with modifications to the procedure reported earlier (Botes et al., 1967). Defatted sorghum malt, extracted with acetate buffer (1:10 ratio, 0.05 M, pH 4.8) having 13.6 mM calcium ($CaCl_2.2H_2O$), following centrifugation to remove coarse particles, was subjected to ammonium sulphate precipitation (0-40%). After centrifugation, the precipitate, redissolved in Tris–HCl (0.1 M, pH 7.0) having 13.6 mM calcium, was dialyzed and applied to a DEAE-Cellulose column (20 x 1.5 cm, 35 ml) and eluted with the same buffer to eliminate contaminating proteins. Bound proteins were eluted with a buffer containing 0.3 M NaCl to recover amylase activity. The pooled (active) fractions were precipitated by ammonium sulphate (0-70%). The precipitate was equilibrated in Tris–HCl (0.1 M, pH 7.0) having 13.6 mM calcium and chromatographed on a gel filtration column (G-75 superfine, 135 x 1 cm, volume 110 ml) using the same buffer. The active fractions were pooled, reprecipitated using ammonium sulphate (0-70%) to recover the active protein in pure form.

Homogeneity and molecular weight determination

Homogeneity of the enzyme was checked by 12.5% SDS-PAGE (Laemmli, 1970) PAGE, under native conditions, was done without SDS and β-mercaptoethanol. The gels were stained for protein with silver nitrate (Blum et al., 1987) while enzyme activity was stained using iodine (Acevedo and Cardmelli, 1997). Markers in molecular weight range 14.2 to 66 kDa were used to determine the molecular weight of α-amylase on SDS-PAGE.

Determination of α-amylase activity

A modification of the procedure devised by Bernfeld (Bernfeld et al., 1962) was used to determine enzyme activity. Gelatinized soluble starch (2%) was incubated with enzyme appropriately diluted with acetate buffer (0.05 M, pH 4.8) having 13.6 mM calcium, for 5 minutes at 60°C. The reaction was stopped by the addition of reagent (3, 5-Dinitrosalicylic acid). The tubes were kept in boiling water for 5 min to develop the color and cooled. The absorbance was read at 540 nm after making up the volume to 20 ml. One unit of enzyme activity is defined as µmole of maltose equivalent released per minute under the assay conditions. Specific activity was expressed as activity units per mg of protein.

Isothermal equilibrium measurements followed by activity measurements

Aliquots of enzyme (~0.4 µM), in buffer A and B, were incubated at 27°C, separately with GuHCl and urea, to attain equilibrium, prior to measuring enzymatic activity. The concentration range and duration of incubation for GuHCl and urea, were (0.1 – 6 M, 4 h) and (1 – 9 M, 12 h), respectively. Enzyme samples, withdrawn from the incubation mixture, were used to perform activity measurements under standard conditions. Corresponding blanks for all samples were treated in the same manner, except that buffer replaced the enzyme.

Determination of kinetic constants of sorghum amylase

To determine the effect of substrate concentration on enzyme activity, substrate concentrations, ranging from 0.26 to 2.1%, were used. Kinetic constants, K_m and V_{max}, were calculated from Lineweaver-Burk plot.

pH optimum

The activity of α-amylase was tested in different pH values in 0.05 M acetate buffer (3.5 – 6.0), Tris-HCl buffer (7.0 – 8.0) and borate buffer (9.0) with 2% gelatinized soluble starch as a substrate. All buffers contained 13.6mM Ca^{2+}, unless otherwise indicated. The relative activity at different pH was calculated (Taking the maximum activity obtained as 100%).

Temperature optima

To determine the temperature optimum of sorghum malt α-amylase, activities were determined in the temperature range 30-80 °C. The assay was carried out in the same manner as described under activity measurements.

Separation and identification of products by HPLC

To determine the mode of action of the enzyme, DP (Degree of Polymerization) of the products was determined by HPLC (Nirmala, 2001). 100 ml of soluble maltodextrin (30% in 0.05 M acetate buffer, pH 4.8 having 13.6 mM calcium and 0.02% sodium azide) was incubated with 5400 U of α- amylase for different time intervals (2, 6, 12, 24 & 72h) at 60°C. At the end of incubation time, 10 ml of reaction mixture was drawn and 3 volumes of absolute ethanol added to stop the reaction. The mixture was kept for 6 h at 4°C for precipitation and

settling of the undigested maltodextrin. The precipitate was separated by centrifugation at 10,000 rpm and discarded. The supernatant was concentrated by vacuum evaporation as reported. The concentrated products, dissolved in ultra pure water, filtered through 0.22µm membrane, were injected (20µl) and analyzed by HPLC on µBondapak NH_2 column (3.9 x 300 mm, 10 µm) using acetonitrile-water solvent system (70:30) at a flow rate of 1 ml/ min. The amounts of oligosaccharides (DP1 – DP7) were quantified by peak integration, with standards detected using a refractive index detector. Glucose (DP1), Maltose (DP2), maltotriose (DP3), maltotetraose (DP4), maltopentaose (DP5), maltohexaose (DP6) and maltoheptaose (DP7) were used as standards.

Entrapment of amylase

For entrapment procedures, defatted sorghum malt was extracted with acetate buffer (0.05 M, pH 4.8, 1:10 ratio) for 2 h and centrifuged to remove coarse particles.

Entrapment of amylase was carried out as described previously by Gupta et al. (Gupta et al., 1993). In short, alginate (3%, w/v) was dissolved in acetate buffer (0.01 M, 5.0 pH). One millilitre of this alginate solution was mixed with 0.5 ml of 1% amylase solution (amylase solution prepared by dissolving 1 g of Fungamyl1 or sorghum amylase extract or Termamyl1 in 100 ml of same buffer). The above solution was incubated at 25 °C for 30 min. The solution, dropped through a pipette into 0.02 M $CaCl_2$ solution (in water) with gentle stirring, was

incubated for 2 h at 25 °C to generate entrapped enzyme in calcium alginate beads. For all the subsequent experiments these beads were considered to be entrapped enzyme. Protein concentration was calculated by taking $E^{1\%}$ as 24.2 at 280 nm (Whitaker and Granum, 1980)

Enzyme characteristics of free and entrapped *A.Oryzae* α-amylase

pH optimum

pH optima of both entrapped and free enzyme were tested by assaying activity in the pH range of 3.0–9.0. Buffers (0.1 M) used included acetate (3.0–5.5), phosphate (6.0–8.0) and borate (9.0). Relative activities at the test pH were calculated assuming the maximum activity observed during the experiment to be 100%

Temperature optimum

Temperature optima of free and entrapped enzymes were determined by assaying activity in the temperature range of 10–75 °C. The temperature at which maximum activity was observed was taken to be 100% and relative activities (at different temperatures) calculated.

Thermal stability of entrapped amylase

Midpoint of thermal inactivation, T_m

Activity loss, as a function of temperature, was followed in acetate buffer (0.1 M, pH 5.0). Both free and entrapped enzyme samples were incubated for 15 min at different temperatures in the range of 20–80 °C. After cooling, residual activity was measured (at 55 °C) by transferring an aliquot to the assay mixture.

The midpoint of thermal inactivation (T_m), where the activity was diminished by 50%, was calculated from the plot of percent residual activity versus temperature. Activity of the unincubated enzyme was taken to be 100%.

Thermal inactivation kinetics

Kinetics of thermal inactivation of free and entrapped enzyme were studied at different temperatures in the range of 52–65 °C. Enzyme samples (in 0.1 M acetate buffer, pH 5.0) were incubated at the test temperature. Aliquots withdrawn at appropriate time intervals were immediately cooled in an ice bath before measuring residual activity. Activity of the unincubated enzyme was taken as 100%. From a semilogarithmic plot of residual activity versus time, the inactivation rate constant, kr, was calculated. The temperature dependence of kr was analyzed from the Arrhenius plot to obtain the inactivation parameters.

Reusability of the entrapped amylase

To test the reusability, amylase entrapped alginate beads of different sizes <1, 2.5–3, 3.5–4.1 mm) were used to assay enzyme activity. After incubation, the beads were removed from the reaction mixture and reused after washing thrice with double distilled water. Activity was determined in the same manner as described for enzyme assay. The decrease in activity for each cycle was determined assuming activity of beads in the first cycle to be 100%.

Buffers and samples used for conformational stability studies of sorghum α-amylase

All the buffers were prepared in double distilled water.

Acetate buffer **-** 0.05 M, pH 4.8 with 13.6 mM Ca^{2+} (Buffer A)

Hepes buffer - 0.05 M, pH 7.0 with 13.6 mM Ca^{2+} (Buffer B)

The other buffers used for α –amylase stability studies were: glycine – HCl for pH 3.0 and 4.0; sodium acetate for pH 4.8; succinate buffer pH 6.0; Tris-HCl for pH 7.0 and 8.0; borate buffer for pH 9.0; glycine – NaOH for pH 10.0, 11.0 and 12.0. Molarities of the buffers prepared were 0.05 M. All buffers contained 13.6 mM of Ca^{2+}, unless indicated otherwise.

GuHCl and urea stock solutions were prepared fresh (in buffer). pH of the stock solutions at high denaturant concentrations was corrected by the addition of HCl or NaOH. The Ca^{2+}concentration in the working solutions was 13.6 mM. The denaturant concentrations of stock solutions were determined at 27°C by refractive index measurements using Eq. 1 and 2 for GuHCl and urea respectively (Pace and Scholtz, 1987).

$$\text{GuHCl molarity} = 57.147(\Delta N) + 38.68(\Delta N)^2 - 91.60(\Delta N)^3 \quad (1)$$

$$\text{Urea molarity} = 117.66(\Delta N) + 29.753(\Delta N)^2 + 185.56(\Delta N)^3 \quad (2),$$

where ΔN is the difference in the refractive index between the denaturant solution and water (or buffer) at the sodium D line.

α–Amylase stock (10 times the requisite final protein concentration), was prepared in buffer B. No change in pH was observed on dilution. Buffer,

denaturant stock and 10-20 µl of protein stock solution were added to yield the desired final protein concentrations with the GuHCl and urea concentrations in the range 0.1-6 M and 1-9 M, respectively. Samples containing GuHCl and urea were vortexed and incubated at 27°C for 4 h and 12 h, respectively, to equilibrate. Longer incubation times produced identical signals. Corresponding blanks for all samples were treated in the same manner as described above, except that buffer replaced the enzyme.

Fluorescence spectra

Fluorescence measurements were carried out using a Shimadzu RF-5000 Spectrofluoremeter attached with a constant temperature circulating water bath (Huber, Germany). Excitation and emission slit widths were set at 5 nm and 5 nm, respectively, using a 10 mm path length cuvette containing 13.6 mM calcium. Protein concentration used was less than 0.1 O.D at 280 nm to minimize inner filter effects.

Isothermal equilibrium measurements followed by fluorescence

Protein concentrations of ~ 0.6 µM (< 0.1 O.D at 280 nm) were used. The excitation wavelength was set to 280 nm. The change in fluorescence intensity at 346 nm (emission maxima) was recorded to monitor the (unfolding) transition. The experiment was performed at 27°C, using a 10 mm path length cuvette with excitation and emission slit widths set at 5 nm and 5 nm (or 10 nm), respectively.

GuHCl induced isothermal unfolding curves were obtained in the temperature range 297 – 323 K in buffer A and 293 – 323 K in buffer B, respectively.

Circular dichroism measurements (CD)

CD spectra were recorded on Jasco J-810 spectropolarimeter, calibrated with d-(10) camphor sulfonic acid, ammonium salt using protein concentrations of 0.094 mg/ml and 0.83 mg/ml, for far-UV and near-UV regions, respectively. All the enzyme solutions were prepared in acetate buffer (0.05M, pH 4.8) containing 13.6mM calcium, unless indicated otherwise. Molar ellipticity values were expressed as mean residue ellipticity using a residue weight of 110 g mol^{-1}. Far –UV CD was recorded in the range 200-260 nm using a 1 mm path length cell while near-UV CD (230 – 320 nm) was recorded using a 10 mm cell. The scan speed was 10 nm/min using a bandwidth of 1 nm. An average of 3 runs was recorded. The secondary structure of α-amylase was analyzed using the computer program of Yang et al. (Yang et al., 1986).

Isothermal equilibrium measurements followed by CD

Protein concentrations of 3.4 μM were used. Molar ellipticity per mean residue, [θ] in deg. $cm^2.dmol^{-1}$ was calculated from the equation: $[\theta] = [\theta]_{obs}.mrw/10lC$, where $[\theta]_{obs}$ is the ellipticity measured in degrees, mrw is the mean residue molecular weight (110 g mol^{-1}), *C* is the protein concentration in g L^{-1} and *l* is the optical pathlength of the cell in cm. The scan speed was 10 nm/min at a bandwidth of 1 nm. An average of 3 runs was recorded. Cell

cuvette thickness and protein concentration were chosen in such a way that the maximum high-tension voltage of the photomultiplier does not exceed 600 V at the lowest wavelength. GuHCl and urea induced equilibrium unfolding curves were obtained, for the samples, by recording the CD signal at 27°C and 222 nm.

Thermal unfolding of α-amylase followed by CD

Thermal unfolding curves, in presence of denaturants at various concentrations, were obtained at 222 nm by CD spectroscopy in the temperature range 25 - 85°C. The scan rate was maintained at 1°C min^{-1}. The concentration of the enzyme was ~3.5 µM. All the signals were normalized before plotting the data.

Temperature-scanning spectroscopy

Absorbance measurements were carried out using spectrophotometer from Amersham Biosciences model-ultrospec4300*Pro*, fitted with a temperature programmer to control the speed of temperature change in thermal melting experiments. The concentration of the enzyme was ~3 µM. Thermal unfolding profiles at 280 nm (for enzyme) and at 400 nm (turbidity due to aggregation) were recorded in the temperature range 25 - 85°C.

Thermal stability

The enzyme was incubated at different temperatures in the range 30-85°C for 15 minutes. Enzyme aliquots were immediately cooled in an ice bath and assayed for activity as described under enzyme assay. T_m, the midpoint of

thermal inactivation, was calculated as the temperature at which 50% loss of activity occurred under the test conditions. Inactivation kinetics was carried out by incubating the enzyme at the test temperature. Aliquots of the enzyme were drawn at different time intervals, cooled to 4°C and residual activity assayed. Activation energy (E_a), enthalpy (ΔH^*), entropy (ΔS^*) and free energy change (ΔG^*) were obtained from the Arrhenius plot.

Interactions involved in α-amylase activity, conformation and stability

Non-covalent and covalent interactions contributing to the thermal stability of α-amylase were followed by incubating the enzyme (0.3 μM) in buffer A at 70°C, (T_m of sorghum α-amylase) in absence and presence of different additives such as NaCl, calcium, substrate, ethylene glycol, glycerol and copper. Aliquots of the enzyme were drawn at different time intervals, cooled in ice before subjecting to residual activity assay at 60°C, the optimum temperature for activity. No significant pH change was observed for sample following heating to 70°C. The activity of the unincubated enzyme was considered to be native with 100% activity and corresponding catalytic half-lives of thermally treated enzyme calculated.

Refolding of the chemically unfolded enzyme

Refolding was conducted by rapid dilution (~ 25 fold) of the denaturant-protein mixture using either buffer A or buffer B freshly prepared with any one among - (i) 50 mM α-cyclodextrin (ii) 10 % PEG (iii) 10% glycerol and (iv) 15 mM

DTT . The resulting mixture following incubation at 27 °C for 2 h was used for fluorescence and activity measurements. To measure the recovery of activity, aliquots of the enzyme were drawn at different time intervals. Residual activity was assayed under standard conditions.

Far - UV CD spectra of the refolded enzyme were recorded after dialyzing the unfolded enzyme against buffer B (instead of dilution) and adjusting the protein absorbance corresponding to that of native enzyme absorbance at 280 nm**.** DTT (15 mM) was added before measuring the far – UV CD spectra. The aggregation kinetics during refolding of chemically unfolded α-amylase were measured as reported earlier (Khodarahmi and Yazdanparast, 2004). Buffer A or buffer B with 15 mM DTT were used for dilution (20 fold) to follow renaturation.

Refolding of thermally unfolded enzyme

Thermally unfolded enzyme was first disaggregated by dilution with an equal volume of GuHCl (final concentration of 3M) at 27 °C for 30 min, followed by further 10 fold dilution with buffer B having 15 mM DTT and incubation for 2 h. Enzyme assay was carried out under optimal conditions.

Reactivity of thiol groups

Determination of thiol content was performed according to the procedure reported earlier (Riddles et al., 1983). The thiol groups exposed during the course of unfolding of α-amylase in buffer B, by chemical denaturants, were

quantified by measuring their reactivities with DTNB as a function of the denaturant concentration.

The environment around the cysteine residues of α-amylase was probed by modifying the thiols with MIANS. Solutions of MIANS, stored in the dark at -20°C, were used within 48 h after preparation. Samples containing α-amylase (0.8 μM) in 0.1 M Tris – HCl buffer (pH 7.4) with various concentrations of GuHCl and 1 mM MIANS were incubated for 1 h. The fluorescence spectra of the labeled α-amylase were recorded by exciting the sample at 280 nm for protein and 328 nm for MIANS.

Acetylation of α-amylase

Acetylation of α-amylase was carried out according to the procedure reported earlier (Fraenkel, 1957). The extent of modification was determined by measuring the residual free amino groups in the enzyme using 2, 4, 6-trinitrobenzenesulphonic acid (Fields, 1971). The purified, unmodified α-amylase was used as a reference standard.

Amino acid analysis

Amino acid analysis was performed according to the method of Bidlingmeyer et al., (Bidlingmeyer et al., 1984) using a Waters Associate Pico-Tag amino acid analysis system.

N-terminal sequencing of sorghum α-amylase

Sorghum α-amylase was reduced and blotted onto a PVDF membrane after SDS-PAGE. Electrophoretic transfer (Mini Trans-Blot®; Bio-Rad) was carried out using 10 mM CAPS (3- [cyclohexylamine]-1-propane sulfonic acid) (pH 11.0) with 0.1 % SDS and 10% (v/v) methanol (100 V, 3 h at 25 °C). The membranes were stained with coomassie brilliant blue. Protein bands were excised and subjected to N-terminal sequencing by automated Edman degradation using an Applied Biosystems 491A automated gas phase protein sequencer (Procise 491A).

Sequence alignment and modeling studies

Cereal α-amylase sequences were aligned using 'MultAlin' option from proteomics and sequence analysis tools, obtained from http://expasy.org/tools/, which also gives information about sequence similarity. Sequences of cereal α-amylases used in the multi alignment taken from http://www.expasy.org/enzyme/3.2.1.1 included *Hordeum jubatum* (barley isoenzyme 1 - P00693, isoenzyme 2 - P04063), *Oryza sativa* (rice - P17654) and *Triticum aestivum* (wheat - P08117). *Sorghum bicolor* α-amylase (Sorghum - sb04g034150; Protein ID: 5039071). These sequence data were produced by the US Department of Energy Joint Genome Institute 'http://www.jgi.doe.gov/'.

The modeling study was performed using the Geno3D server, available at http://geno3d-pbil.ibcp.fr (Combet et al., 2002). Barley AMY 1 (Robert et al.,

2003), sharing > 75 % identity with sorghum α-amylase was used as templates to construct a homology model. Structures were visualized using Invitrogen Vector NTI advance 10.3, 3D molecule viewer.

Analysis of unfolding curves

Pre- and post- transition baselines were linearly extrapolated to general forms, $y_F = ax + b$ and $y_U = cx + d$, where x is the denaturant concentration, and y_F and y_U are the ellipticity at 222 nm (CD data) or intensity at 346 nm (fluorescence data) of the folded (F) and unfolded (U) molecules, respectively.

GuHCl and urea unfolding transition curves were analyzed with the assumption of a two state transition.

Folded (F) ↔ Unfolded (U) (3)

For a two state transition, the conformational transition constant (K) can be measured directly from the average fraction of unfolding (f_U) in the transition region.

$$f_U = (y_F - y) / (y_F - y_U) \quad (4)$$

where y_F and y_U represent the values of y characteristic of the folded and unfolded states, respectively, under the same conditions for measurement of y.

The conformational transition constant (K) was calculated with the equation

$$K = f_U / (1 - f_U) \quad (5)$$

The free energy change (ΔG) of the transition was calculated with the following equation

$$(\Delta G) = -RT \ln K, \quad (6)$$

where R is the gas constant (1.987 calories/deg/mol) and T is the absolute temperature.

Conformational stability of the enzyme in the absence of a denaturant, $\Delta G(H_2O)$, was measured by means linear extrapolation model (LEM). The LEM model assumes the standard denaturation Gibbs energy change to be a linear function of denaturant concentration according to the equation.

$$\Delta G = \Delta G(H_2O) - m\,[\text{denaturant}] \quad (7)$$

The plot of ΔG values versus denaturant concentrations was extrapolated to zero denaturant concentration using experimental points from the transition region to obtain $\Delta G(H_2O)$, m is a measure of the dependence of ΔG on denaturant concentration.

Denaturant concentration at the mid point of the unfolding curve were calculated from

$$[\text{denaturant}]_{1/2} = \Delta G(H_2O) / m \quad (8)$$

Linear least - squares regression analysis was carried out to estimate the unknown parameters associated with the unfolding transition. After obtaining

the $\Delta G(H_2O)$ at each temperature, the values were fitted to the following equation (9) to obtain ΔH_g, ΔC_p and T_g

$$\Delta G(T) = \Delta H_g(1-T/T_g) + \Delta C_p [T-T_g-T \ln (T/T_g)], \quad (9)$$

where T_g is the temperature at which $\Delta G = 0$ and H_g is the unfolding enthalpy at T_g and C_p is the change in excess heat capacity.

Role of calcium

Calcium free buffer

To get a Ca^{2+} free buffer, buffer prepared in double distilled water was left for ~ 48 h, with chelex 100 filled in dialysis tubing. All the glassware were thoroughly rinsed with HCl and dried before using for experimentation.

Removal of calcium

Calcium was removed from α-amylase was performed by dialyzing the enzyme against Ca^{2+} free HEPES (50 mM, pH 7.0) buffer for ~ 48 h with 8-10 changes. The resulting preparation had a specific activity of ~2000 U/mg protein.

Determination of Ca^{2+} content

The quantitative determination of Ca^{2+} content of α–amylase was performed by Shimadzu AA-6701F atomic absorption spectrophotometry. The system was calibrated using metal calibration standard solutions in the range of 0-4 ppm. The buffer was used as control and its value was subtracted from the

sample values. Determination of Ca^{2+} content was done in three runs, each in triplicate.

Trypsin digestion

Proteolytic degradation of amylase (20 µg) was performed in Hepes buffer, pH 7.0 with trypsin (5 µg) at 27 °C for 30 min. Proteolysis was performed both in presence of EDTA, as well as in presence and absence of added Ca^{2+}. The reaction was stopped by the addition of phenylmethylsulfonylfluoride and the reaction products were examined by SDS-PAGE.

Renaturation of EDTA inactivated amylase: Role of metal ions

Renaturation studies of 1 mM EDTA α-amylase was performed at pH 7.0 in presence of different 5 mM divalent metal ions, namely, Ca^{2+}, Ba^{2+}, Mg^{2+}, Zn^{2+} and Sr^{2+}. Solutions were incubated for 2 h at 27°C. Activity measurements were performed as mentioned above.

Important terms

The term "native amylase" or "native protein" or "native enzyme" refers to the amylase in presence of 13.6 mM Ca^{2+}, whereas "amylase" refers to the enzyme in absence of added Ca^{2+}.

RESULTS AND DISCUSSION

RESULTS AND DISCUSSION

Part–I. Isolation, purification and characterization of α-amylase from malted sorghum and comparison with other cereal amylases with respect to the products of starch degradation

This section describes the sorghum α-amylase purification as well as physico-chemical properties and comparison with other cereal amylases with respect to the products of starch degradation.

RESULTS

Thermal stability of cereal amylases

Amylase activities have been extracted from germinated barley, ragi and jowar in 0.05 M acetate buffer (pH 4.8). Residual activities of the enzyme, incubated in the range 40 -75°C for 15 min, is shown in Figure 12. Amylase from malted jowar is found to be the most thermostable (Figure 12) with a T_m of 70 ± 0.5°C compared to barley (T_m 57 ± 0.6°C) and ragi amylase (T_m 67 ± 0.3°C). Hence, the major amylase from germinated jowar has been further purified and characterized.

Entrapment and purification of sorghum α-amylase has been attempted using sodium alginate. Entrapment of α-amylase from bacterial, fungal and cereal sources has been explored. Bacterial and plant α-amylases did not get

entrapped into the alginate under experimental conditions. The binding of enzymes to alginate is reported to be critically dependent on the pH.

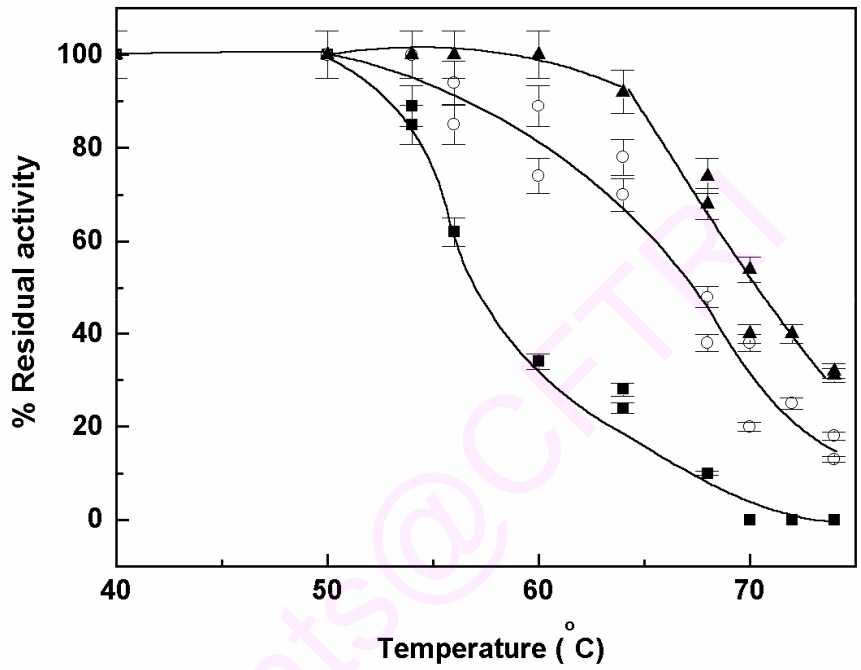


FIGURE 12. Thermal stability of cereal amylases.

Barley (■), Ragi (O) and Jowar (▲). samples in 0.05 M acetate buffer containing 13.6 mM Ca^{2+}, pH 4.8 were incubated at the test temperature for 15 min, cooled rapidly to 4°C and assayed for residual activity at 40°C.

However, it is found possible to entrap the fungal α-amylase from *Asperzillus oryzae*. Purification and thermostability properties of entrapped *Asperzillus oryzae* are given in part II of this chapter.

Sorghum α–amylase could be purified to homogeneity by conventional chromatographic methods (Figure 13).

Extraction and purification of sorghum malt α-amylase

Alpha-amylase is present in trace amounts in ungerminated sorghum but increases greatly during germination (~10 times). α-Amylase, has been extracted from sorghum malt, precipitated by ammonium sulfate and fractionated using DEAE–Cellulose column as given under materials and methods. This step has helped remove large amounts of unbound and contaminating proteins. Bound proteins have been eluted with 0.3 M sodium chloride with a recovery of 19.3 and a fold purification of 18.1 (Table 5). The active fractions have been pooled, concentrated and loaded on a G-75 superfine column to recover the pure protein with a purification factor of 24.7 and a specific activity of 2,741 U/ mg. The overall yield of amylase activity from germinated jowar is 17.1 %. The homogeneity of the preparation has been checked by SDS – PAGE (Figure 14A), and activity staining (Figure 14B). The enzyme appears to move as a single band indicating the absence of hetero-subunits. The molecular weight of the protein is determined to be 47 kDa by SDS-PAGE.

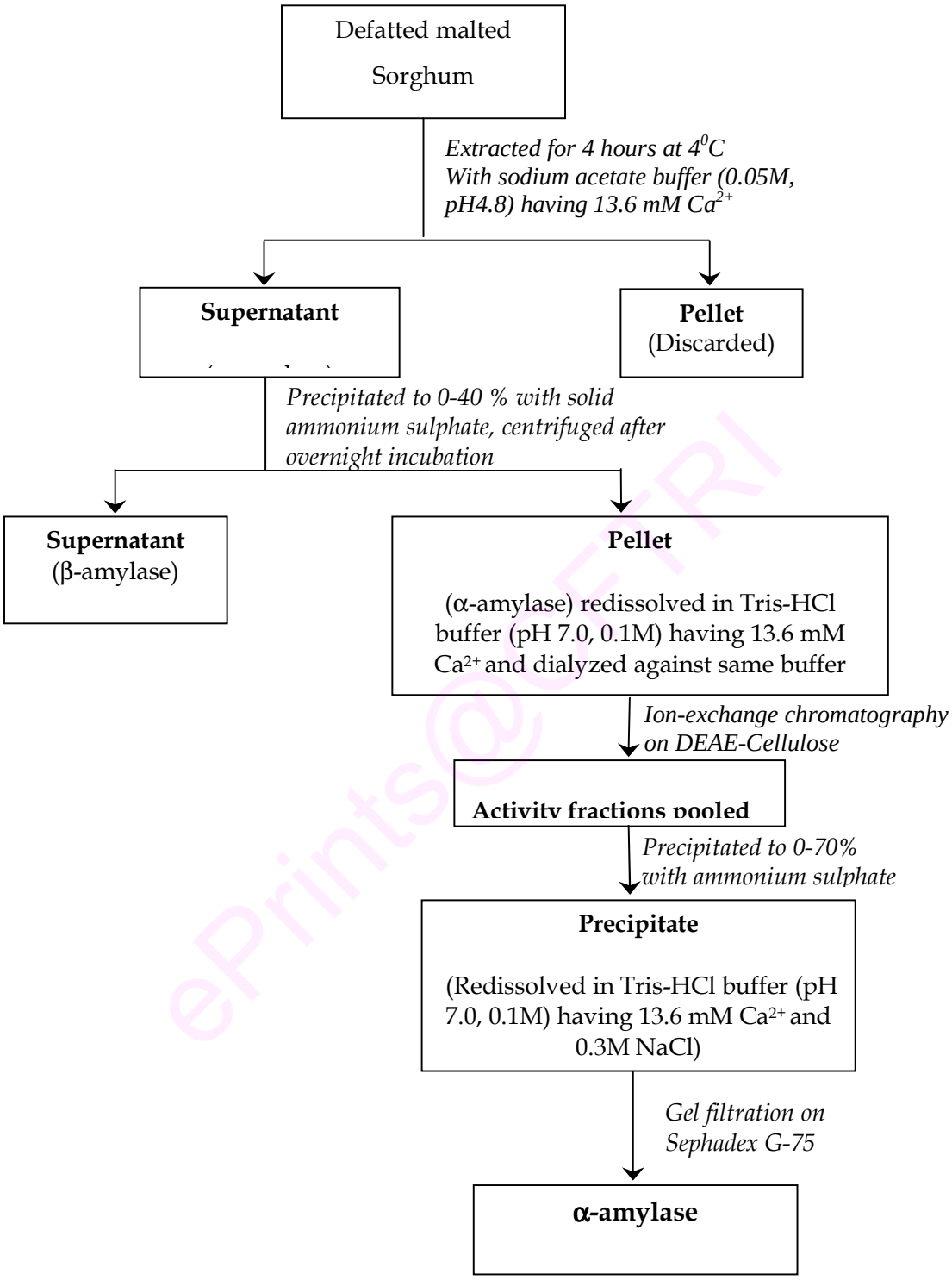


Figure 13. Flow diagram for the purification of sorghum α-amylase

Table - 5. Purification and recovery of α- amylase from malted sorghum

	Total activity (U)	Total protein (mg)	**Specific activity (U/mg protein)**	Purification factor	Overall yield (%)
Crude	139320	1257	111	1	100
0-40% Precipitation	36438	61.3	594	5.3	26.1
DEAE-Cellulose Chromatography	27000	13.4	2014	18.1	19.3
Sephadex G-75	23850	8.7	2741	24.7	17.1

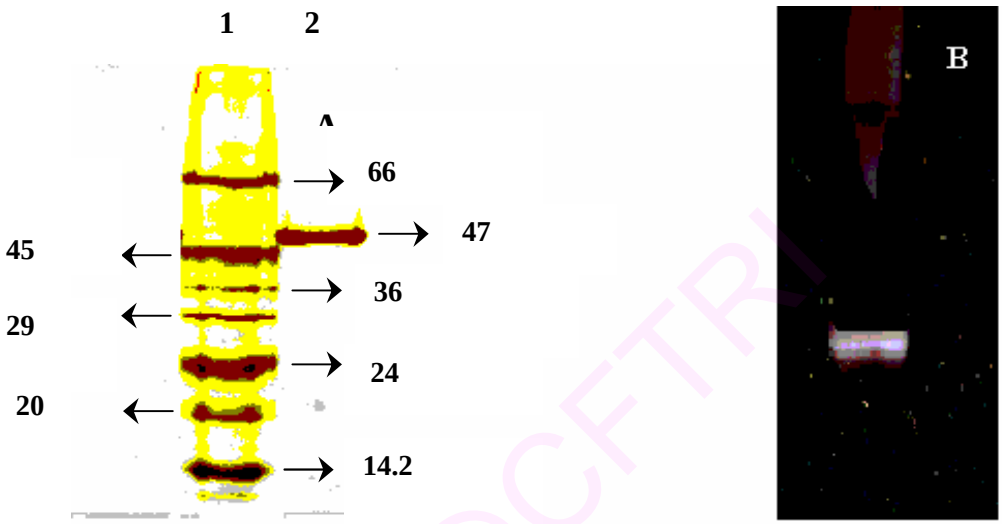


Figure 14. Homogeneity of purified sorghum α-amylase

A = SDS – PAGE of sorghum α-amylase: lane 1: molecular weight markers (Sigma), 14 – 66 kDa. lane 2: jowar α- amylase.

B = PAGE of purified α-amylase from malted jowar: activity staining

It is difficult to quantitate the content of α-amylase in sorghum because of the presence of different isomers with different specific activities. However, an attempt has been made by measuring the relative proportions of different proteins extracted in the medium and correlating with activity stain of α-amylase. It was estimated to be 1.3% of the total proteins in sorghum.

Enzyme characteristics

Determination of $K_{m \text{ and }} V_{max}$

The apparent Michaelis constant (K_m) and V_{max} for sorghum α-amylase were determined for gelatinized starch to be 1.55% and 6535 μmol/min/mg protein, respectively.

pH and temperature optimum of sorghum amylase

The enzyme was active over a wide pH range with a pH optimum lying between 4.5 – 5.0, in similarity with other cereal amylases. Sorghum α-amylase was found to have temperature optimum of 60 ± 0.5°C, which is higher than that reported for ragi (Nirmala and Muralikrishna, 2003), barley (MacGregor, 1978), wheat (Tkachuk and Kruger, 1974), pearl millet (Adelaide Beleia and Varriano-Marston, 1981) and immature barley (Greenwood and MacGregor, 1965).

Product identification by HPLC

Products of enzymatic hydrolysis of maltodextrin were identified using oligomers of maltose as standards. Glucose and oligosaccharides, varying in their DP ranging from 2 to 7, released during the course of the reaction were trapped

at different intervals of time by stopping the reaction with ethanol. Higher oligosaccharides (DP 5, 6 and 7) were the major products released in the early stages of hydrolysis (2 – 6 h) (Figure 15). An increase in the concentrations of low molecular weight oligosaccharides (1 – 4) with a corresponding decrease in the higher oligosaccharides (>4), was observed by 24 h. The final products of the reaction were maltose, glucose and maltotriose.

Spectral properties

The enzyme in acetate buffer (0.05 M, pH 4.8), with 13.6 mM Ca^{2+}, exhibited intrinsic fluorescence when excited at 280 nm. The enzyme exhibited emission maximum at 346 nm, indicating that the tryptophan residues were in a fairly exposed environment (Figure 16).

The near UV CD spectra of the enzyme revealed minima at 284 and 272 nm and maxima at 250 nm with shoulders at 263 (negative band) and 255 nm (positive band) (Figure 17A). The far UV CD showed a minima at 213 nm, indicating predominantly β structure (Figure 17B). The analysis of CD data shows a helix content of 19%, beta structure of 48%, beta turn of 12% and aperiodic of 21%.

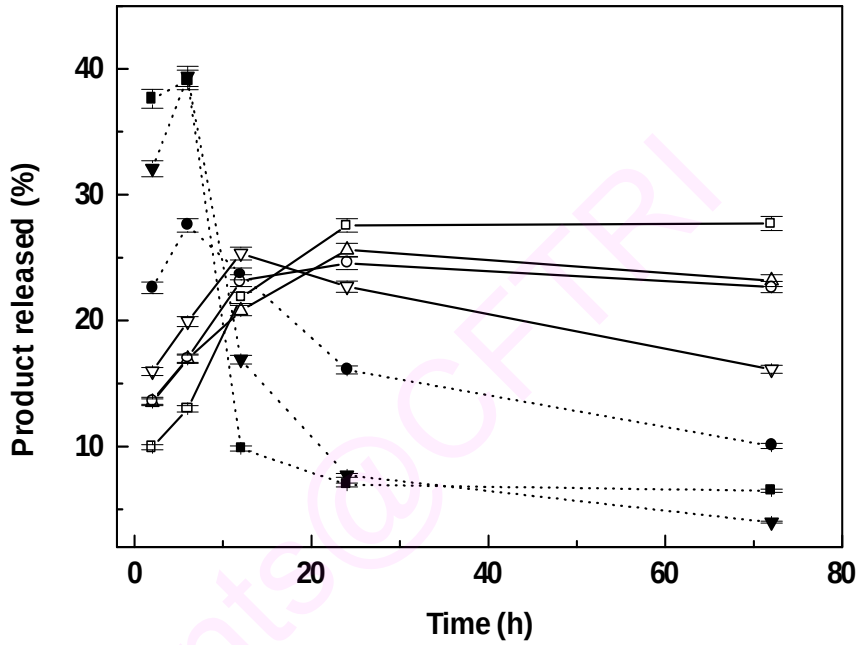


Figure 15. Product distribution curves in the hydrolysis of maltodextrin catalyzed by α-amylase.

Hydrolysis was carried out at pH 4.8 and 60°C for different time intervals, products were concentrated and analyzed by HPLC on a Waters® μBondapak™ NH_2 column (3.9 x 300 mm, 10 μm) using acetonitrile: water solvent system (70 : 30) at a flow rate of 1 ml/ min as given under materials and methods. The amounts of products (DP1 – DP7) released were quantified by peak integration, using standards for refractive index detection. —△—, glucose; —□—, maltose; —○—, maltotriose; —▽—, maltotetraose; ----●----, maltopentaose; ----▼----, maltohexaose; ----■----, maltoheptaose.

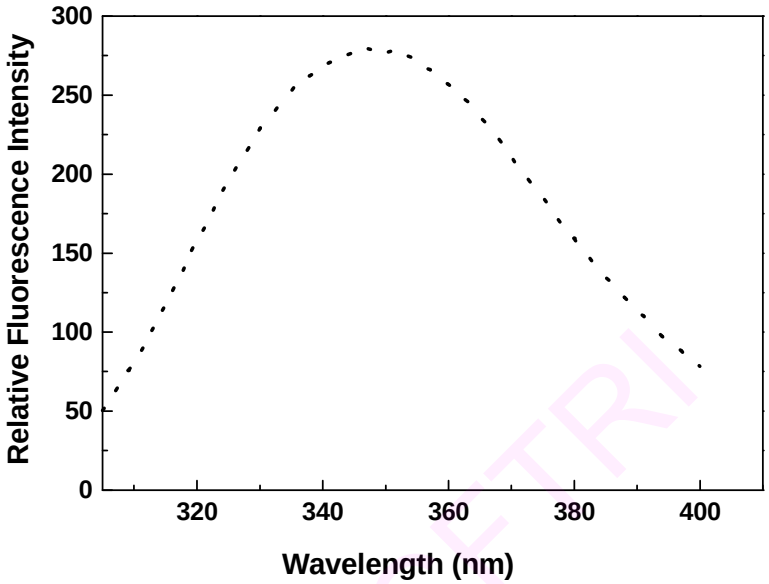


Figure 16. Fluorescence emission spectra of sorghum α-amylase

Protein concentration of ~ 0.6 μM (< 0.1 O.D at 280 nm) was used in 0.05 M acetate buffer, pH 4.8, containing 13.6 mM calcium. The excitation wavelength was set to 280 nm. Excitation and emission slit widths were set at 5 and 5 nm, respectively, using a 10 mm path length cuvette.

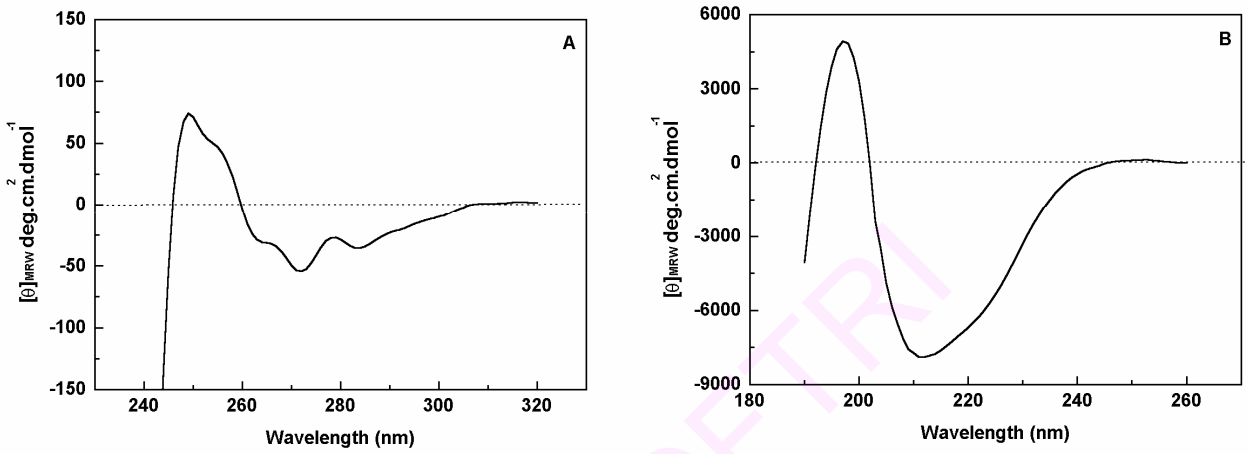


Figure 17. CD spectra of jowar α-amylase.

Protein concentrations used for near UV CD were 0.83 mg/ml and 0.094 mg/ml for far UV CD. The spectra were run in the range 320 – 240 nm (for near UV CD) and 260 – 190 nm (for far UV CD) at a scan speed of 10 nm/ min in a cell with pathlength of 1 cm (near UV CD) or 1 mm (far UV). An average of 3 runs were taken. Mean residue weight of 110 was considered for calculating mean residue ellipticity.

A = Near-UV CD spectra of sorghum α-amylase.

B = Far –UV CD spectra of sorghum α-amylase.

Amino acid composition and N-terminal sequencing data

The amino acid sequence of sorghum α-amylase (Name - sb04g034150; Protein ID: 5039071) has been recently produced by the US Department of Energy Joint Genome Institute (http://www.jgi.doe.gov/). The amino acid composition of the published sequence is very similar to the present study (Table 6).

N-terminal sequence (first twelve residues) of sorghum α-amylase is determined to be Gln-Val-Leu-Phe-Gln-Gly-Phe-Asn-Trp-Glu-Ser-Trp-Xaa-Gln-Asn. This is identical to the reported sequence of sorghum and other cereal α-amylases (in absence of their signal peptide) (Figure 18).

Midpoint of thermal inactivation, T_m

The thermal stability of α-amylase was tested by incubating enzyme for 15 minutes at different temperatures (30-85°C) and measuring the residual activities. The enzyme was stable up to 60°C with a T_m of 69.6 ± 0.3°C.

Effect of salts and sugars on thermal stability of α-amylase from sorghum

Addition of NaCl decreased the thermal stability of α-amylase. The T_m of α-amylase decreased to 66.3 ± 0.3, 58.1 ± 0.2 and 48.1 ± 0.5°C in presence of 0.1 M, 0.5 M and 1 M NaCl, respectively (Figure 19A). Sucrose was a stabilizer of α-amylase. The T_m in presence of 1 M sucrose shifted from 69.6 ± 0.3 to 77.3 ± 0.3°C (Figure 19B).

Table 6. Amino acid composition of sorghum α-amylase (g of amino acid per 100 g of protein)

Amino acid	Sorghum α-amylase	Sorghum α-amylase*
Asp/Asn	14.4	12.7
Glu/Gln	11.3	8.0
Ser	4.3	2.9
Gly	8.7	5.1
His	4.1	4.1
Arg	5.9	5.3
Thr	4.2	3.0
Ala	8.2	5.3
Pro	3.9	3.9
Tyr	3.0	4.5
Val	5.4	5.1
Met	0.4	1.1
Cys	0.6	0.7
Ile	6.5	6.7
Leu	8.0	7.2
Phe	5.6	5.9
Lys	5.6	7.6

* calculated from sorghum α-amylase sequence (Name - sb04g034150; Protein ID: 5039071)

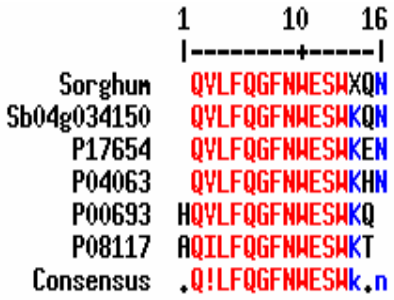


Figure 18. N-terminal sequence similarity among cereal α-amylases.

(A)N-terminal sequence of sorghum α-amylase aligned with cereal amylases using 'MultAlin' option from proteomics and sequence analysis tools, obtained from http://expasy.org/tools/. Sequences of cereal α-amylases including *Hordeum jubatum* (barley isoenzyme 1 - P00693, isoenzyme 2 - P04063), *Oryza sativa* (rice - P17654) and *Triticum aestivum* (wheat - P08117), were taken from http://www.expasy.org/enzyme/3.2.1.1. *Sorghum bicolor* α-amylase (Name - sb04g034150; Protein ID: 5039071) sequence was obtained from the US Department of Energy Joint Genome Institute 'http://www.jgi.doe.gov/'. Consensus sequence indicating (!) is anyone of I or V and (+) is anyone of F or *Y*.

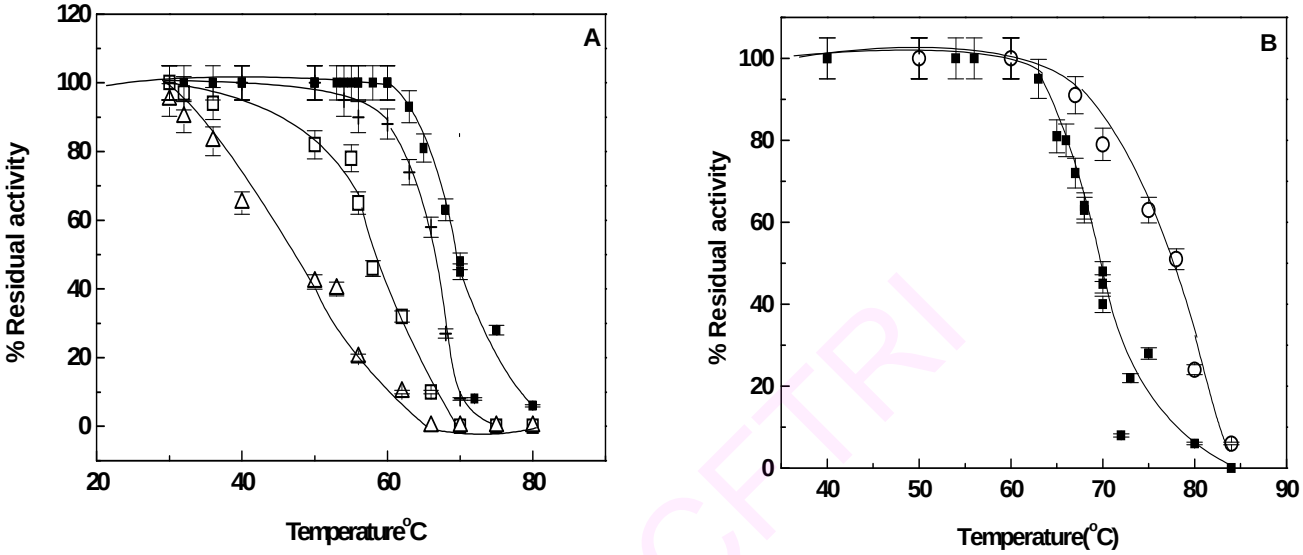


Figure 19. Thermal stability of α-amylase in the presence of additives.

A = Effect of NaCl on thermal stability of α-amylase. Enzyme concentration (4.9x 10^{-3} mg/ml) with no salt (■), 0.1 M NaCl (+), 0.5M NaCl (□), 1 M NaCl (Δ).

B = Effect of sucrose on thermal stability of α-amylase. Enzyme concentration (4.9x 10^{-3} mg/ml) with no salt (■), and 1M sucrose (O) were incubated for 15 minutes at the test temperature. Samples were cooled to 4°C and residual activity was assayed as described under enzyme activity assay.

DISCUSSION

It is well known that germination of cereals causes a marked increase in their amylase activities (Andrew and Bailey, 1934). The chief function of starch splitting enzymes in the resting grain is one of saccharification, but an additional property is imparted by germination which is referred to as liquefaction. This results in the conversion of thick starch pastes into thin watery liquids making it easier for consumption. Liquefaction also results in the food absorbing less water rendering it more 'nutrient dense'.

One of the objectives of this work is to find a suitable and thermostable source of α- amylase for addition to supplementary foods in an effort to enhance their nutrient density. Thermal stability of α- amylase is a desirable feature as the supplementary food is reconstituted with hot water/milk before consumption. Some of the α- amylases are derived from cereals such as barley (MacGregor, 1978), rye (Baker and Hulton, 1921), wheat (Tkachuk and Kruger, 1974) and ragi (Nirmala and Muralikrishna, 2003).

In the present study, cereal amylase activities from barley, ragi and jowar with respect to thermal stability have been studied. Jowar amylase has better thermal stability compared to other cereal sources, with a T_m of 70 ± 0.5°C. Malted sorghum (jowar) is also shown to have ~10 times greater amylase activity compared to ungerminated grain. Thus, isolation and characterization of the pure amylase is important to make future work to understand the basis for thermal stability feasible.

α-Amylase has been purified from malted sorghum to homogeneity with a molecular weight of 47 ± 0.2 kDa. Molecular weights of most cereal α-amylases are in the range 42 – 46 kDa. The α-amylases from wheat (Tkachuk and Kruger, 1974) and ragi (Nirmala and Muralikrishna, 2003) have a molecular weight of 42 kDa and 47 kDa, respectively. However, there are exceptions wherein the molecular weight ranges from 20 to 57 kDa in the case of amylases isolated from wheat (Tkachuk and Kruger, 1974) and barley (MacGregor, 1978).

In the present study, k_m and V_{max} values for gelatinized starch were found to be 1.55 % and 6535 μmol/min/mg, respectively. K_m values for ragi α_3 - amylase were found to be in the range of 0.53% - 1.0% for cereal starches and the V_{max} values were found to be in the range of 2381 – 2778 U/mg/min (Nirmala and Muralikrishna, 2003). α-Amylase was found to have predominant β-structure (~60 %) and α-helical content of 19%. The CD spectra of Ca^{2+} depleted and Ca^{2+} saturated molecules of barley α-amylase was reported (Bush et al., 1989). The α-helical content of both the forms was estimated to be 27%. Ca^{2+} depletion decreased the mean residue ellipticity between 200 and 208 nm, indicating an increase in the random structure, emphasizing the role for Ca^{2+} in stabilizing the structure of the enzyme.

α-Amylase from sorghum was active over a wide pH range with a pH optimum of 4.5 – 5.0, akin to other cereal amylases. Optimum pH values for pearl millet (Adelaide Beleia and Varriano-Marston, 1981) and malted barley (MacGregor, 1978) were reported to be 4.4 – 4.8 and 5.5, respectively. In the

present study, α-amylase was found to have temperature optimum of 60 ± 0.5°C, which is higher than that reported for ragi (Nirmala and Muralikrishna, 2003), barley (MacGregor, 1978), wheat (Tkachuk and Kruger, 1974), Pearl millet (Adelaide Beleia and Varriano-Marston, 1981) and immature barley (Greenwood and MacGregor, 1965). A summary of enzyme characteristics from different plant sources is given in Table 7.

Aminoacid composition of α-amylases from sorghum, barley (Rodaway., 1978) and wheat (Tkachuk and Kruger, 1974) bear close similarity to one another. Sorghum α-amylase is found to have high content of Asp and Glu residues compared to *ragi* α-amylase (Nirmala and Muralikrishna, 2003). Sulphur containing amino acids (Cys and Met) are less compared to other amino acids. Such quantitative similarities exist in other α-amylases also (Fischer and Stein, 1960).

NaCl and sucrose are added to supplementary foods to modify and improve the acceptability of the food. Sucrose is found to have a stabilizing effect on the enzyme, while NaCl is found to destabilize the enzyme activity without significant change in the enzyme structure.

Malted jowar could become a commercially viable source of α-amylase for viscosity reduction and to increase the nutrient density of supplementary foods. The thermal stability of the enzyme could be an added advantage.

Table 7. Summary of α-amylase characteristics from different cereal sources

Source		Specific activity (U/mg protein)	pH Optima	Temperature Optima (˚C)	Mol.Wt.(kDa)SDS-PAGE	K_m	Vmax (µmol maltose /mg/min)
						(%)	
Malted Sorghum		2741	4.5 – 5.0	60 ± 0.5	47 ± 0.2	1.55	6535
Malted Ragi[a]	α_1	1500	5.0	45	47	0.59	2381
	α_2	974	5.5	50		1.1	1111
	α_3	1773	5.0	45		0.53	2778
Immature barley[b]		-[g]	5.5	45-50			
Malted barley[c]	α_1	-	5.5	55	52		
	α_2						
Malted wheat[d]	α_1	1480	5.5	55	41.5 – 42.5		
	α_2	1300	5.7				
	α_3	1510	5.5				
						(g/ml)	
Immature wheat[e]	α_1		3.6-5.75		52 - 54	$2.5x10^{-4}$	
	α_2		3.6-5.75			$5.3x10^{-4}$	
	α_3		3.6-5.75			$2.3x10^{-4}$	
Pearl millet[f]			4.4 – 4.8	55	22 – 53		

[a] Nirmala and Muralikrishna, 2003

[b] Greenwood and MacGregor., 1965

[c] MacGregor., 1978

[d] Tkachuk and Kruger., 1974

[e] Marchylo, B.; Kruger, J. E.;

[f] Adelaide Beleia and Varriano-Marston., 1981

[g] -, not given.

Part–II. Entrapment of α-amylase in alginate beads: Single step protocol for purification and thermal stabilization

Bacterial amylases, used in industrial applications, are highly thermostable with optimum temperatures greater than 90°C (Richardson et al., 2002; Pandey et al., 2000). Such high temperature optima could lead to deleterious changes in foods and development of burnt flavors. Further, high temperature optima (near 100°C) mean that the enzymes cannot be used at lower temperatures (60°C) with good efficiency. Fungal enzymes (being mesophilic in nature) have the advantage of high catalytic rates at moderate temperatures of 50–60°C, without affecting the sensory appeal.

Microbial enzymes, including commercial enzymes, are a mixture of enzymes. The presence of a contaminating protease, for example, considerably reduces the efficiency as well as reusability of the enzyme. Even partial purification can improve the specific activity of the enzyme and eliminate unwanted side reactions or products. Purification of the enzyme will aid the characterization with respect to its kinetics and inhibitor sensitivity, besides reducing or eliminating undesirable by-products, thereby enhancing the yields of desired products.

A single step purification protocol for α-amylase from *Asperzillus oryzae* has been developed.

RESULTS

Entrapment of α-amylase in alginate beads

Entrapment of α-amylase in alginate beads from three different sources—bacterial, fungal and plant—was attempted. While bacterial and plant amylases did not bind to alginate, the fungal amylase could bind. This proved to be a simple method for both purification and entrapment of α-amylase from *A. oryzae*.

Purification of α-amylase

The commercial fungal enzyme from *A. oryzae* has an activity of approximately 80 U/ml (specific activity of 205 U/mg). In the present investigation, it has been possible to purify α-amylase to homogeneity by affinity precipitation using alginate. The supernatant, discarded after affinity precipitation, has 4.9% of total activity. The enzyme has been eluted from the beads using 0.5 M sodium chloride containing 0.2 M Ca^{2+}. Enzyme recovery is found to be 76% with ~9-fold purification and a specific activity of 1764 U/mg (Table 8). Homogeneity of the sample, followed by SDS-PAGE, reveals a single band corresponding to 47 kDa (Figure 20).

Table 8. Activity yield of the enzyme during affinity precipitation

Stage	Total enzyme activity (units)	Yield (%)	Specific activity (units/ mg of protein)	Purification factor
Amylase starting with Fungamyl® enzyme	477	100	205	1
Enzyme released during washing with 0.02 M Ca^{2+} (pH 5) after three washes	23.5	4.9	164	-
Enzyme recovered after 4 elutions with 0.5 M NaCl containing 0.2 M Ca^{2+}, pH 5.0	363.8	75.8	1764	8.6

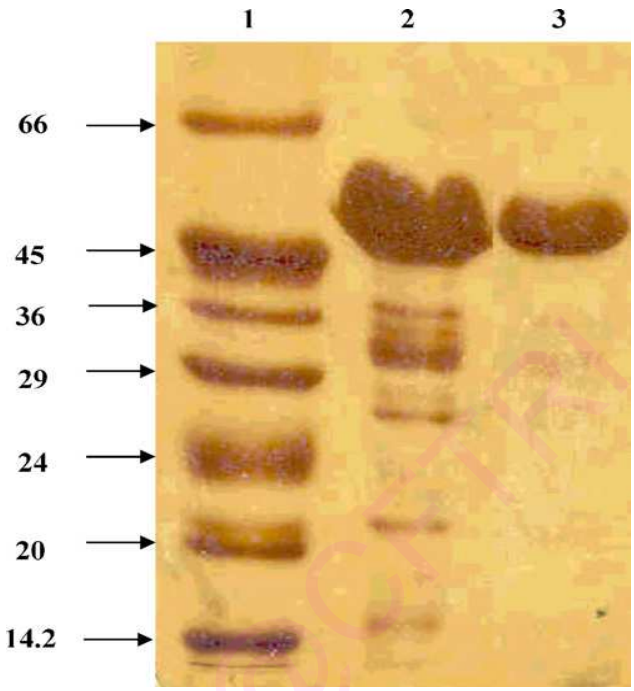


Figure 20. SDS-PAGE of α-amylase from *Aspergillus oryzae*.

Lanes: (1) molecular weight markers (66–14.2 kDa); (2) commercial amylase preparation; (3) α-amylase recovered from the alginate beads.

Enzyme characteristics

K_m and V_{max} of free and entrapped enzyme

Michaelis constant (K_m) for both free and entrapped enzymes remained the same (0.83%), indicating that, alginate entrapment did not have any effect on the binding of substrate to the enzyme (Figure 21). However, V_{max} of the entrapped enzyme was affected, with >10-fold decrease from 1764 U mg^{-1} (for free enzyme) to 140 U mg^{-1}. Probably, decreased flexibility of the entrapped enzyme has affected its turnover.

pH and temperature optima for amylase activity

The free enzyme has pH optimum of in the range of 5.5–6.0, while that of the entrapped enzyme shifted towards alkaline by 0.5 units to 6.0–6.5 (Figure 22).

The entrapped enzyme was active at a higher temperature range (compared to free enzyme) and the optimum temperature for activity got shifted by 6 ± 1 °C. The temperature optimum was found to shift from 54 for free enzyme to 60 °C for the entrapped enzyme (Figure 23).

Thermal stability of entrapped amylase

Midpoint of thermal inactivation (T_m)

T_m, defined as midpoint of thermal inactivation, shifted from 57 °C for free enzyme to 63 °C for the entrapped amylase, reflecting increased temperature stability (+6 °C) on entrapment (Figure 24).

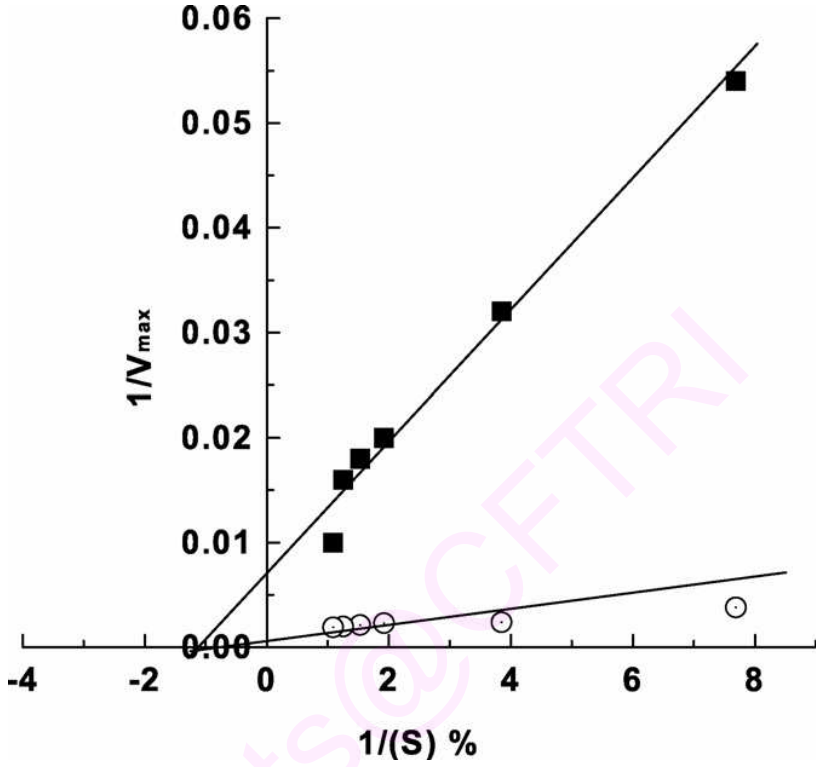


Figure 21. Effect of amylase entrapment on substrate concentration and reaction velocity. (○) Free enzyme; (■) entrapped enzyme. Gelatinized starch in water (0.2–2%) was used as substrate. The activity was assayed as given for *A.Oryzae* under materials and methods.

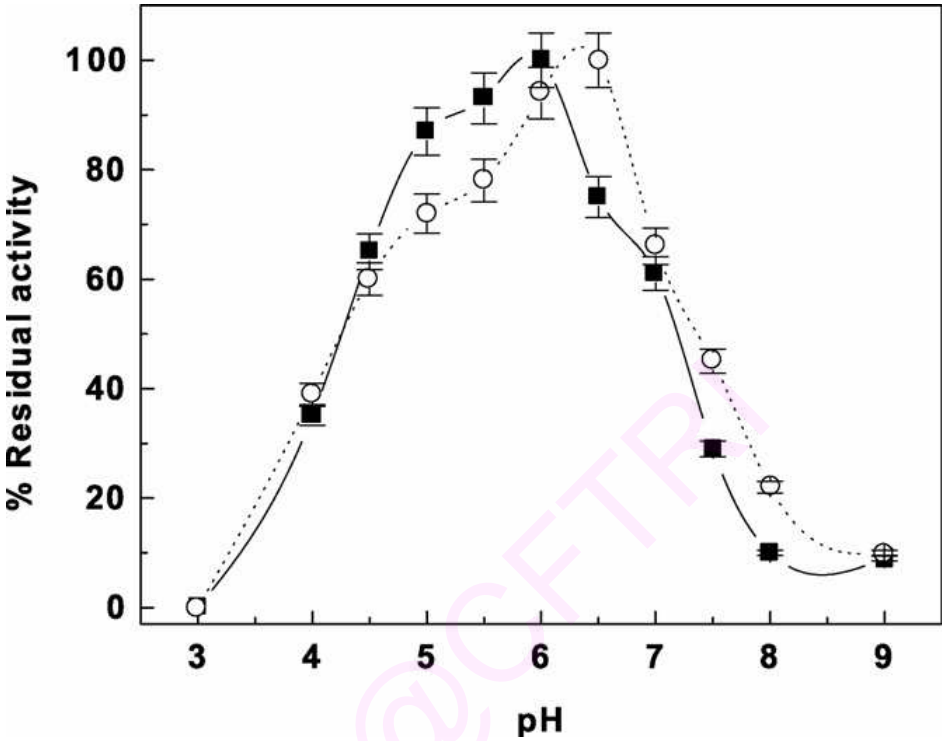


Figure 22. Change in pH optimum of α-amylase after entrapment.

(■) Free enzyme; (○) entrapped enzyme. The substrate was prepared in 0.1 M buffer at different pH (3–9) and activity determined. The various buffers used were acetate (pH 3.0–5.5), phosphate (pH 6.0–8.0) and borate buffer (pH 9.0). The highest activity obtained (during the experiment) was taken as 100% for calculating the relative activities.

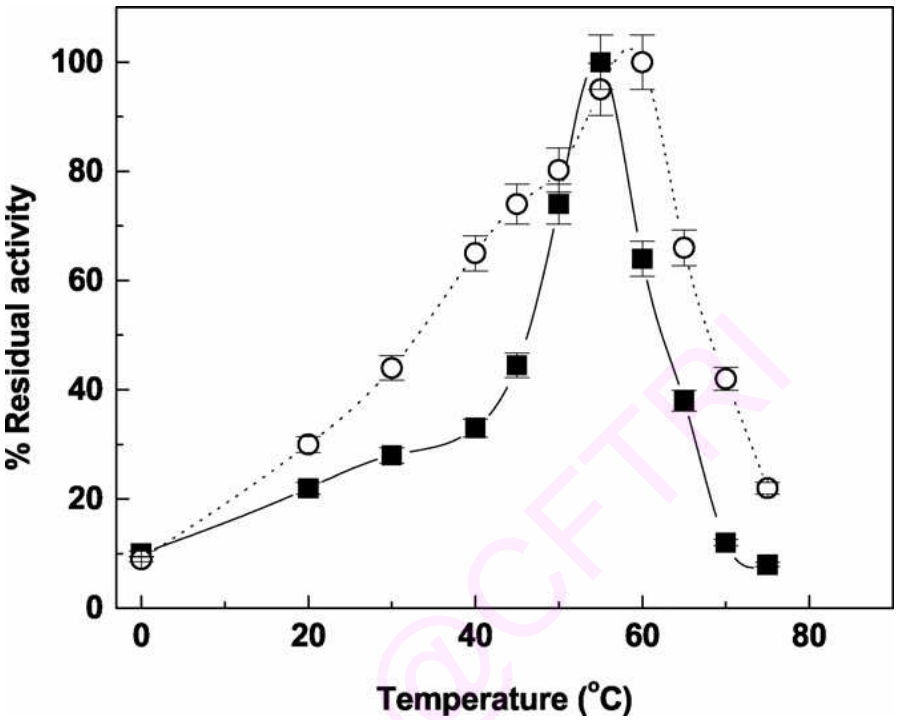


Figure 23. Change in temperature optimum for activity of free and entrapped amylase. Temperature optimum was checked by assaying activity, at the pH optimum, in the range of 10–75 °C. (■) Free enzyme; (○) entrapped enzyme. Activity was assayed at the pH optimum. The maximum activity obtained was taken as 100% and relative activities at different temperatures calculated.

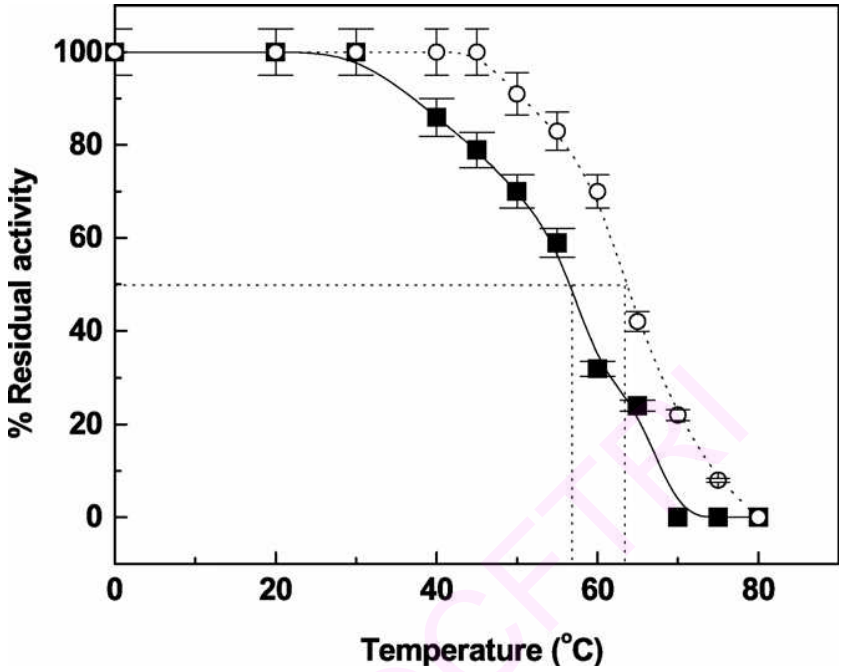


Figure 24. Midpoint of thermal inactivation (Tm) for free and entrapped α-amylase. (■) Free enzyme; (○) entrapped enzyme. Free or entrapped enzyme was incubated at different temperatures for 15 min, cooled immediately in an icebath and residual activity assayed. The activity of unincubated enzyme was taken as 100% for calculating the residual activity at different temperatures.

Thermal inactivation parameters

Thermal inactivation parameters for the free and entrapped enzyme were followed by incubation at different test temperatures in the range 52–65°C and assaying for residual activity as a function of time (Figure 25A and 25B). Enzyme inactivation followed first-order kinetics. The Arrhenius plot derived from the slopes of the plot of log% residual activity versus time is shown (Figure 25C). E_a values of the entrapped enzyme were higher (51.7 kcal mol^{-1}) compared to free enzyme (40.9 kcal mol^{-1}). Activational enthalpy (ΔH^*), entropy (ΔS^*) and free energy change (ΔG^*) were calculated to be 40.2 ± 0.1 kcal mol^{-1}, 48.9 ± 0.5 cal mol^{-1} K^{-1}, 24.1 ± 0.3 kcal mol^{-1} for free enzyme and 51 ± 0.1 kcal mol^{-1}, 80.8 ± 0.6 cal mol^{-1} K^{-1} and 24.7 ± 0.4 kcal mol^{-1}, respectively, for the entrapped enzyme (Table 9). Half-life of the enzyme increased from 8.5 to 32.7 min (~4 times higher) after entrapment at 60 °C indicating stabilization of the enzyme.

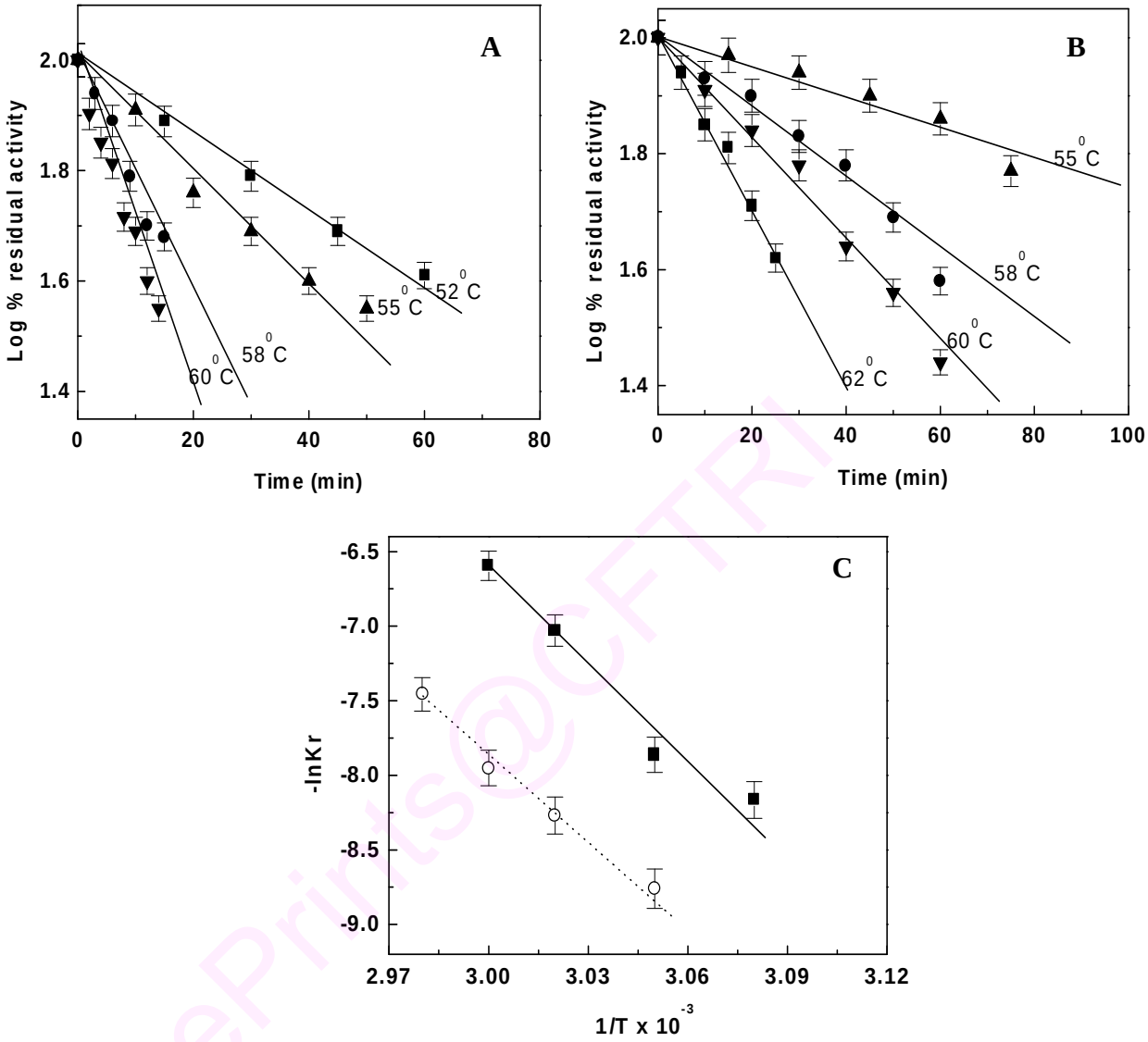


Figure 25. Thermal inactivation kinetics of free and entrapped α-amylase.

(A) Free enzyme; (B) entrapped enzyme; (C) Arrhenius plots for free (■) and entrapped enzymes (○). For determination of thermal inactivation parameters, free enzyme or entrapped amylase in alginate beads were incubated at the test temperatures. Aliquots were drawn at different time intervals and the activity assayed. The unincubated enzyme activity was taken as 100% for calculating the residual activities.

Table 9. Thermodynamic parameters of native and immobilized α- amylase

Temperature (K)	kr ($X10^{-1}$ s^{-1})	Half-life (min)	Ea (kcal mol^{-1})	ΔH* (kcal mol^{-1})	ΔG* (kcal mol^{-1})	ΔS* (cal mol^{-1} K^{-1})
Free enzyme						
325	2.84	40.7	40.9	40.3	24.3	49.1
328	3.56	32.3	40.9	40.2	24.3	48.4
331	8.84	13.1	40.9	40.2	23.9	49.1
333	13.7	8.5	40.9	40.2	23.8	49.2
Entrapped						
328	1.13	102.4	51.7	51.1	25.1	79.2
331	2.55	45.3	51.7	51.1	24.8	81.3
333	3.53	32.7	51.7	51.0	24.7	81.1
335	6.83	20.9	51.7	51.0	24.4	81.3
338	11.74	9.8	51.7	51.0	24.3	81.1

DISCUSSION

Most biotechnological products are proteins and need to be prepared in large volumes with high a degree of purity. Commercial production of proteins is possible by advances in genetic engineering. However, separation and purification techniques for these products have been slow in development. Conventional purification methods are tedious, time consuming and expensive. Affinity precipitation is an alternative to conventional protein purification techniques. It has several advantages over the other bio-separation strategies for enzymes, being easily scalable and economically viable. The recovery of active protein is high and often the method results in a purification factor of 5–10 (sometimes greater). Further, the process uses inexpensive and easily obtainable materials thus making it suitable for scale-up or commercialization.

Enzymatic conversion of starch includes its gelatinization by dissolution in hot water. Use of thermostable bacterial amylases for hydrolysis of gelatinized starch has helped avoid cooling time. Fungal amylase loses 50% residual activity on heating for 15 min at 54°C, and is therefore, is not as stable as bacterial amylases. Stabilization of fungal amylase would enable its utility at higher reaction temperatures. The fact that the enzyme can also be purified by a single step of eluting with sodium chloride and calcium chloride helps in the large-scale purification of the enzyme. Purification of the enzyme helps in characterization of the enzyme with respect to its kinetics, inhibitor sensitivity as well as in

reduction or elimination of undesirable by-products, resulting in higher yields of the desired end products.

In the current study, attempts were made to entrap of α-amylase from different sources. The bacterial and plant α-amylases did not get entrapped into alginate under the conditions used. However, it was possible to entrap the fungal α-amylase from *A. oryzae*. The binding of enzymes to alginate is reported to be critically dependent on the pH (Teotia, Lata et al., 2001). The entrapped enzyme, being active, could be used without eluting the enzyme out. Further, the entrapped enzyme had a higher temperature optimum and stability. Higher temperature optima in enzymes found attraction because the rates of reactions are faster, in addition to low microbial contamination of food materials. Kennedy (Kennedy, 1978) suggested that an increase in temperature optimum could result from a lower temperature in the gel microenvironment compared to the bulk solution.

Immobilization of α-amylase from *B. licheniformis,* on glass beads, has been reported (De Cordt et al., 1992). α-Amylase, bound to silica, polystyrene or polyacrylamide, has shown biphasic kinetics compared to the soluble enzyme (Ulhrich and Schellenberger, 1986). However, in the current study, the entrapped enzyme has exhibited monophasic kinetics, similar to the soluble enzyme. In the presence of 1 M maltose, the enzyme is found to dissociate from the alginate beads and retain only ~57% activity at the end of one cycle (Figure 26).

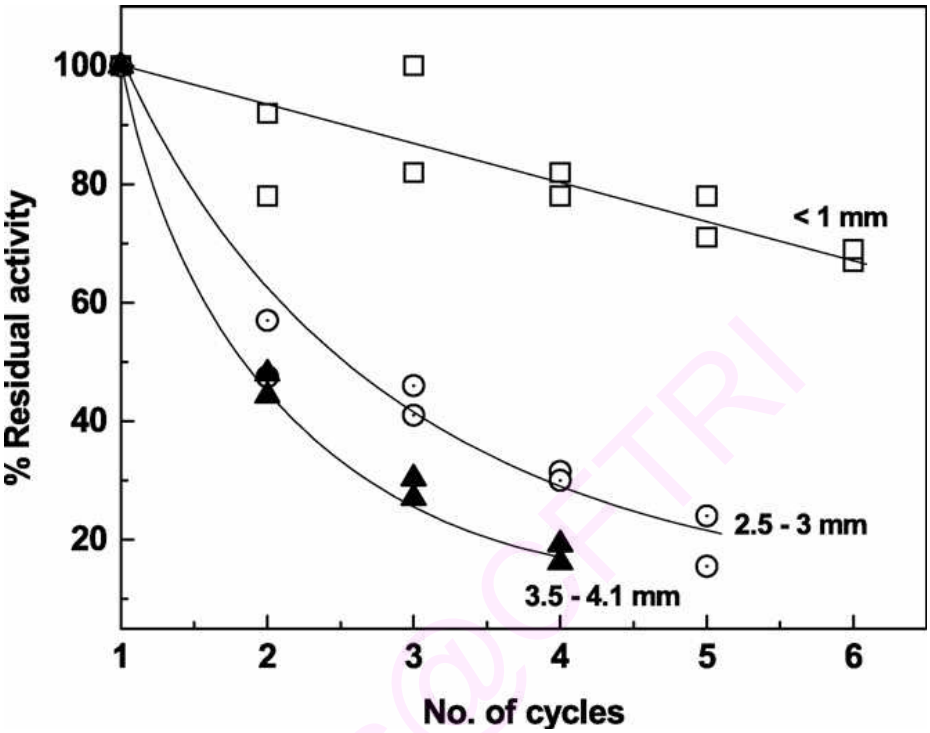


Figure 26. Effect of bead size on the reusability of the entrapped amylase in alginate beads. Entrapped amylase in alginate beads were used for assaying activity. The beads (<1–4.1 mm) were washed thrice in double distilled water and reused for assay with 2% gelatinized starch as substrate.

Reusability and efficiency of entrapped amylase apparently depends on the amount of maltose released.

Increased thermal stability for immobilized α-amylase from *A. oryzae* was reported (Raviyan et al., 2003). The Ea value of the enzyme entrapped in polyacrylamide gel increased to values between 200 and 275 kJ mol^{-1} from 156 kJ mol^{-1} for the free enzyme. In the current study, half-life of the enzyme increased by ~4 times from 8.5 to 32.7 min, at 60 °C. However, the extent of stabilization was still lower than reaction temperatures required for bacterial amylases (95 °C). High temperature might become a limiting factor for utilization of the entrapped enzyme.

Entrapped enzyme in beads of diameter <1 mm has greater stability, retaining ~70% activity even after six cycles. Higher stability ensures that the enzyme is active for many more cycles compared to enzymes that are less stable. Immobilized enzymes are generally more stable compared to free enzymes, due to the curtailment of their degrees of freedom (of rotation). Entrapped enzymes are unable to rotate freely. Prevention of the unfolding of its structure may be preserving its function. Entrapping the fungal enzyme has resulted in an increase of 6–7 °C in the T_m with the added advantage of recyclability.

Our intention was to have a stable enzyme system that can be used and recycled for industrial processes either singly or in combination with other enzymes. Using enzymes at higher temperatures would help enhance the reaction rates in addition to controlling microbial contamination, especially when

the substrates are food products. We could demonstrate that it is possible to purify the *A.oryzae* α-amylase in a single step without using conventional chromatographic methods that are both laborious as well as expensive, with low yields.

Conformational stability measurements of α-amylase – effect of pH, salts, ionic strength, denaturants and temperature

The structure-function and structure-stability relationships as well as the folding and refolding behavior of sorghum α-amylase were studied, employing different spectroscopic and activity measurements.

RESULTS

Unfolding / refolding of sorghum α-amylase

The enzyme could be unfolded completely by incubating either for 4 h with 4 M GuHCl for 12 h or with 8 M urea in buffer A (pH 4.8). Buffer B (pH 7.0) required incubation with 5 M GuHCl for 4 h to completely unfold the enzyme. The enzyme refolded on diluting 25 times with buffer B containing 15 mM DTT at 27°C, resulting in the recovery of ~85 % of the activity. The recovery of activity as a function of time, was compared with the native enzyme (Figure 27A). Maximum activity was recovered, after 2 h of incubation, without further regain even after prolonged incubation (> 6 hours). Refolding of the unfolded α-amylase was also studied using CD and fluorescence spectral changes. It was observed that the refolded enzyme, with buffer B having 15 mM DTT, regained secondary (Figure 27B) and tertiary structure (Figure 27C), similar to the native enzyme. However, dilution of the unfolded enzyme with buffer A, containing 15 mM DTT, did not yield active enzyme but led to protein aggregation as evidenced by the increase in optical density at 400 nm (Figure 27D). Aggregation

was not observed when renatured with buffer B, in presence of DTT. Inactivation of enzyme, in absence of DTT, could be due to the oxidation of free thiols to form sulfite (see thiol reactivity). Attempts to refold the enzyme using protein folding agents, α-cyclodextrin, 10% PEG (MW. 8000) or 10% glycerol, did not meet with success.

Refolding studies of sorghum α-amylase at two different pH (4.8 and 7.0) revealed that pH plays an important role in maintaining the proper structure for activity. This was further confirmed by studying the effect of pH on the structure and activity of sorghum α-amylase.

pH induced changes in structure and activity of sorghum α-amylase

Structural perturbations, at extreme pH conditions, occurred mainly due to disruption of electrostatic interactions, which play a significant role in protein stability. Enzyme stability was examined by measuring molar ellipticity at 222 nm and tryptophan fluorescence at 346 nm, as a function of pH, after incubating the samples for 24 h at 27 °C, at the test pH. The results were expressed as percentage of residual structure at pH 7.0. Over 90% of secondary and tertiary structural elements were retained in the pH range 4.8 - 8 (Figure 28A).

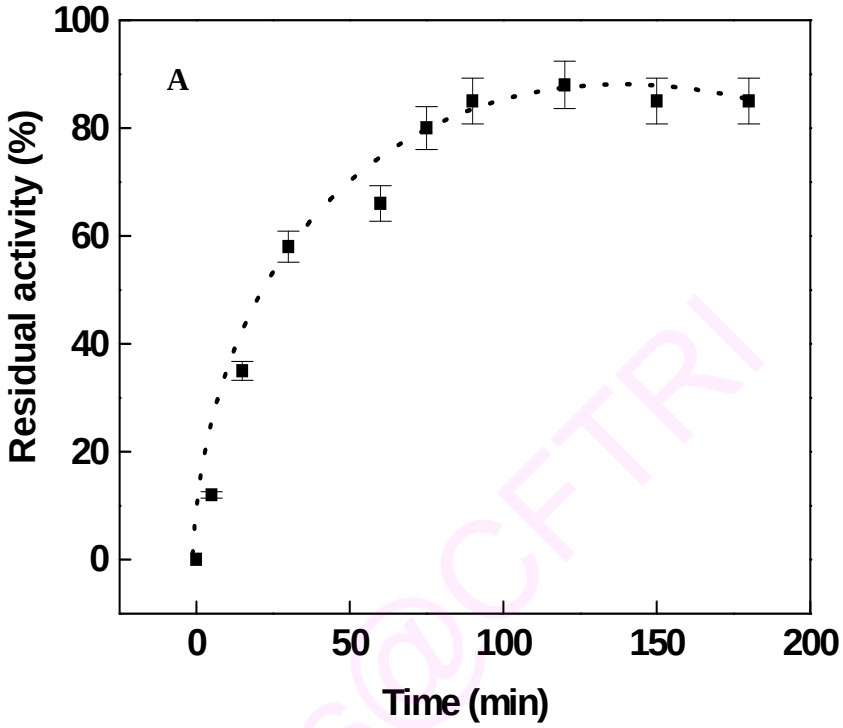


Figure 27. Refolding of the GuHCl unfolded enzyme

A. Activity regain of the GuHCl unfolded enzyme.

Activity recovery as a function of time. The unfolded α-amylase (0.75 mg/ml) was diluted to 25 times with buffer B having 15 mM DTT. Activity measurements were performed as mentioned under materials and methods.

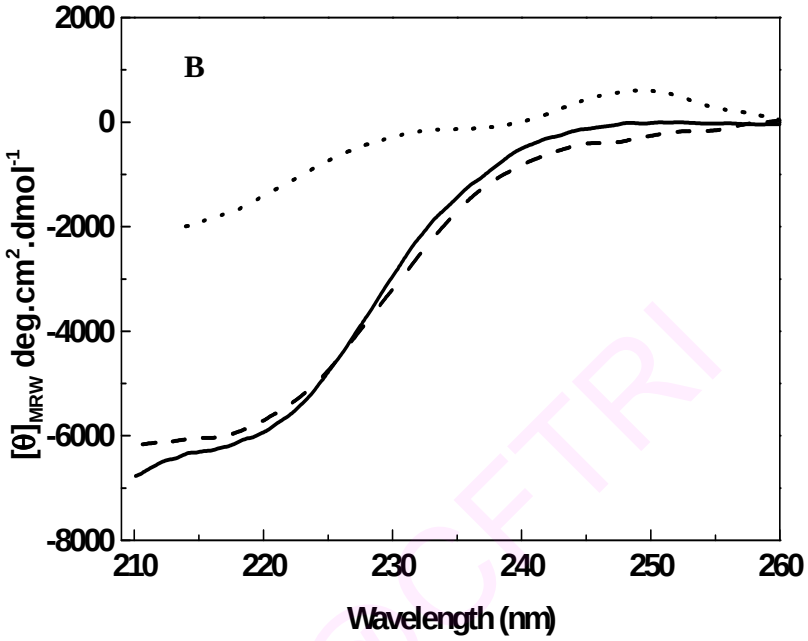


Figure 27B. Refolding of the GuHCl unfolded enzyme monitored by CD

CD spectra of native protein (*solid line*), unfolded (*dotted line*) and refolded protein (*dashed line*). Refolding of the enzyme was recorded after dialyzing the unfolded enzyme against buffer B (instead of dilution) and adjusting the protein absorbance corresponding to that of native enzyme absorbance at 280 nm. DTT (15 mM) was added before measuring the far – UV CD spectra.

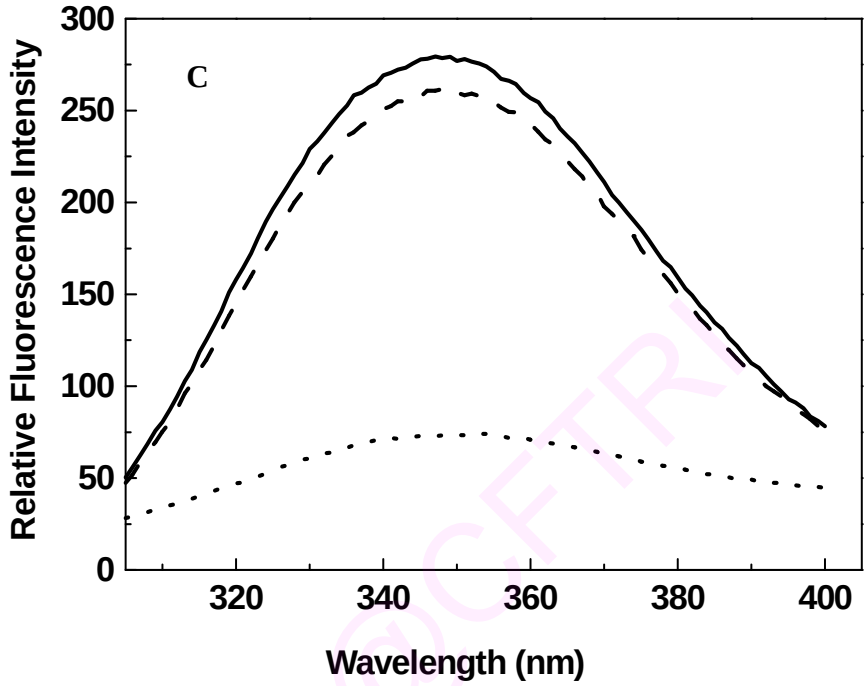


Figure 27C. Refolding of the GuHCl unfolded enzyme monitored by fluorescence.

Intrinsic fluorescence spectra of native protein (*solid line*), unfolded (*dotted line*) and refolded protein (*dashed line*). The unfolded α-amylase (0.75 mg/ml)was diluted to 25 times with buffer A and B each having 15 mM DTT .The fluorescence spectra were recorded after 2 h of incubation at 27°C.The enzyme samples have been excited at 280 nm.

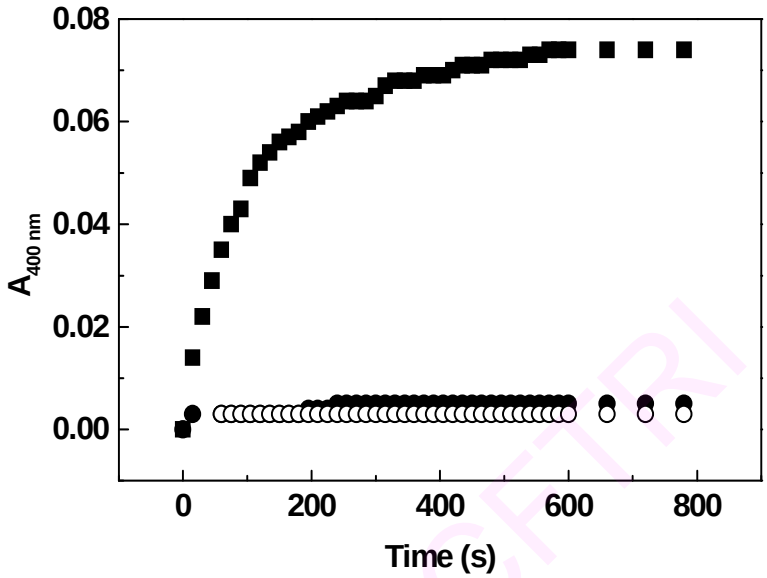


Figure 27D. Aggregation kinetics of unfolded α-amylase during refolding with buffers A and B. Native α-amylase (10 μM) was treated with 6 M GuHCl for 4 h at pH 7.0 and 27°C. This was diluted 20 fold (final protein and GuHCl concentrations were 0.5 μM and 0.3 M, respectively) by buffer A (■) or B (●) containing 15 mM DTT. Absorption was followed at 400 nm after addition of denatured enzyme to the refolding buffer at 27°C. α-Amylase, treated in the same manner (except for the addition of denaturant), was taken as control (○).

Shift in the emission maxima exhibited a bell-shaped profile, indicating unfolding of the enzyme under both acidic as well as basic pH conditions. The enzyme retained its native conformation in the pH range 4.8-8. Beyond this pH range (below 3.0 or above pH 10.0), it unfolded with loss of both structure and activity (Figure 28B). The mid-point of transition for the pH induced unfolded states ($pH_{1/2}$) is 4.1 and 8.9 (Figure 28C). Repulsive electrostatic energies between charged groups and the changes associated with the burial of ionized groups could result in destabilization of enzyme at extreme pH values (Fitter et al., 2001).

The enzyme retained > 90% of the activity in the pH range 4.8 to 8. When preincubated at pH < 4.8 or pH >8.0, irreversible inactivation occurred. Maximum stability for structure as well as activity of sorghum α-amylase was observed at pH 7.0. It was also evident from denaturant unfolding studies that α-amylase is more resistant to unfolding at pH 7.0 in comparison to unfolding at pH 4.8 (Figure 29). Sorghum α-amylase showed no significant unfolding at urea concentrations below 9 M at pH 7.0 and at 27°C. In order to compare the unfolding of α-amylase by urea and GuHCl, we investigated the conformational stability at pH 4.8 (the optimum pH for activity) in buffer A at 27°C. Chemical denaturants (GuHCl and urea) were used as perturbants to understand the contributions of non-covalent and covalent interactions to the conformational stability of the enzyme.

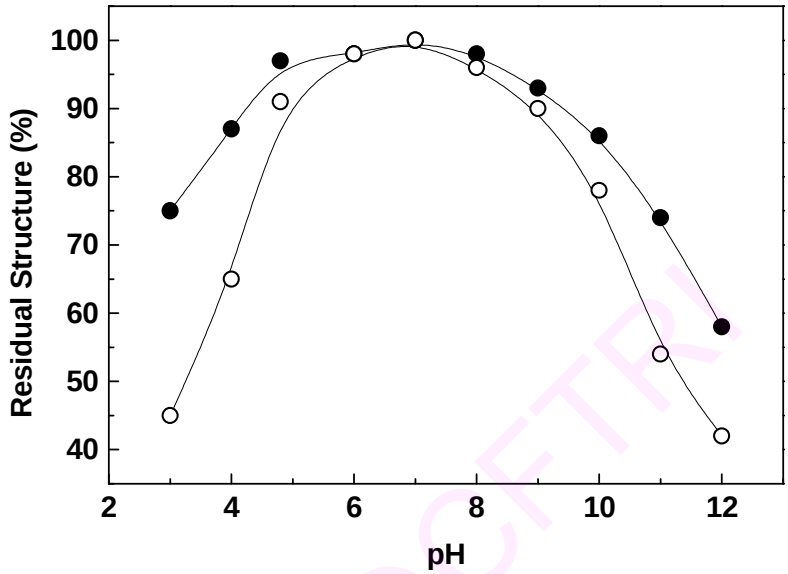


Figure 28A. Effect of pH on the structure of α-amylase

The structural stability was examined by measuring molar ellipticity at 222 nm (●), the tryptophan fluorescence at 346 nm (○), as a function of pH after incubating the samples for 24 h at 27 °C in buffers of various pH's. The concentration of the enzyme used for secondary structure analysis was ~3.5 μM and for tertiary structure analysis ~ 0.6 μM (< 0.1 O.D at 280 nm) was used.

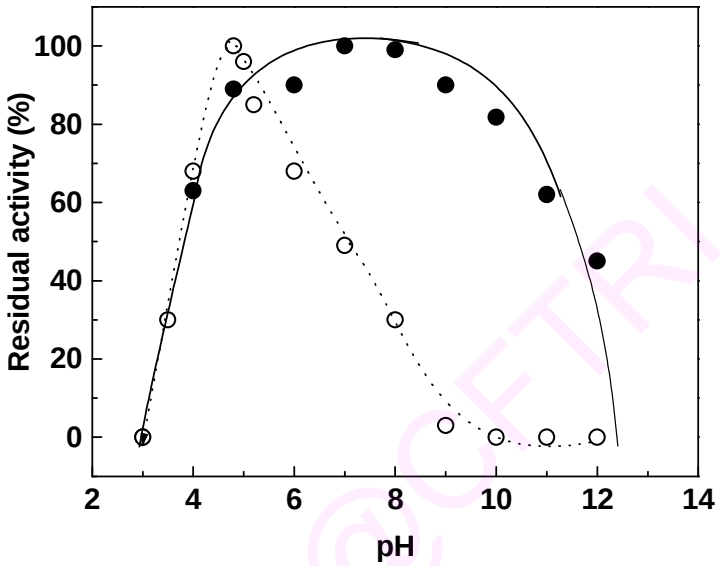


Figure 28B. Effect of pH on α-amylase activity and stability.

Effect of pH on activity was studied by performing enzymatic activity measurements at different pH values in the range of 3-12.

Stability measurements were performed by incubating the enzyme samples at different pH values in the range of 3-12 for 24 h at 27°C and then measuring enzymatic activity under standard conditions.

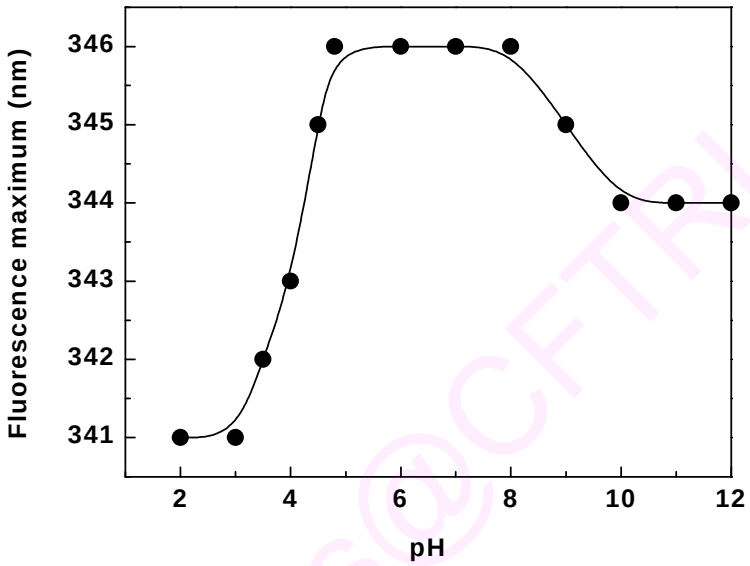


Figure 28C. Effect of pH on emission maximum of sorghum α-amylase.

Fluorescence maximum (λ_{max}) of α-amylase, as a function of pH, was recorded at 27°C. The excitation wavelength was 280 nm with slit widths of 5 and 10 nm for excitation and emission, respectively. The protein concentration was ~ 0.5 μM.

Effect of denaturants on α-amylase activity

Activity measurements are a very sensitive probe, to study the changes in the enzyme conformation during various treatments, as they reflect trivial readjustments at the active site, enabling the detection of very small conformational variations of the enzyme structure (Killenberg-Jabs et al., 2002). The changes in α-amylase activity, CD and fluorescence with both GuHCl and urea are presented in Figures 29A and 29B. Increasing concentrations of GuHCl, has resulted in decreased activity with complete unfolding at 4 M and 5 M in buffer A and B, respectively. α-Amylase activity does not change significantly in buffer B at urea concentrations below 9 M. However, with buffer A, a slight change in α-amylase activity till 5 M urea has been observed, while a gradual decrease in activity is noticed with urea between 5.0 - 9 M. This variation in activity measurements observed in presence of both the denaturants against α-amylase can be due to the difference in their mechanism of action (Monera et al., 1994). The slight increase in the activity observed for α-amylase in presence of denaturant may be due to the stabilizing effect of the denaturant at low concentrations. The stabilizing mechanism of chemical denaturants, at lower concentrations, in respect of other enzymes, has been reported (Kumar et al., 2004; Bhuyan, 2004).

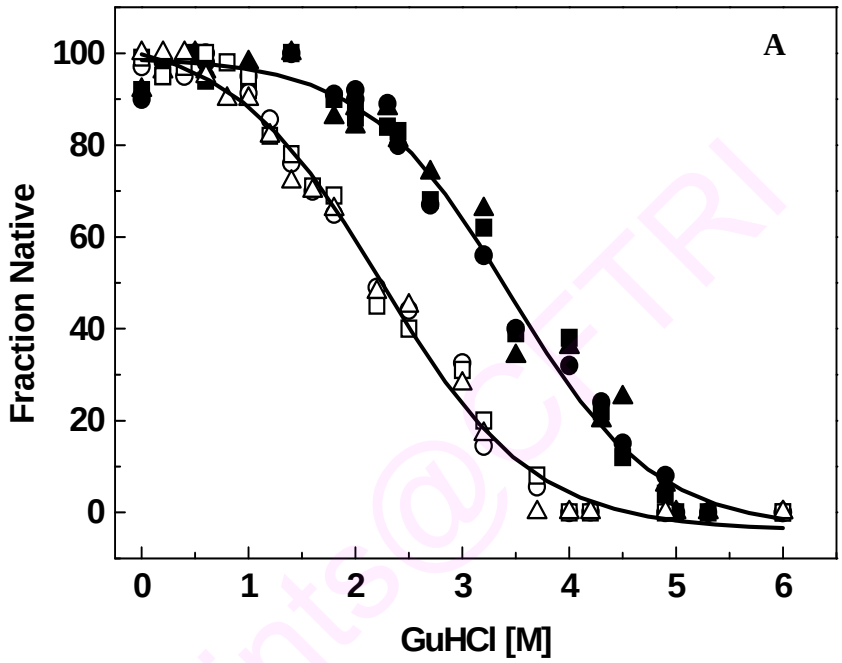


Figure 29. Unfolding of α-amylase by chemical denaturants

(A) The overlay of GuHCl induced unfolding curves of α-amylase at pH 4.8 (open) and 7.0 (solid), at 27°C monitored by two spectroscopic probes - Far- UV CD at 222 nm (*circles*) and fluorescence intensity change at 346 nm (*squares*). Effect of denaturant on α-amylase activity (*up triangles*) was overlaid on the stability curve.

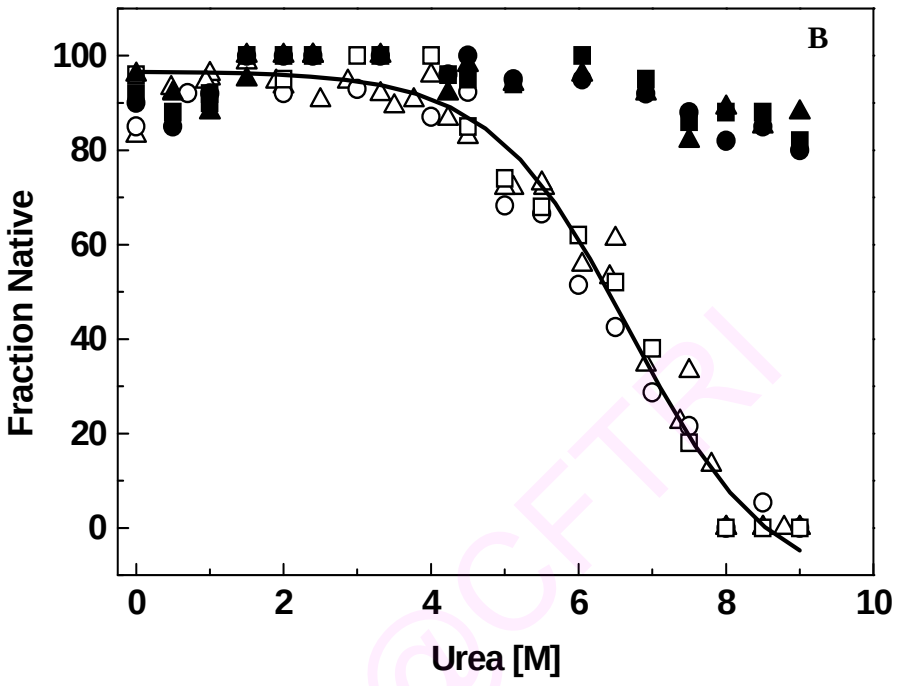


Figure 29. Unfolding of α-amylase by chemical denaturants

(B) The overlay of urea induced unfolding curves of α-amylase at pH 4.8 (open) and 7.0 (solid), at 27°C monitored by two spectroscopic probes - Far- UV CD at 222 nm (*circles*) and fluorescence intensity change at 346 nm (*squares*). Effect of denaturant on α-amylase activity (*up triangles*) was overlaid on the stability curve.

Denaturant induced unfolding of α-amylase

Aliquots of enzyme (0.3 μM for fluorescence and 3.4 μM for CD), in buffer A and B, were incubated separately at 27°C for 4 h and 12 h, respectively, with GuHCl and urea, to attain equilibrium, prior to measuring structural changes. The fluorescence emission spectra, of sorghum α-amylase, exhibited maximum intensity at 346 nm, characteristic of Trp residues exposed to aqueous solvent. It was noticed that the signal intensity decreased with the increasing concentration of denaturant. The enzyme, unfolded with urea, revealed a red shift of 6nm (346 – 352 nm) while the shift with GuHCl was 8 nm (346 –354 nm).

α-Amylase, which did not completely unfold with urea even at 9 M and pH 7, completely unfolded at 8 M and pH 4.8. GuHCl completely unfolded the enzyme at (5 M, pH 7.0) and (4 M, pH 4.8). Within experimental errors, unfolding curves had a sigmoidal shape, regardless of the spectroscopic probe used to monitor the unfolding. Denaturant concentrations at half-completion of the $[\text{denaturant}]_{1/2,}$ were: (6.8 M, pH 4.8) for urea and (2.3 M, pH 4.8) and (3.4 M, pH 7.0) for GuHCl. Interestingly, activity measurements for unfolded samples coincided with the loss in secondary and tertiary structure, reflecting a good correlation between activity and structural integrity of the molecule. This finding could be an indication of GuHCl and urea induced unfolding of sorghum α-amylase being a two step transition, involving F↔U. The enzyme structure was found to be resistant to the unfolding action of urea compared to GuHCl at both the pH values 4.8 and 7.0.

The strong difference in the unfolding action of GuHCl and urea on sorghum α-amylase may be attributed to the ionic character of GuHCl. It has been reported that urea and GuHCl provide a different estimate of protein stability as a consequence of the role played by electrostatic interactions (Monera et al., 1994). With a view to mimicking the screening effect of charge-charge interactions exerted by GuHCl, we have studied urea-induced unfolding in presence of 1 M NaCl at pH 4.8 and 7.0. Although NaCl is not a denaturing agent of globular proteins, the presence of Na^+ and Cl^- ions, in aqueous solution, causes a general screening effect of favorable electrostatic interactions among the charged groups on the protein surface. Unlike sodium ion, GuHCl binds mainly to the exposed groups of the protein, causing a marked weakening of the electrostatic interactions on the protein surface (Vecchio et al., 2004). In presence of 1 M NaCl, when followed at 346 nm, $[\text{urea + 1M NaCl}]_{1/2,}$ = 3.9 compared to $[\text{urea}]_{1/2}$ = 6.8 M in absence of NaCl at pH 4.8. At pH 7.0, $[\text{urea + 1M NaCl}]_{1/2}$ = 6.4 M, suggesting that the conformational stability of sorghum α-amylase depends on the ionic strength of the aqueous solution, specifying the role of electrostatic interactions (Figure 30).

Analysis by LEM of GuHCl and urea transition curves indicates the respective $\Delta G(H_2O)$ values in buffer A to be 16.5 kJ mol^{-1} and 25.2 kJ mol^{-1}, while the *m* values are 7.2 kJ $mol^{-1}M^{-1}$ and 3.7 kJ $mol^{-1}M^{-1}$. $\Delta G(H_2O)$ value of 22 kJ mol^{-1} and *m* value of 6.5 kJ $mol^{-1}M^{-1}$ is obtained from GuHCl unfolding in buffer B .

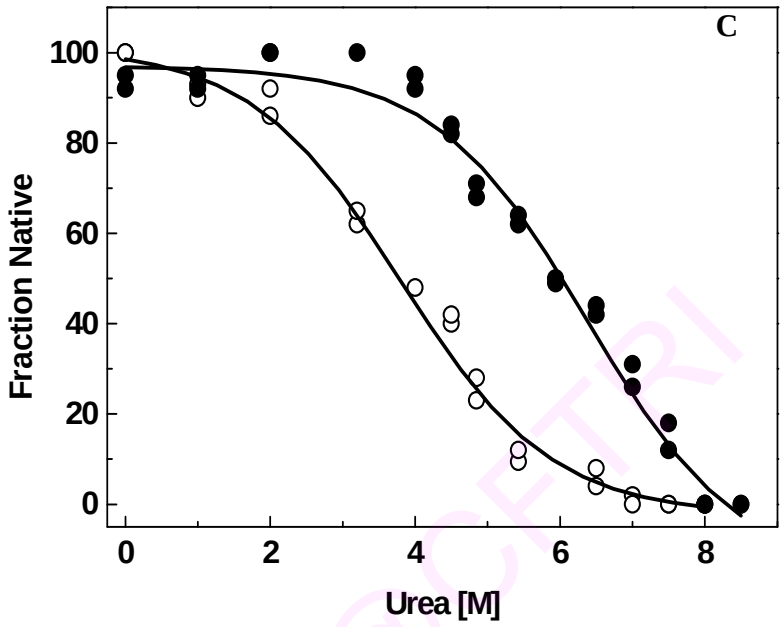


Figure 30. Unfolding of α-amylase by urea in presence of NaCl

Urea induced transition curves of α-amylase at pH 4.8 (○) and 7.0 (●) at 27°C recorded by measuring fluorescence intensity changes at 346 nm in the presence of 1 M NaCl.

Since LEM analysis is too crude to account fully for the complex action of GuHCl and urea, there is no agreement between the $\Delta G(H_2O)$ estimates obtained from GuHCl and urea transition curves (Vecchio et al., 2002). The transition curve of sorghum α-amylase, as a function of denaturant concentration, is a direct evidence that the enzyme which is resistant to the urea unfolding, is easily unfolded by GuHCl. To obtain the conformational stability parameters, unfolding was carried out as a function of temperature.

Conformational stability of sorghum α-amylase: unfolding by GuHCl as a function of temperature

The effect of temperature on free energy of unfolding with GuHCl has been followed by fluourescence measurements at 346 nm in the temperature ranges 297–323 K and 293-323 K at pH 4.8 and 7.0, respectively. From each of the isothermal melts, $\Delta G(H_2O)$ of unfolding at each temperature is calculated and a stability curve is drawn by fitting the values of $\Delta G(H_2O)$ and T to equation 9, to generate the values of ΔH_g , ΔC_p and T_g. The curve is shown in Figure 31. These thermodynamic parameters obtained from the curves at both the pH values are shown in Table 10.

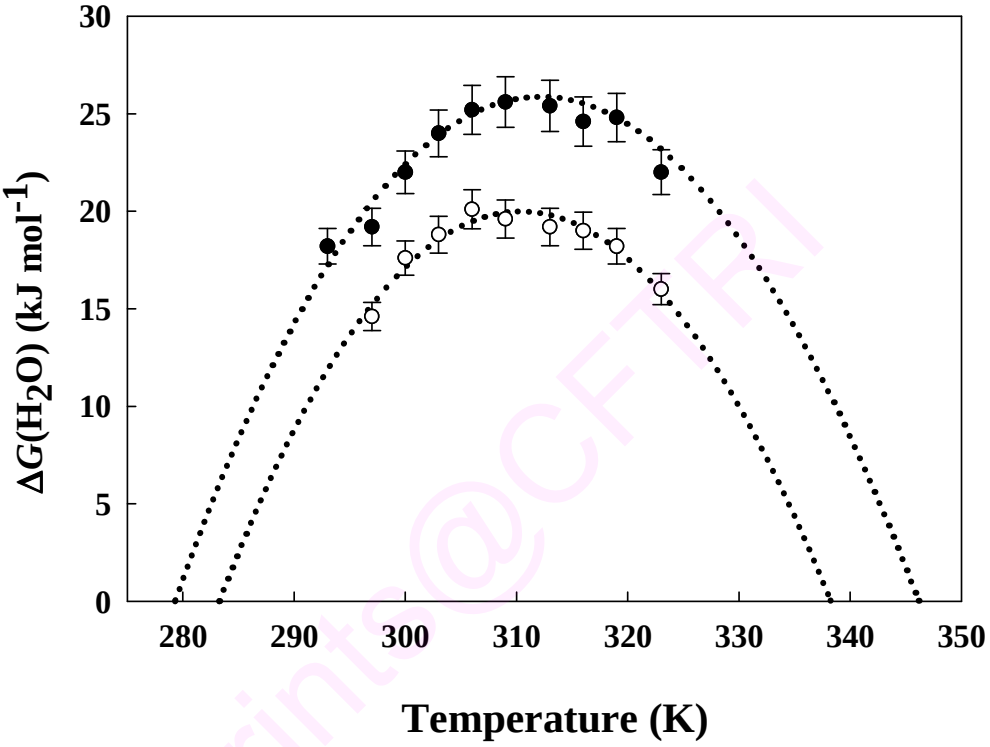


Figure 31. Stability curve of sorghum α-amylase

Data points for the conformational stability parameters of α-amylase were obtained from individual unfolding experiments carried out at different temperatures in the range 297–323 K at pH 4.8 (○) and 293 – 323 K at pH 7.0 (●). The broken lines passing through the points were fitted using equation (9).

Table 10. Conformational stability parameters of sorghum α-amylase analyzed from stability curves

pH	ΔHg (kJ mol^{-1})	ΔC_P (kJ mol^{-1} K^{-1})	Tg (K)
4.8	501.2 ± 18.2	17.9 ± 0.7	337.3 ± 6.9
7.0	509.3 ± 21.7	14.3 ± 0.5	345.4 ± 4.8

Curves were fitted using equation (9)

Thiol reactivity during unfolding and refolding

Accessibility of thiols to DTNB, in the presence of denaturant, reflected denaturant-induced structural changes in sorghum α-amylase. To assess the state of cysteine residues during the unfolding process, α-amylase in buffer B was unfolded at different concentrations of urea and GuHCl, at 27 °C. The thiol content of the unfolded enzyme was assayed using DTNB. In the native state, only 1.2 moles of cysteine residues were accessible to DTNB. The available thiols remained constant till 6 M urea, while only a small increase is observed with 8 M urea (1.5 moles/mole of protein). In case of α-amylase unfolded with 0.5, 0.8, 2, 4 and 6 M GuHCl, the number of cysteine residues assayed were 2.0, 2.2, 1.8, 0.9 and 0.6, respectively (Figure 32A). These results suggested that the thiol groups in α-amylase get oxidized during the course of unfolding by GuHCl. The decrease in cysteine content could be either due to the formation of disulphide bridge or due to the oxidation of cysteine residues to sulfite. Disulfide bond formation led to aggregation of protein (Sudharshan and Rao, 1999). This was checked by electrophoretic mobility of modified residues on non-reducing gel electrophoresis (results not shown). Before loading on to the gel, α-amylase was unfolded at different concentrations of GuHCl (0.5, 0.8, 2 and 6 M) and dialyzed against buffer B. The absence of higher aggregates clearly pointed to the formation of sulfite.

The role of cysteine residues, during refolding, was also examined. Thiol groups, when blocked with iodoacetamide (0.5 mM) in presence of different concentrations of GuHCl (0 M, 0.5 M, 0.8 M and 6 M), became unavailable to DTNB**.** The regaining of secondary structure, by the cysteine blocked enzyme, allowed to refold following dialysis, was evident from the far UV CD spectra (Figure 32B). The enzymatic activity regained was ~ 69 %, 55 %, 40% and 8 % for the enzyme whose cysteine residues are blocked in presence of 0 M, 0.5 M, 0.8 M and 6 M GuHCl, respectively. These results indicated that though thiol groups play no role in the regaining of the enzyme structure, yet are essential for catalysis.

The environment around the cysteine residues was probed by using MIANS, which reacts covalently with cysteine residues and exhibits fluorescence in a hydrophobic environment (Sudharshan and Rao, 1999). α-Amylase was labeled with MIANS in the presence of different concentrations of GuHCl and the corresponding emission spectra were recorded. Initially, the fluorescence emission was checked after exciting at 328 nm for MIANS fluorescence (Figure 32C). The relative fluorescence intensity at 425 nm increased with 0.5 M and 0.8 M GuHCl, pointing to the hydrophobicity of cysteine residues. Further, to check the proximity of these cysteine residues to aromatic amino acid side chains of α-amylase, the samples were excited at 280 nm. Two emission maxima were observed: one for sorghum α-amylase (346 nm) and the other for MIANS (425 nm) (Figure 32D).

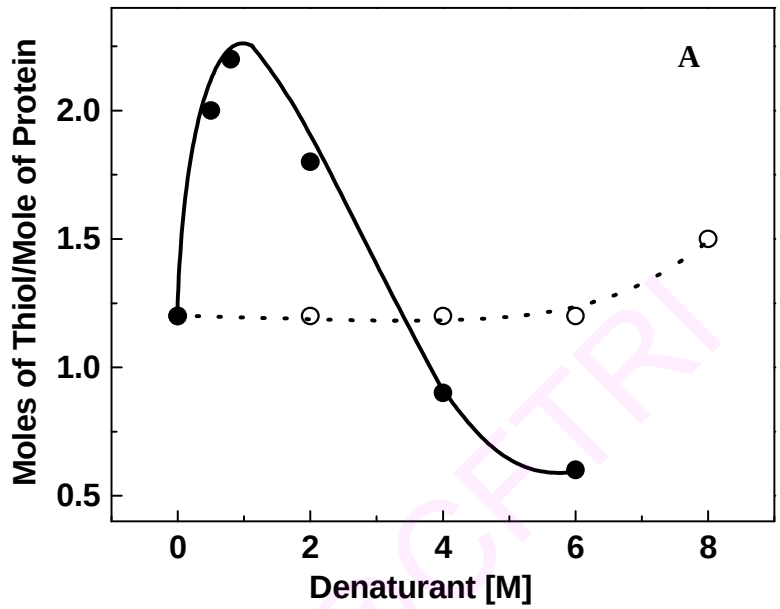


Figure 32. Reactivity of cysteine residues during unfolding

(**A**) Cysteine accessibility to DTNB in presence of various concentrations of GuHCl (●) and urea (○). To the assay mixture of 0.9 ml containing 0.1 M Tris HCl pH 7.4, 1 mM DTNB and various concentrations of denaturants, sorghum α-amylase (1.5 mg in 50 mM Hepes, pH 7.0) was added and the increase in absorbance at 412 nm was measured. The number of thiol groups exposed at various denaturant concentrations was estimated using the value of 12,500 M^{-1} cm^{-1}.

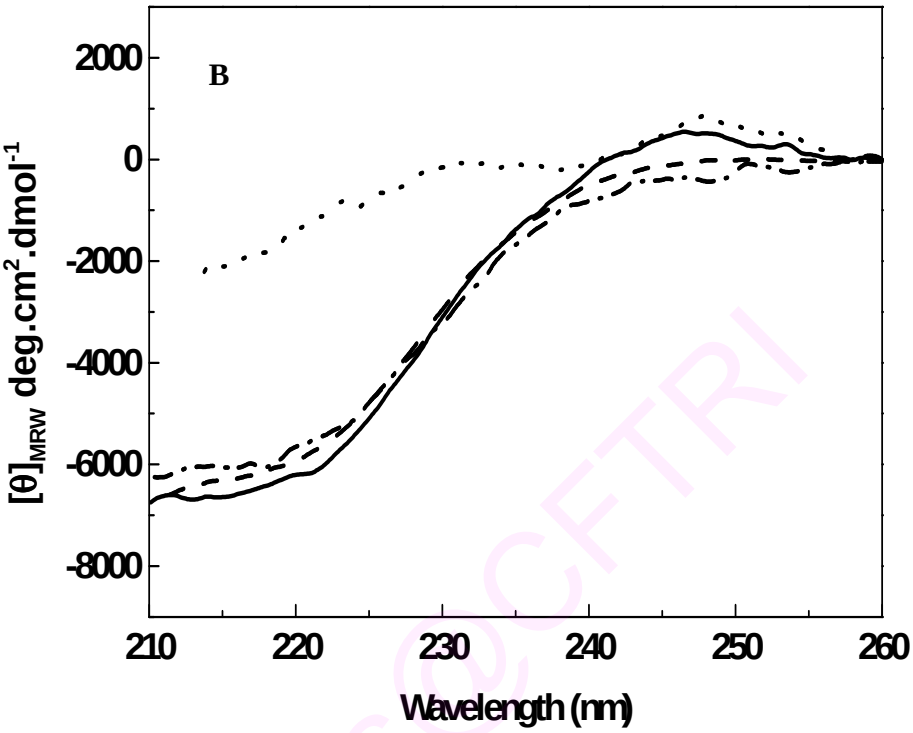


Figure 32. Reactivity of cysteine residues during unfolding

(**B**) Unfolding and refolding of sorghum α-amylase treated with iodoacetamide and DTT. Native protein (*solid line*), unfolded with 6 M GuHCl (*dotted line*), refolded amylase in presence of DTT (*dashed line*) and refolded protein in presence of iodoacetamide (*dashed and dotted line*).

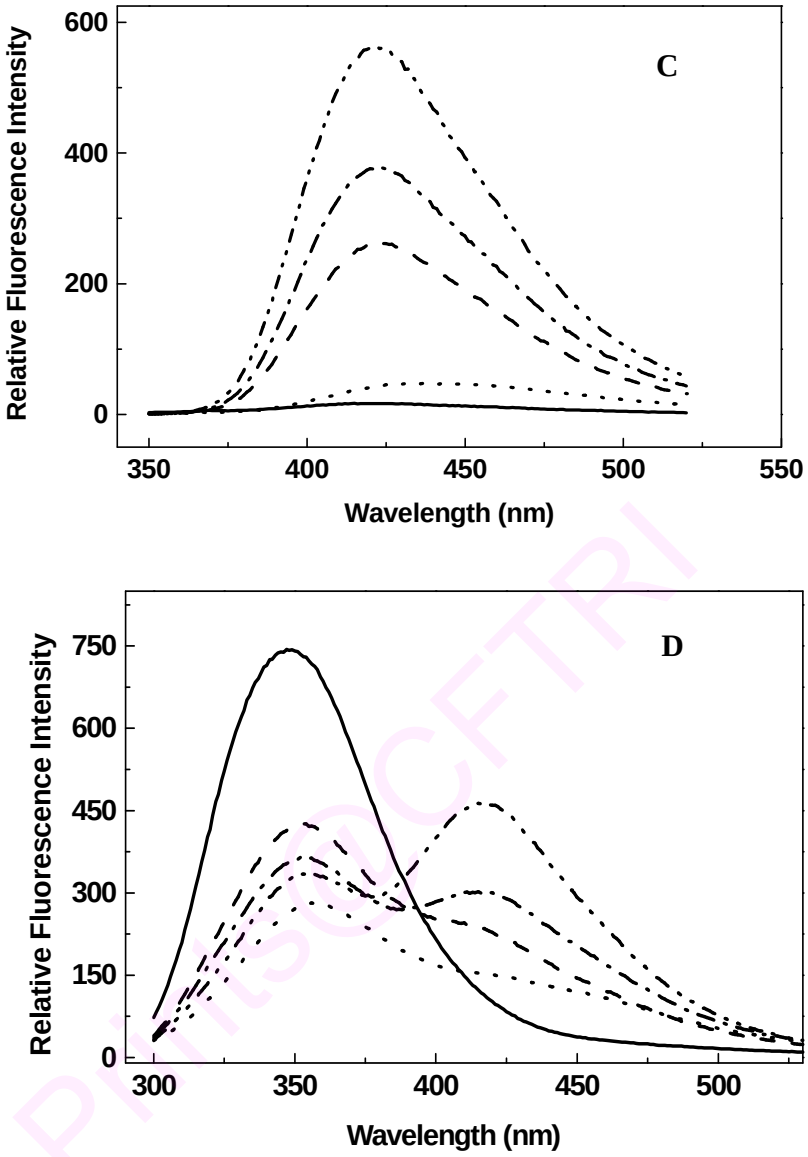


Figure 32. Reactivity of cysteine residues during unfolding

Fluorescence spectra of the MIANS labeled sorghum α-amylase (0.8 μM) were recorded by exciting at 328 (C) and 280 nm (D). Sorghum α-amylase labeled with MIANS in the presence of native (----) , 0.5 M GuHCl (—·—) , 0.8 M GuHCl (—··—) and 6 M GuHCl (.....). Native amylase without MIANS is represented by (——).

The emission intensity at 425 nm increased with 0.5 and 0.8 M GuHCl, indicating an energy transfer from aromatic side chains to MIANS. The decrease in area in the first peak was almost completely compensated in the second peak.

These observations indicated that cysteine residues, exposed at 0.5 and 0.8 M GuHCl, are in close proximity to aromatic amino acid side chains of α-amylase. The low efficiency of energy transfer, observed at higher concentrations of GuHCl (>2 M), could probably be due to the oxidation of cysteine residues during unfolding.

Thermal unfolding of α-amylase

Sorghum α-amylase, which did not unfold at pH 7.0 in the temperature range 25-85°C (Figure 33A), unfolded at pH 4.8. Thermal unfolding curves of sorghum α-amylase in buffer A observed by absorbance spectroscopy at 280 nm and turbidity measurements at 400 nm revealed the unfolding being accompanied by aggregation, leading to irreversibility, which precludes thermodynamics of unfolding. The experimental curves are shown in Figure 33A. The presence of denaturants (6 M urea or 2 M GuHCl) allowed to study thermal unfolding of α-amylase without aggregation. Below this denaturant concentration range, the midpoint temperature could not be calculated due to enzyme precipitation. During sample preparation, sorghum α-amylase did not unfold in 6 M urea at 27°C. With 2 M GuHCl, a small decrease in the CD signal was observed at 27°C. The midpoint of ellipticity change, $T_{m[urea]}$ and $T_{m[GuHCl]}$ at 222 nm, occurred at

49.3°C and 47.3°C, in presence of 6 M urea and 2 M GuHCl, respectively (Figure 33B). Obviously, the presence of GuHCl was more effective than urea, during thermal unfolding. Thermal unfolding in presence of these denaturants was found to be irreversible for sorghum α-amylase, since the reheating of the cooled enzyme failed to give a superimposable melting profile. However, partial refolding with reference to activity was observed for the thermally unfolded and aggregated enzyme. Treatment with 3 M GuHCl followed by dilution with buffer B having 15 mM DTT resulted in the disaggregation of the enzyme sample, with ~35 % activity recovery. Failure to recover activity completely could be due to side reactions that occur at higher temperatures (See study on the contribution of various interaction on the stability of sorghum α-amylase)

The thermal transition curves for the native enzyme revealed only a small loss in secondary structure: $[\theta]_{222}$ = -5000 deg. cm^2. dmol^{-1} at 65 °C . However, α-amylase at 65°C, in presence of denaturants, revealed the complete loss of secondary structure with molar ellipticity close to zero, in presence of 2 M GuHCl and ~ -1200 deg. cm^2. dmol^{-1} in presence of 6 M urea (Figure 34). This observation indicated that the end states are different for the different denaturants.

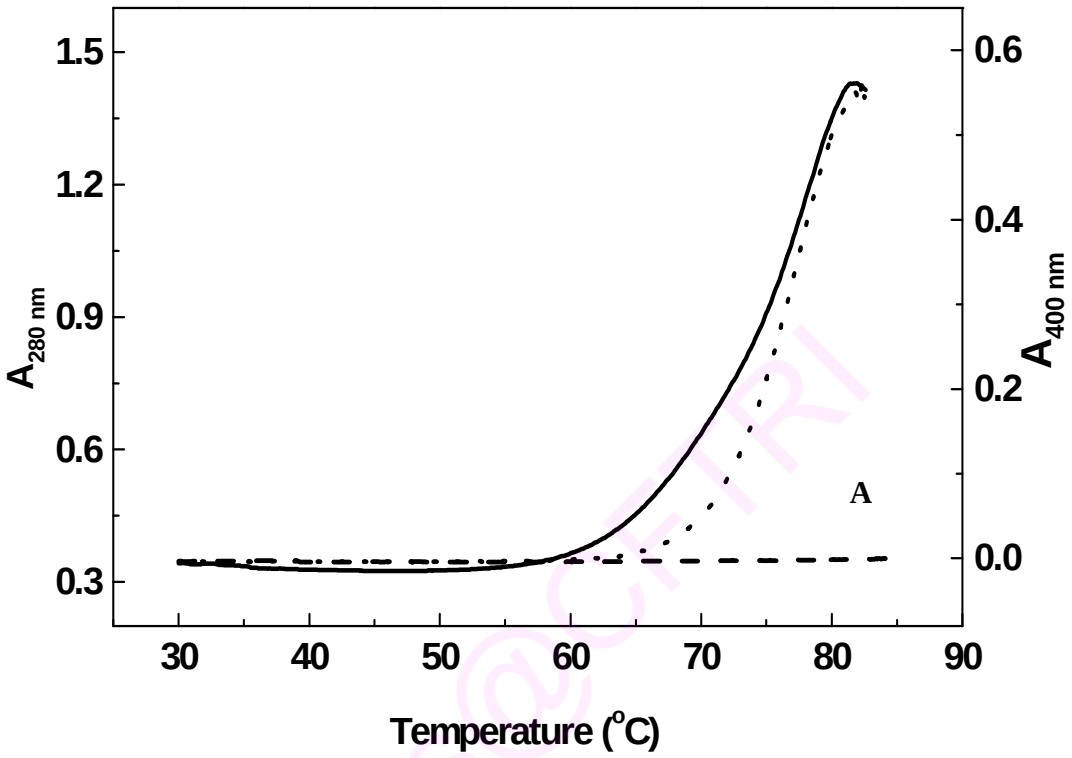


Figure 33. Thermal unfolding of α-amylase

(**A**) Absorbance spectra was obtained at 280 nm and 400 nm (to measure aggregation) as a function of temperature. Amylase unfolded at pH 4.8 (*solid line*), protein unfolded at pH 7 (*dashed line*) and aggregation of thermally unfolded protein followed at 400 nm and pH 4.8 (*dotted line*)

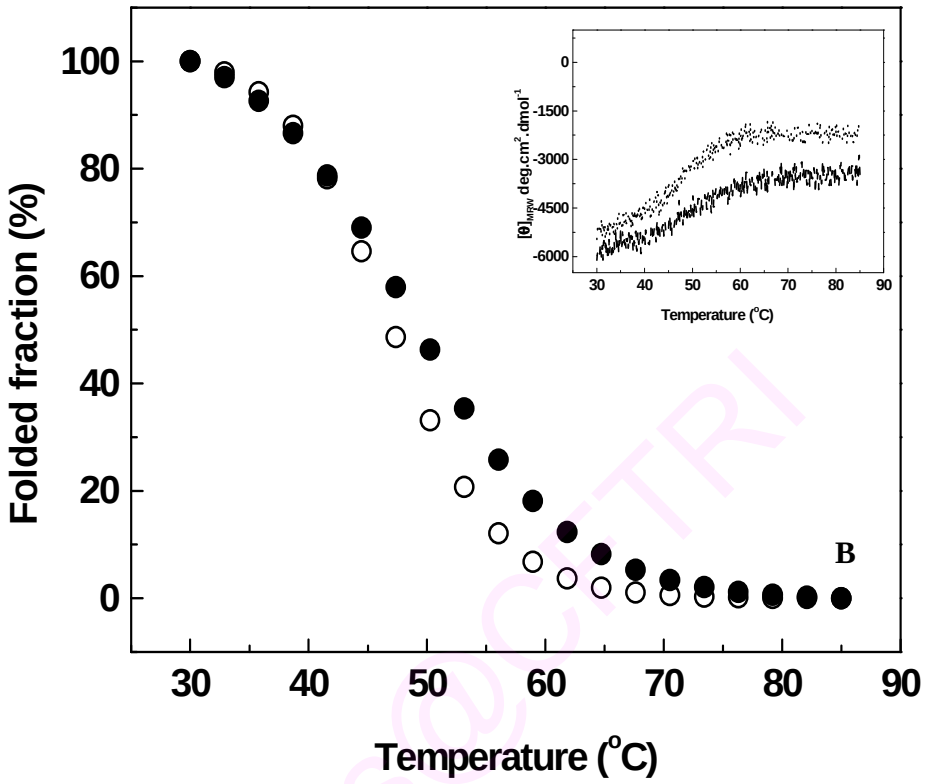


Figure 33. Thermal unfolding of α-amylase

(**B**) Normalized thermally induced unfolding of sorghum α-amylase in buffer A in presence of 2 M GuHCl (○) and 6 M urea (●) was measured by recording molar ellipticity at 222 nm. **Inset** shows the CD curves for thermal unfolding of α-amylase in presence of 2 M GuHCl (……) and 6 M urea (——).

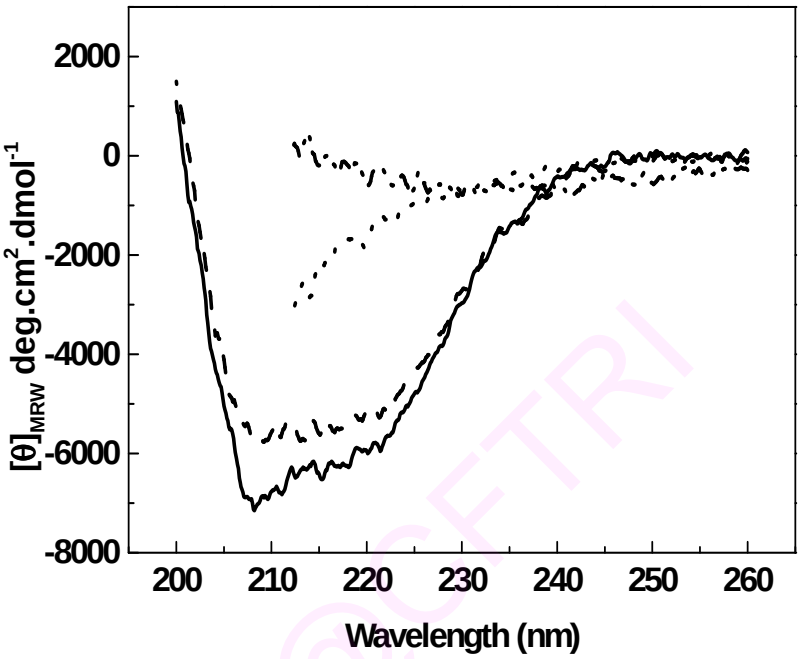


Figure 34. Thermal unfolding of α-amylase in presence of denaturants Temperature induced unfolding of α-amylase at 65°C in the absence and presence of denaturants, (—— ·· ——) 2 M GuHCl and 6 M urea (….), amylase heated at 65°C for 10 min without denaturants (----). α-Amylase was heated at 65°C for 10 min with or without denaturants, cooled to 25 °C and residual structure measured by CD in the far-UV region at scan speed of 10 nm/min. Unheated α-amylase was taken as native enzyme (——).

Measurement of thermal inactivation parameters

Thermal inactivation kinetics of the enzyme was determined by incubation of the enzyme, in buffer A, at the test temperature for varying intervals of time. The thermal inactivation was found to follow first order kinetics, indicating the existence of a single inactivation mechanism. The time at which loss of activity reached 50% was taken as the experimental half-life for the enzyme and the rate constant determined (Figure 35A). Thermodynamic parameters were estimated from the Arrhenius plot constructed using the rate constants at different temperatures. E_a (activation energy) was determined to be 45.3 ± 0.17 kcal.mol^{-1}, from the slope of the Arrhenius plot (Figure 35B). The activation enthalpy (ΔH*), entropy (ΔS*) and free energy change (ΔG*) were calculated to be 44.6 ± 0.01 kcal.mol^{-1}, 57.1 ± 0.25 cal.mol^{-1}.$^{\circ}$K^{-1} and 25.2 ± 0.24 kcal.mol^{-1}, respectively.

Sequence similarity and homology modeling

The amino acid sequence of sorghum α-amylase (Name - sb04g034150; Protein ID: 5039071) has been recently produced by the US Department of Energy Joint Genome Institute (http://www.jgi.doe.gov/). The amino acid composition of the published sequence is very similar to the present study (Table 10). Hence, this sequence is used as the template for building the model. There are 3 invariant cys residues present in all cereal amylases (Figure 36). Two of the cysteines are present in domain B (irregular fold), while the third residue is situated in domain A (Figure 37).

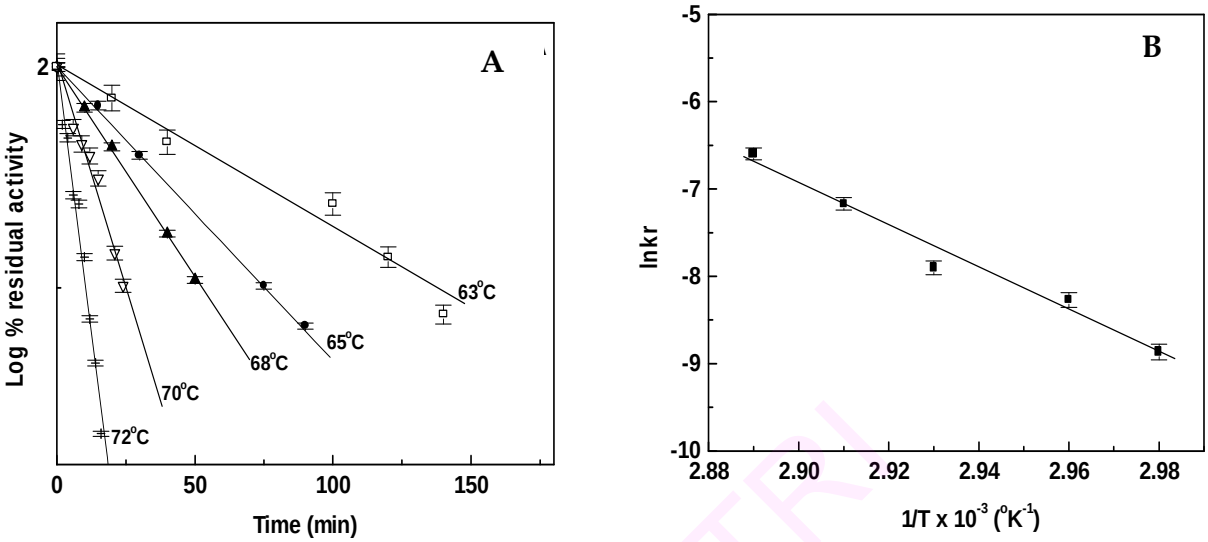


Figure 35. Thermal inactivation kinetics

A = Thermal inactivation profile of sorghum α-amylase.

Enzyme (4.93x 10^{-3} mg/ml) was incubated at 63°C (□), 65°C (●), 68°C (▲), 70°C (▽) and 72°C (+),aliquots were drawn at different intervals of time, cooled to 4°C and the remaining activity was measured under standard assay conditions.

B = Arrhenius plot for the thermal inactivation of sorghum α-amylase.

The slope of the plot equals $-E_a/R$.

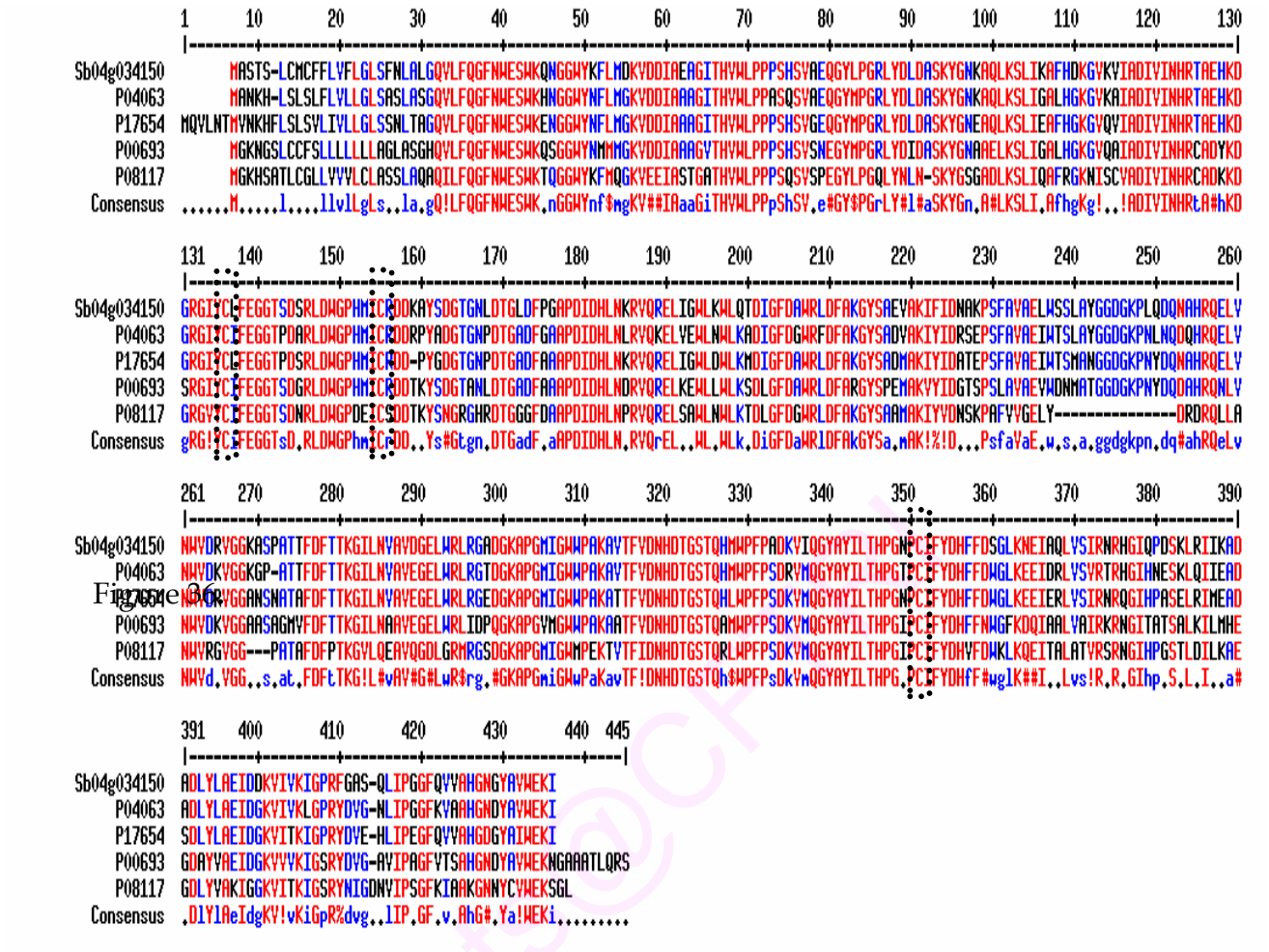


Figure 36

Multiple sequence alignment of entire sequences of cereal α-amylases showing homology and conserved cysteines in dotted boxes. Sequences of cereal α-amylases including *Hordeum jubatum* (barley isoenzyme 1 - P00693, isoenzyme 2 - P04063), *Oryza sativa* (rice - P17654) and *Triticum aestivum* (wheat - P08117), were taken from http://www.expasy.org/enzyme/3.2.1.1. *Sorghum bicolor* α-amylase (Name - sb04g034150; Protein ID: 5039071) sequence was obtained from the US Department of Energy Joint Genome Institute 'http://www.jgi.doe.gov/'. Consensus sequence indicating (!) is anyone of I or V, ($) is anyone of L or M, (+) is anyone of F or Y and (#) is anyone of N, D, Q , E , B or Z.

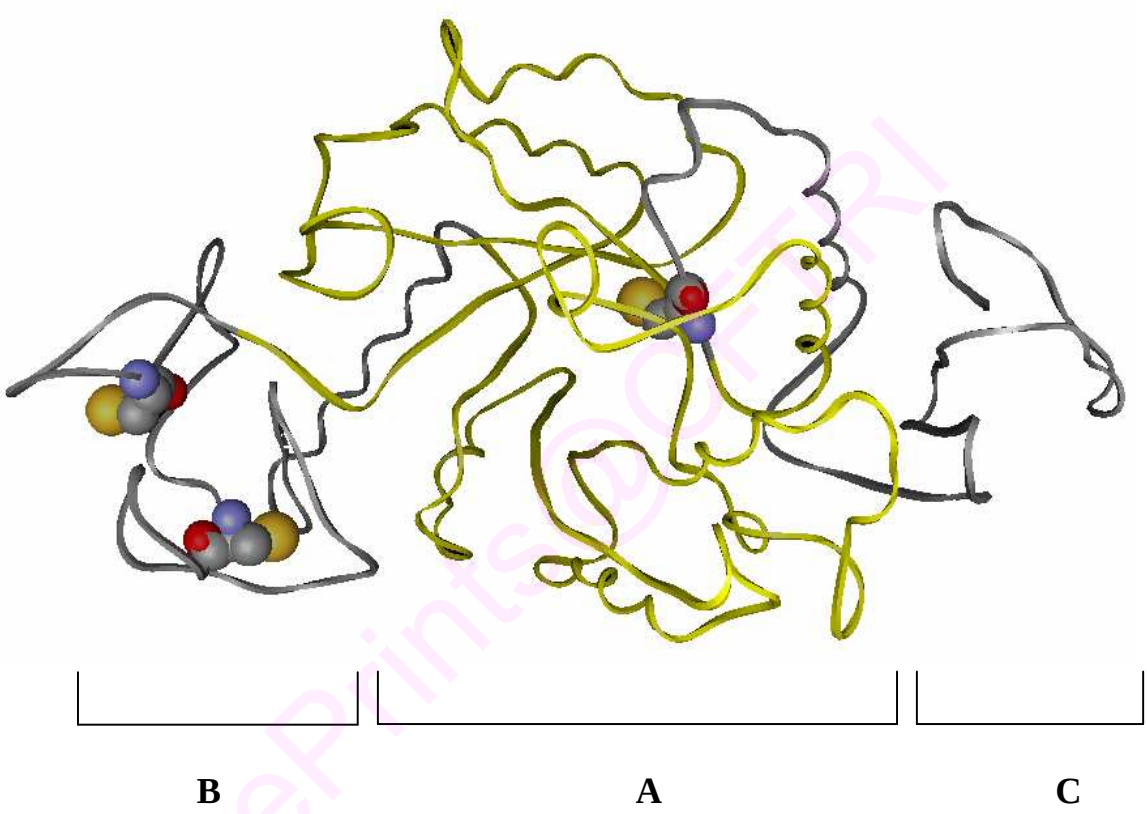


Figure 37. Homology model of sorghum α-amylase was predicted based on barley AMY1 (Robert et al., 2003) (PDB ID: 1HT6). The three domains are represented by A, B and C respectively. The invariant cys residues – two in domain B and one in domain A- are highlighted.

DISCUSSION

In the present study, we have delved into the unfolding and refolding of sorghum α-amylase as a function of pH and denaturants (chemical and thermal). The roles of non-covalent interactions as well as covalent interactions have been investigated.

Numerous side reactions, such as aggregation and sulfhydryl oxidation, contribute to the irreversible unfolding of proteins (Sudharshan and Rao, 1999). α-Amylases, typically, unfold irreversibly (psychrophilic α-amylase from A*lteromonas haloplanctis* is an exception) (Duy and Fitter, 2005). However, refolding of the α-amylase from bacterial and fungal sources is observed under specific conditions (Strucksberg et al., 2007). The efficient renaturation is hampered by local interactions and oxidative events that either inactivate the enzyme or lead to aggregation (Sudharshan and Rao, 1999).

Unfolding of sorghum amylase using denaturants

The unfolding of sorghum α-amylase by denaturants exhibited a superimposable profile for activity, secondary ($[\theta]_{222}$) and tertiary (fluorescence) structural changes, suggesting that the unfolding is a two-state process ($F \leftrightarrow U$). Sorghum α-amylase is stable against the unfolding action of urea but not against that of GuHCl, at both the pH values examined. GuHCl is ~2 times more effective as denaturant than urea in unfolding proteins (Vecchio et al., 2002). However, GuHCl is ~ 3 times more effective than urea in unfolding sorghum α-

amylase. GuHCl, which exists in a fully dissociated form, i.e., Gu^+ and Cl^-, at pH below 11 can effectively interfere with favorable electrostatic interactions among the charged groups on the protein surface, evidencing the role of electrostatic interactions in the stability of enzyme. Urea, being uncharged, does not affect the inter and intramolecular electrostatic interactions in the protein. Hence, $[urea]_{1/2}$ or free energy of unfolding by urea, reveals the net stability of the protein contributed by hydrophobic and electrostatic interactions (Monera et al., 1994). In addition, the urea induced unfolding of enzyme in presence of NaCl has revealed the importance of electrostatic interactions for the stability of sorghum α-amylase. Similar observations have been made in case of barley α-amylase (Jensen et al., 2003). The ratio of $[urea]_{1/2}$ to $[GuHCl]_{1/2}$ > 2 for thermophilic proteins (Vecchio et al., 2002). For sorghum α-amylase, the calculated ratio for $[urea]_{1/2}$ to ($[GuHCl]_{1/2}$ is ~3.1, suggesting the thermophilic nature of protein, whose stability is determined primarily by electrostatic interactions.

Conformational stability parameters obtained from the isothermal denaturation curves (Figure 3) are shown in Table 9. The temperatures of maximum stability (T_s) at pH 4.8 and 7.0, are 306 K and 309 K, respectively. The T_g value obtained at pH 7.0 is ~ 8°C more than that at pH 4.8, indicating that sorghum α-amylase is more stable at pH 7.0 compared to pH 4.8. The ΔC_p values for the isothermal unfolding at pH 4.8 and 7.0 are 17.9 ± 0.7 kJ $mol^{-1}K^{-1}$ and 14.3 ± 0.5 kJ $mol^{-1}K^{-1}$, respectively. The exposure of hydrophobic core is the prominent feature in protein unfolding. This exposure is reflected in the positive

change in heat capacity of the protein during the unfolding process (Sinha et al., 2005). Sorghum α-amylase shows a positive change in heat capacity upon unfolding, confirming the exposure of nonpolar surfaces to the polar aqueous environment.

α-Amylase is resistant to unfolding near pH 7.0 compared to pH 4.8, the optimum pH for activity. $\Delta G(H_2O)$ values obtained for GuHCl are higher at pH 7.0 compared to 4.8. The pH dependence of the conformational stability of a globular protein depends mainly on two factors: (1) Electrostatic interactions involving the charged groups and (2) Differences between the p*K* values of the ionizable groups in the folded and unfolded conformations. The difference between the sum of the attractive and repulsive interactions in the folded and unfolded conformations of the protein surely contribute to the conformational stability (Pace, 1990; Pace et al., 1990)

Refolding of unfolded sorghum amylase

The refolding of sorghum α-amylase at pH 7.0, in presence of DTT, indicates the role of electrostatic interactions and free thiol groups in the refolding process. The reversible nature of unfolding established in the present study, helps in the determination of conformational stability parameters. DTT is essential for regaining enzyme activity, as it aids in the selective reduction of sulfite and disulfide bonds to thiols. α-Amylase regains its secondary and tertiary structure, when cysteine residues are blocked with iodoacetamide.

However, the refolded enzyme lacks the enzymatic activity. Thus, it is evident that the cysteine residues in sorghum α-amylase, though not essential for refolding of the molecule, are necessary for biological activity.

Multiple sequence alignment of cereal α-amylases has revealed a high degree of homology, wherein cysteine residues are highly conserved at positions 136, 156 and 351 (Figure 7B). During the unfolding of sorghum α-amylase with 0.5 M and 0.8 M concentration of GuHCl, 2 and 2.2 moles of cysteine residues are, respectively, exposed to DTNB. Cysteine residues corresponding to C-136 and C-156 are present in irregular fold of domain 'B'. Structural analysis of B domains of fungal amylase and pig pancreatic amylase has indicated similarity with respect to a disulfide bond formed between C-140 and C-150. However, cysteine residues in the B domain of barley α-amylase is found to remain free without participating in disulfide formation. The other conserved residue, equivalent to C-351, is located in the central 'A' domain. Our experimental data using 3D construct of sorghum α-amylase (Figure 7C), suggests that the cysteine residues, exposed at lower concentrations of GuHCl, must be from the domain B. The cysteine residue present in the central A domain is buried and may not be accessible to DTNB at lower concentrations of GuHCl. Unfolding of sorghum α-amylase studied by CD, fluorescence and activity measurements has shown a single transition, indicating that the domains interact strongly during the cooperative unfolding of all the domains. The unfolding of sorghum α-amylase,

which starts from domain B located near the N-terminal end, continues towards the C- terminal.

The refolding of sorghum α-amylase at pH 7.0, but not at pH 4.8, suggests the importance of proper ionization of charged amino acids for the enzyme stability, fold and function. Generally, majority of protein contains Asp, Glu, Arg, and Lys to about a quarter of the total residues. They account for almost half of active site residues while about another fifth are His (Kim et al., 2005). α-Amylases are known to contain two Asp and one Glu in their catalytic site and two His residues in their substrate-binding site, essential for their activity (Ann MacGregor et al., 2001). Electrostatic interactions, among the ionized groups, play a role in stability and functioning of α-amylase. Improper folding of sorghum α-amylase at pH 4.8 may be due to the net charge on the molecule being insufficient to restrict the formation of correct intra- and intermolecular electrostatic interactions.

α-Amylase from bacterial sources requires α-cyclodextrin for proper refolding (Khodarahmi and Yazdanparast, 2004), while *Bacillus licheniformis* α-amylase requires glycerol for its refolding (Strucksberg et al., 2007). α-Cyclodextrin, glycerol and PEG are found to promote refolding through preventing aggregation by interfering with intermolecular hydrophobic interactions (Strucksberg et al., 2007; Karuppiah and Sharma, 1995). Activity recovery of sorghum α-amylase is almost insignificant when renatured in

presence of these folding aids, ruling out the possibility of aggregation due to hydrophobic interactions.

Thermal unfolding of sorghum amylase

Sorghum α-amylase is more resistant to thermal unfolding at pH 7.0 compared to pH 4.8. The irreversibility of thermal unfolding of sorghum α-amylase in buffer A due to aggregation of the enzyme sample can be represented by the following simple model:

$$F \leftrightarrow U \rightarrow I,$$

where F is the native catalytically active enzyme, U is the unfolded enzyme and I is the irreversibly inactivated enzyme, which quite often forms aggregates. Earlier studies on thermal unfolding of α-amylases from various sources have confirmed that unfolding is accompanied by aggregation, where the first reversible unfolding is the rate limiting step (Duy and Fitter, 2005. Addition of denaturants (6 M urea or 2 M GuHCl) is found to prevent aggregation of sorghum α-amylase during thermal unfolding. Chemical denaturants such as GuHCl and urea act as destabilizing agents as well as protect proteins in the unfolded state from aggregation. Hence, thermal unfolded states are more prone to aggregation than chemically induced unfolded states (Strucksberg et al., 2007).

Thermal unfolded states of sorghum α-amylase obtained at 65°C (where aggregation is not observed) in presence and absence of denaturants are not

similar. Thermal unfolded state in absence of denaturants is more compact in comparison with the unfolded states in presence of chemical denaturants. Principally, more compact unfolded states lower the conformational entropy change, ΔS, between the folded and unfolded state and result in the stabilizing effect with respect to thermal unfolding (Fitter and Pohlmeier, 2004).

Bacterial α-amylases from *Bacillus licheniformis* and *Bacillus amyloliquefaciens* (T_m: 86°C and 102°C) lack cysteine residues and disulphide bridges (Fitter, 2005), which prevent them from undergoing oxidation and reduction processes. Presence of free cysteines and disulphide bridges in fungal alpha amylases and free cysteine residues in cereal α-amylases (lack disulfide bridges) make them vulnerable to oxidation and reduction processes.

Many factors such as purity of preparation, presence of calcium as well as substrate and other stabilizers can effect the thermal stability of the α-amylase. The stabilizing effect of substrate can be attributed to the presence of small amounts of Ca^{2+} present as impurity in the starch and this can stabilize conformation of the enzyme in a more rigid and stable form against denaturing conditions (Vihinen and Mantsala, 1989). To determine the activation energy of the reaction catalyzed by α-amylase, an Arrhenius plot has been prepared covering the temperature 63-72°C. The value of E_a, obtained from the slope of the line, is 45.3 ± 0.17 kcal.mol^{-1}. The activation enthalpy (ΔH^*), entropy (ΔS^*) and free energy change (ΔG^*) are calculated to be 44.6 ± 0.01 kcal.mol^{-1}, 57.1 ±

0.25 cal.mol^{-1}.K^{-1} and 25.2 ± 0.24 kcal.mol^{-1}. The values obtained are found to be comparable to ΔH*, ΔS* and ΔG* values (46.1 kcal.mol^{-1}, 55.6 cal.mol^{-1}.K^{-1} and 26.0 kcal.mol^{-1}) of thermophilic bacterium (Hasegawa and Imahori, 1976).

Refolding of the thermally unfolded amylase

Like other α-amylases, even though calcium imparts thermal stability for sorghum α-amylase, the presence of excess calcium in the medium aids the formation of aggregates at higher temperatures (Nielsen et al., 2003; Khajeh et al., 2001). These aggregates can be dissolved by treating the heat inactivated α-amylase with GuHCl, following appropriate dilution to restore ~35% of the original activity. This observation clearly indicates that the major reason for loss of activity of the α-amylase, in the presence of Ca^{2+}, is the formation of non-specific aggregates of thermally unfolded protein. A large number of sequence and structural factors contribute to the greater thermal stability of α-amylase. Stability parameters of different α-amylases are compared in Table 11.

Table 11. Summary of α-amylase characteristics from different sources

Enzyme	$[GuHCl]_{1/2}$	$[Urea]_{1/2}$	T_m(°C)	$\Delta G(H_2O)$ (kJ mol^{-1})
rAMY1[31]	3.6	7.9	75.5	-
AMY2 [31]	3.1	8.2	79	-
TAKA from *Asperzillus oryzae* [30]	0.34	-	-	-
Bacillus licheniformis α-amylase (BLA) [15,41]	0.6	2.4	103	17
BLA with 2 M GuHCl [37]	-	-	~82	-
Bacillus amyloliquefaciens α-amylase (BAA) [15]	0.24	-	86	-
BAA with 2 M GuHCl [37]	-	-	~70	-
Bacillus halmapalus α-amylase (BHA) [39]	-	-	89	-
Alteromonas haloplanctis α-amylase (AHA)[42]	0.8	-	-	10
Porcine pancreatic α-amylase [42]	1.4	-	-	-
	Sorghum α-amylase (present study)			
(i) pH - 4.8 at 27°C	2.3	6.8	-	16.5 & 25.2 (with GuHCl & urea)
(ii) pH - 7.0 at 27°C	3.4	-	-	22
(iii) With 2 M GuHCl	-	-	47.3	-
(iv) With 6M Urea	-	-	49.3	-

In essence, sorghum α-amylase is resistant to the unfolding action of temperature and urea. It is unfolded at relatively low GuHCl concentrations. The unfolding process is also pH dependent. Electrostatic interactions are important for enhanced stability than hydrophobic interactions. Electrostatic interactions among charged groups are crucial for the proper refolding of unfolded α-amylase. Cysteine residues are essential for catalysis. Addition of 15 mM DTT prevents the oxidation of cysteine, during refolding of chemically unfolded enzyme. Presence of two free cysteines in the irregularly folded domain B and their exposure to DTNB at low concentrations of GuHCl indicates that unfolding of amylase starts from domain B in the N-terminus. Aggregation at higher temperatures is due to the oxidation of cysteine and the presence of excess calcium in the medium. These studies on structure function and stability of sorghum α-amylase may lead to the development of enzymes with improved stability and help in understanding the folding and refolding of a multi – domain protein.

Mechanism of inactivation of α-amylase: Role of divalent cations (Ca^{2+}) in stabilization

Earlier reports (Brosnan et al., 1992) have suggested that both non-covalent and covalent interactions play an important role in maintaining the enzyme conformation at higher temperatures. In order to map the contribution of various interactions in the stability of sorghum α-amylase against the unfolding action of temperature, inactivation has been carried out in presence of different additives, namely, NaCl, glycerol, ethylene glycol and metal ions at pH 4.8 (optimum pH for activity) and 70° C, activity T_m for sorghum α-amylase (Kumar et al., 2005). Aggregation of enzyme is not observed in the concentration range (0.3 – 0.5 μM) used for the study.

RESULTS

Role of Non-covalent interactions

Non-covalent interactions include electrostatic interactions, hydrogen bonds and hydrophobic interactions.

Electrostatic interactions

Inorganic salts, at high concentrations, are known to weaken electrostatic interactions in a protein molecule (Tomazic and Klibanov, 1988). The effect of NaCl on the rates of thermoinactivation of sorghum α-amylase has been studied. The half-life of α-amylase at 70°C is found to decrease to 7.9 from 14 ± 1 min in

presence of 0.1 M NaCl (Figure 38A), suggesting that the salt bridges and/or other electrostatic forces play a role in maintaining the enzyme conformation at high temperatures.

Ca^{2+} ions play a very complex role in the thermostability of α-amylases. The effect of added calcium is an additional sign of the effect of electrostatic interactions in the thermo stabilization of this enzyme (Brosnan et al., 1992). The half-life of α-amylase at 70°C becomes 4.6 min, in absence of added Ca^{2+}, compared to native (14 ± 1 min). (Figure 38B)

Hydrogen bonds

The number of potential hydrogen bonds in α-amylase was reduced by chemically acetylating the ε-amino groups of the exposed lysine residues. Modification resulted in the acetylation of 31% amino groups, as determined by a spectrophotometric titration with trinitrobenzene sulfonate. The activity of the acetylated α-amylase was 45% of the activity of the native enzyme. Acetylated α-amylase had a half-life of 7.8 min, compared to 14 ± 1 min of native (Figure 39). Therefore, hydrogen bonds also play an important role in stabilizing sorghum α-amylase at high temperatures.

Enzyme-substrate interactions

The tertiary structure of many enzymes, particularly in the region of active site, is protected at higher temperatures by the substrate. α-Amylase has exhibited approximately 4 fold increase in half-life at 70°C in the presence of 10% maltodextrin (Figure 40). The protective effect of the excess substrate bolsters the

fact that maintaining a tightly coiled conformation and protecting the role of non-covalent interactions, at high temperatures, are vital for stability (Brosnan et al., 1992).

Hydrophobic interactions

Ethylene glycol and glycerol are known to strengthen hydrophobic interactions among non-polar amino acids in a protein molecule (Brosnan et al., 1992). In the presence of 10 % ethylene glycol and glycerol, the half-lives increase substantially to 34.4 and 48.1 min, respectively, as compared to 14 ± 1 min in control (Figure 41), underlining the role of hydrophobic interactions in the conformational stability of sorghum α-amylase.

Role of covalent interactions - Air oxidation of thiols

Spontaneous air oxidation of thiols, known to occur at high temperatures, often leads to the formation of aggregates. The possibility of this reaction contributing to the thermoinactivation of sorghum α-amylase has been investigated by incubating the enzyme in the presence of Cu^{2+}(0.01 M). Copper ions are known to catalyze air oxidation of thiols. It is found that Cu^{2+} greatly reduces the α-amylase activity (Half-life of 7.8 min compared to 14 ± 1 min in absence of Cu^{2+}) (Figure 42A).

Half-life of thermal inactivation increased to 18 min in presence of 15 mM DTT compared to 14 ± 1 min of native enzyme, indicating that cysteine residues are important not only for catalysis but also for maintaining the active conformation of sorghum α-amylase (Figure 42B).

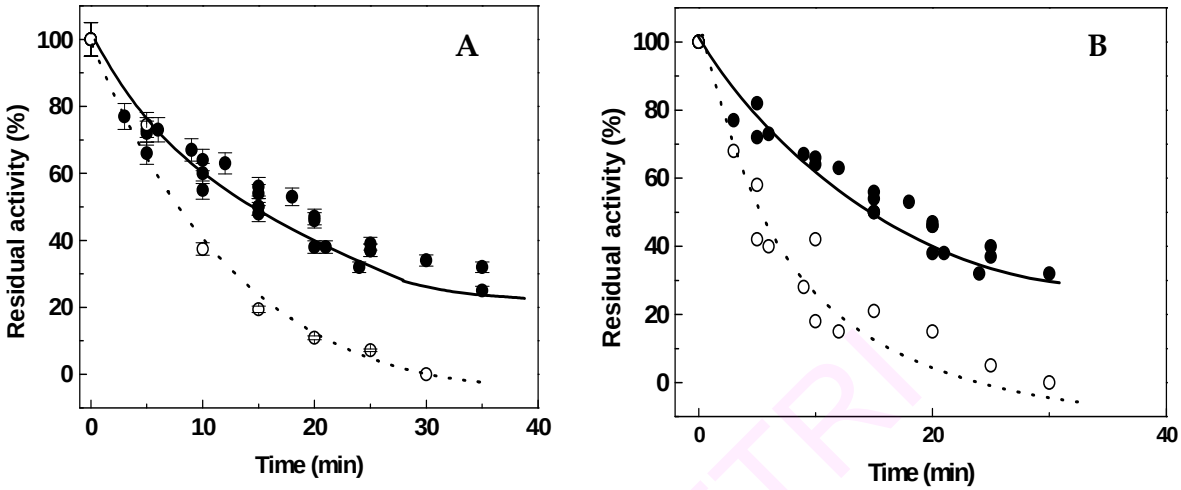


Figure 38. Thermal stability of sorghum α-amylase in NaCl and $CaCl_2$

Thermal stability of α-amylase **(A)** in absence (●) and presence of (○) NaCl at 70°C. **(B)** in absence of added calcium (○) and in presence of (●) $CaCl_2$ at 70°C. Enzyme (~0.4 μM) was incubated at 70°C, aliquots were drawn at different intervals of time, cooled to 4°C and the remaining activity was measured under standard assay conditions.

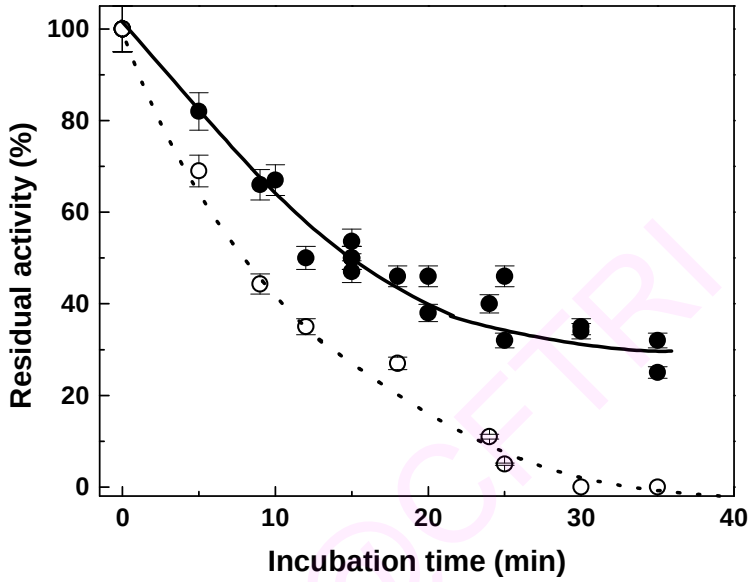


Figure 39. Thermal stability of acetylated sorghum α-amylase

The time course of thermal inactivation of native (●) and acetylated (○) sorghum α-amylase at 70°C. Enzyme (~0.4 μM) was incubated at 70°C, aliquots were drawn at different intervals of time, cooled to 4°C and the remaining activity was measured under standard assay conditions.

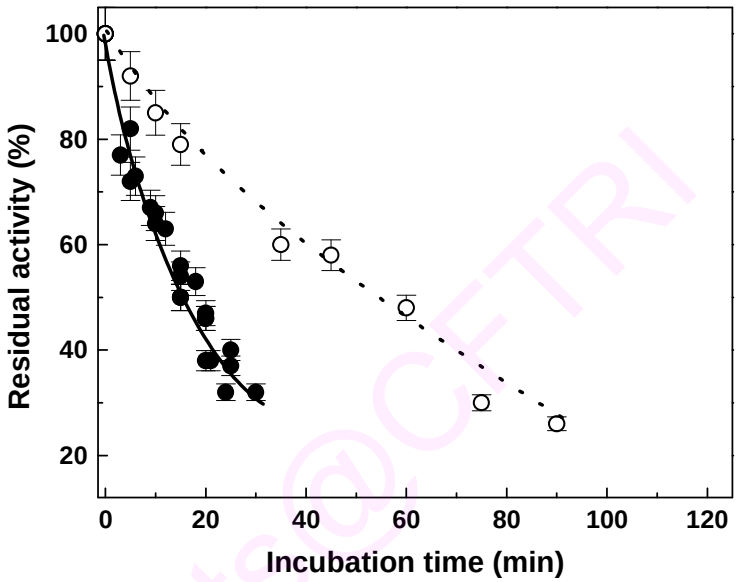


Figure 40. Effect of substrate on the thermal stability of sorghum α-amylase

Enzyme solutions were incubated at 70ºC, pH 4.8 , in absence (●) and in presence of substrate (○) sorghum α-amylase at 70°C. Aliquots were drawn at different intervals of time, cooled to 4ºC and the remaining activity of enzyme was measured under standard assay conditions.

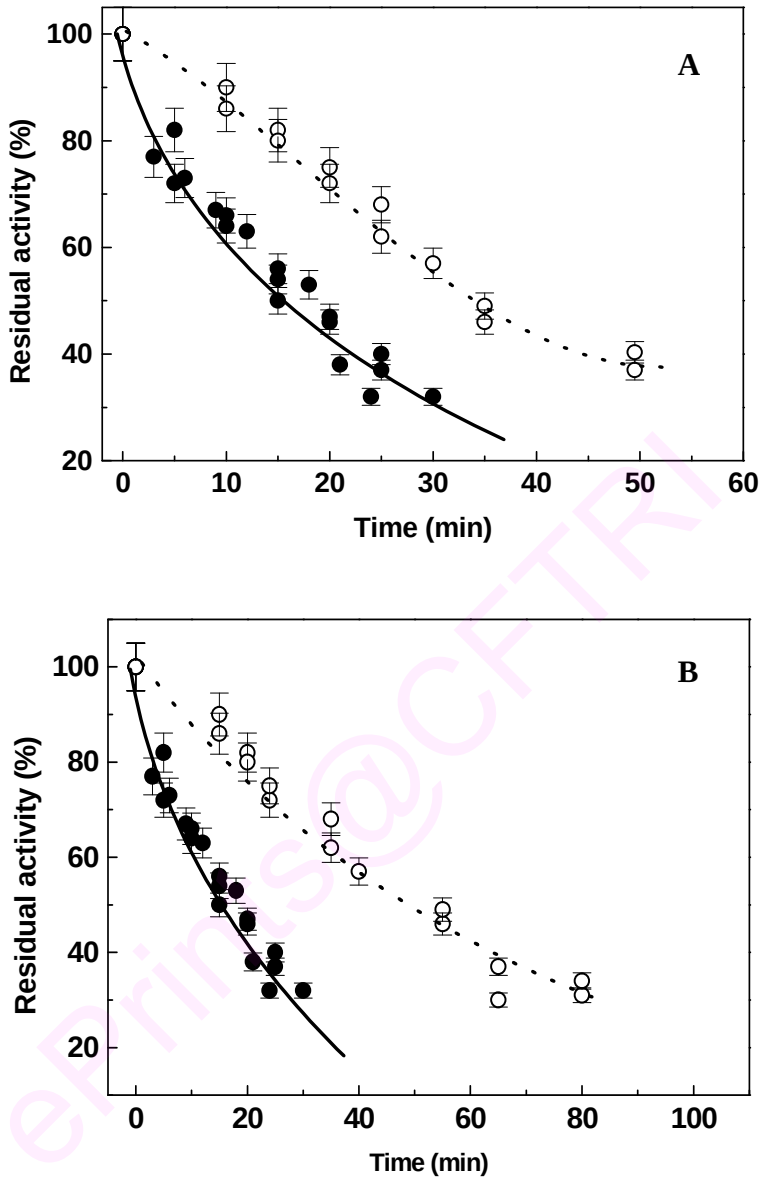


Figure 41. Thermal stability of sorghum α-amylase in presence of additives

Thermal stability of α-amylase **(A)** in absence (●) and presence of (○) ethylene glycol at 70°C. **(B)** in absence (●) and presence of (○) glycerol at 70°C. Enzyme (~0.4 μM) was incubated at 70°C, aliquots were drawn at different intervals of time, cooled to 4°C and the remaining activity was measured under standard assay conditions.

Addition of 15 mM dithiothreitol (reducing agent) prevented aggregation during thermoinactivation. This was demonstrated by non-reducing SDS PAGE (Figure 42C). In contrast, PAGE analysis under native conditions showed a single band corresponding to the same molecular mass, 47 kDa, as the native protein (Figure 42D). Hence, hydrolysis of the polypeptide chain could not be a factor in the mechanism of thermal inactivation.

Thermal unfolding of α-amylase

The temperature induced unfolding of sorghum α-amylase, in Hepes buffer, has been monitored by recording the molar ellipticity at 222 nm in absence and presence of Ca^{2+} and EDTA. The experimental curves are shown in Figure 43. It is more rather difficult to determine the denaturation temperature of sorghum α-amylase in presence of added Ca^{2+}, since the protein is not completely unfolded at the highest experimental temperature. The midpoint of ellipticity change, T_m at 222 nm, occurred at 73.3°C in absence of added Ca^{2+} and at 52.1°C, in presence of 1 mM EDTA (Figure 43). Thermal unfolding profiles were found to be irreversible for sorghum α-amylase, since the reheating of the cooled enzyme failed to give a superimposable melting profile.

The thermal transition curves for the enzyme in absence of added Ca^{2+} and in presence of 1 mM EDTA revealed only a small loss in secondary structure even at high temperatures. The molar ellipticity is not zero: $[\theta]_{222}$ = -3010 deg. cm^2. $dmol^{-1}$ and -2680 at 85 °C, respectively.

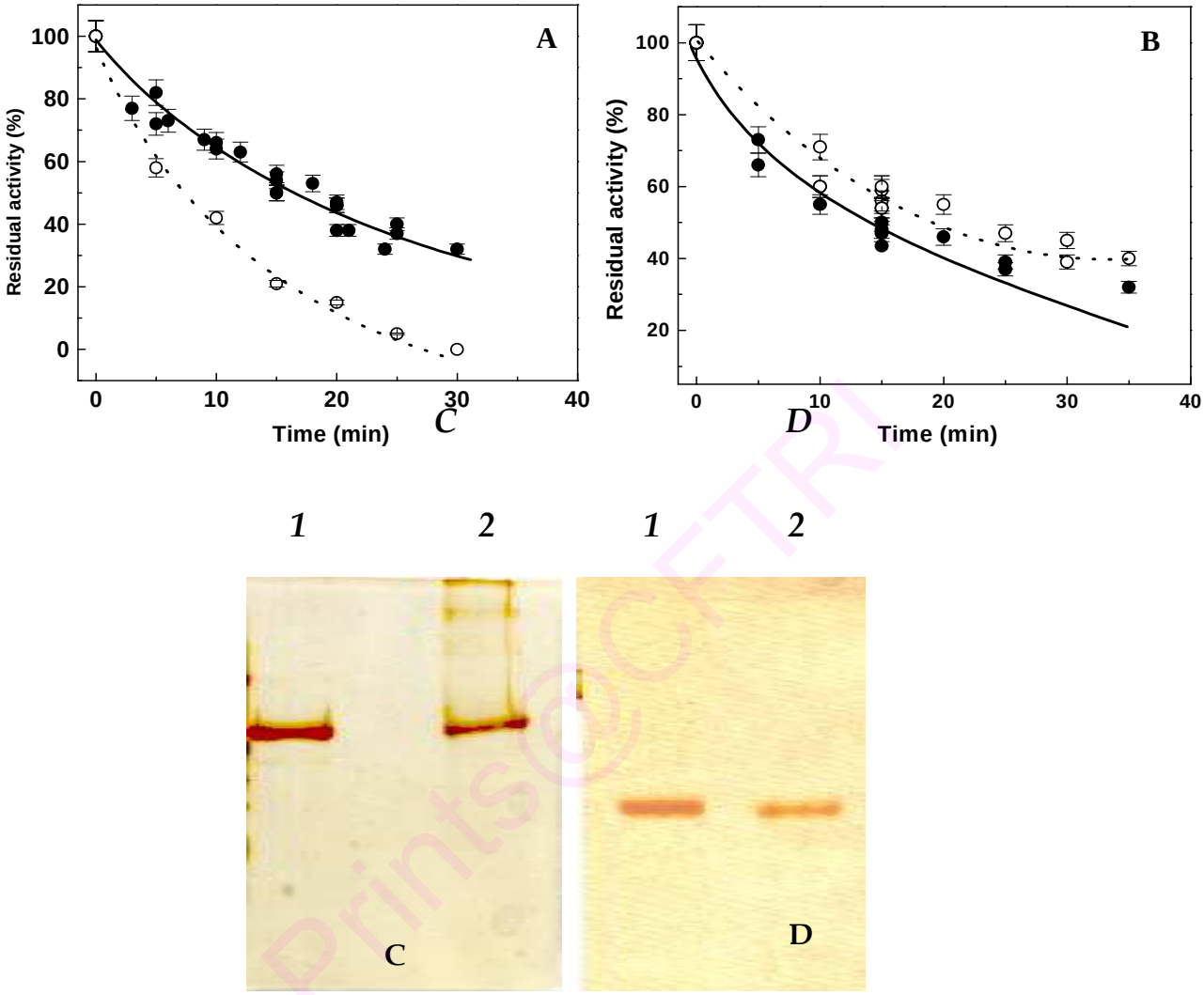


Figure 42. Air-oxidation of thiols

(A) and (B) Loss of enzymatic activity upon heating. Thermal stability of α-amylase **(A)** in absence (●) and presence of (○) Cu^{2+} at 70°C. **(B)** in absence (●) and presence of (○) DTT at 70°C. Aliquots were drawn at different intervals of time, cooled to 4°C and the remaining activity of enzyme was measured under standard assay conditions.

(**C**) Non-reducing SDS and (**D**) native PAGE pattern of heat inactivated α-amylase in presence (1) and absence (2) of 15 mM DTT.

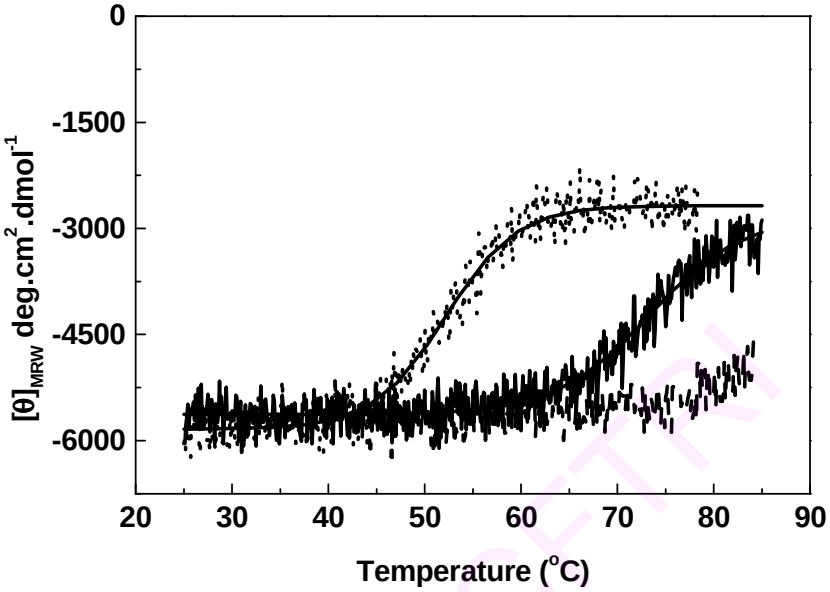


Figure 43. Thermal unfolding of α-amylase

CD curves for thermal unfolding of α-amylase in presence of 1 mM EDTA (*dotted curve*) and 1 mM Ca^{2+} urea (*dashed curve*). Amylase in absence of added Ca^{2+} was represented by (*solid curve*). Best fits for each curve were shown by solid lines.

This result indicated the presence of residual secondary structure even though the most part of the polypeptide chain is unordered.

Metal content of sorghum α-amylase

Sorghum α-amylase contained 0.8 moles of calcium per mole of protein even after extensive dialysis against 50mM Hepes buffer, pH 7.0. This enzyme retained > 80% of activity compared to native enzyme (13.6 mM calcium). The remaining calcium (0.8 moles of calcium per mole of protein) could be removed by 1 mM EDTA resulting in total loss of activity. The calcium content in the samples treated with EDTA was found to be less than 0.02 mol per mol of protein.

The effect of Ca^{2+} on the structure of amylase

Structure, function and stability relationships are interlinked. The loss of enzymatic activity upon calcium removal suggests a role for calcium in amylase activity and therefore in maintaining active conformation of the molecule. The effect of calcium removal on the secondary and tertiary structure of the enzyme observed, suggests a structural role for Ca^{2+} in the amylase molecule.

Information about secondary structure has been obtained from CD spectra of α-amylase. This enzyme has a molar ellipticity: $[\theta]_{222}$ = -5674 deg. cm^2. $dmol^{-1}$ in presence of 13.6 mM calcium and in absence of added calcium, revealed a small loss in secondary structure: $[\theta]_{222}$ = -4509 deg. cm^2. $dmol^{-1}$. α-Amylase in presence of 1 mM EDTA has a molar ellipticity of ~3659 deg. cm^2. $dmol^{-1}$. It is found that α-amylase did not regain its native structure even after addition of

excess calcium (13.6 mM) to the EDTA treated sample ($[\theta]_{222}$ = -3375 deg. cm^2. $dmol^{-1}$) suggesting the irreversible loss of native secondary structure. Effect of Ca^{2+} on secondary structure of α-amylase is shown in Figure 44.

The effect of Ca^{2+} on the tertiary structure of amylase was obtained from fluorescence spectra of the enzyme. Ca^{2+} depletion induced changes in the tryptophan fluorescence spectra of α-amylase. Native enzyme with 13.6 mM Ca^{2+} exhibited emission maximum at 346 nm, indicating that the tryptophan residues are in a fairly exposed environment. Though the enzyme in buffer did not show any change in the emission maxima, fluorescence intensity was reduced, compared with Ca^{2+} containing amylase (Figure 7B), indicating that calcium removal causes changes in the environment of tryptophan residues. Fluorescence intensity of this enzyme (in absence of added calcium) was further reduced with slight red shift of 2 nm in presence of 1 mM EDTA (Figure 45). In contrast to the secondary structure, EDTA treated enzyme partially regained its tertiary structure, upon addition of calcium (13.6 mM).

The susceptibility of amylase to proteolytic degradation by trypsin indicates a change in the stability following Ca^{2+} removal. Incubation of Ca^{2+}depleted amylase for 1 h at 27°C in the presence of trypsin resulted in significant degradation of the enzyme as shown by the disappearance of the 47 kDa protein and the appearance of low molecular mass fragments on SDS-PAGE (Figure 46). Incubation of the native enzyme and the enzyme in absence of added

calcium under identical conditions, however, showed much less amylase degradation.

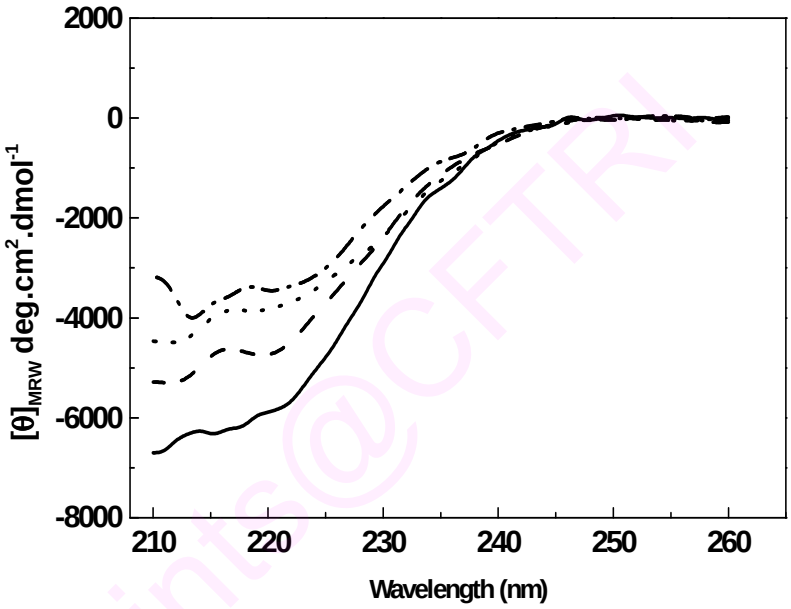


Figure 44. Effect of Ca^{2+} removal on the secondary structure of sorghum α-amylase followed by spectroscopic methods.

(**A**) CD spectra and of native protein (*solid line*), enzyme in absence of any added Ca^{2+} (*dashed line*), unfolded with 1 mM EDTA (*dotted line*), and Ca^{2+} added amylase after EDTA unfolding (*dashed and dotted line*)

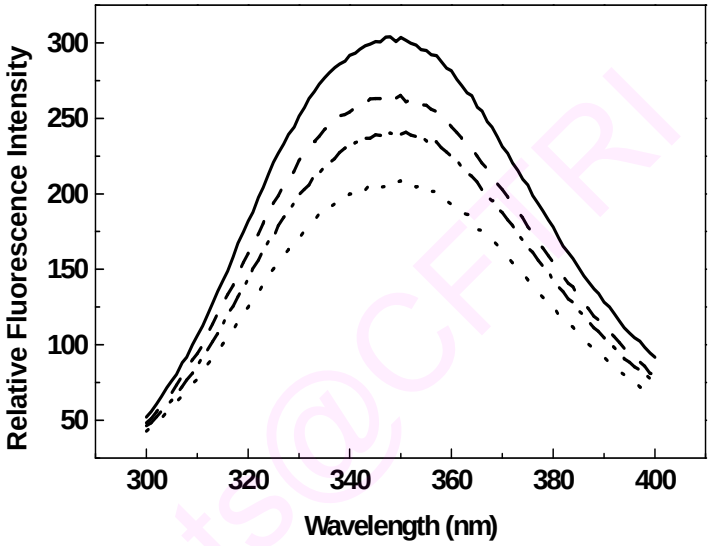


Figure 45. Effect of Ca^{2+} removal on the tertiary structure of sorghum α-amylase followed by spectroscopic methods.

(**B**) Intrinsic fluorescence spectra of native protein (*solid line*), enzyme in absence of any added Ca^{2+} (*dashed line*), unfolded with 1 mM EDTA (*dotted line*), and Ca^{2+} added amylase after EDTA unfolding (*dashed and dotted line*)

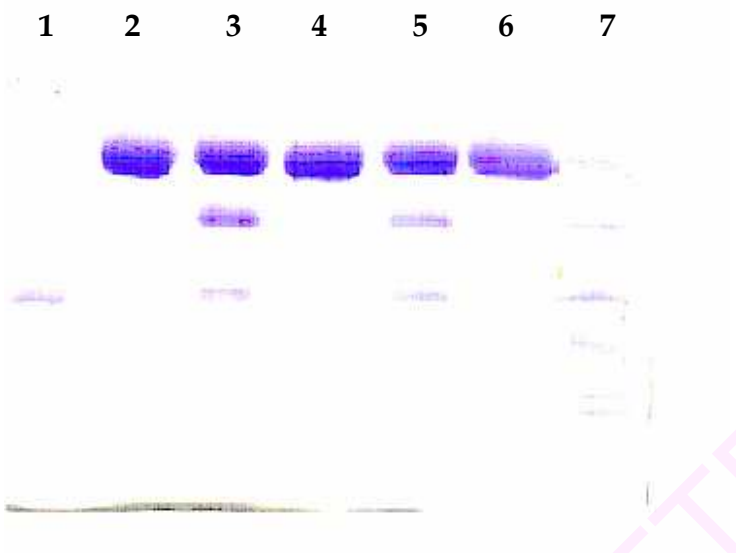


Figure 46. The effect of Ca^{2+} removal from amylase on its stability and its susceptibility to proteolytic degradation by trypsin. Amylase was incubated in 13.6 mM (lane 2**)** and in absence of added Ca^{2+} (lane 4**)** for 1 h in the presence (lane 3 and 5) and absence (lane 2 and 4) of trypsin at 27°C. Lane 7 represents amylase incubated with trypsin in presence of 1 mM EDTA. The products of the reaction were then analyzed by SDS-PAGE. Equal amounts of protein were added to each lane. Trypsin alone was added in lane 1. Lane 6 represents amylase incubated in presence of EDTA.

Calcium requirement for the renaturation of sorghum α-amylase

The enzyme, in absence of added Ca^{2+}, can be unfolded completely by incubating for 4 h with 4 M GuHCl, in Hepes buffer (pH 7.0). The enzyme refolded on diluting 25 times with Hepes buffer, containing 5 mM Ca^{2+} and 15 mM DTT at 27°C, resulting in the recovery of ~85 % of the activity. However, amylase activity recovery was insignificant when allowed to refold in absence of Ca^{2+}.

EDTA inactivation and renaturation of amylase

α-Amylase activity was lost in presence of 1 mM EDTA as shown in Figure 9. After 30 min of incubation, 5 mM Ca^{2+} was added and the reaction mixture was allowed to stand at 27°C. Calcium accelerated the reactivation and about 50 % of the original activity was regained after 2h.

Effect of other metal ions on renaturation

Incubation of α-amylase, in presence of 1 mM EDTA, for 30 min resulted in complete loss of activity. Various divalent cations (Barium, Magnesium, Zinc and Strontium) at 5 mM concentration were added to the EDTA-amylase mixture and allowed to stand for 2 h prior to measuring enzymatic activity. The addition of Ba^{2+} resulted in the recovery of activity similar to Ca^{2+} (Figure 47). On the other hand, Zn^{2+}, Mg^{2+} and Sr^{2+} were not effective.

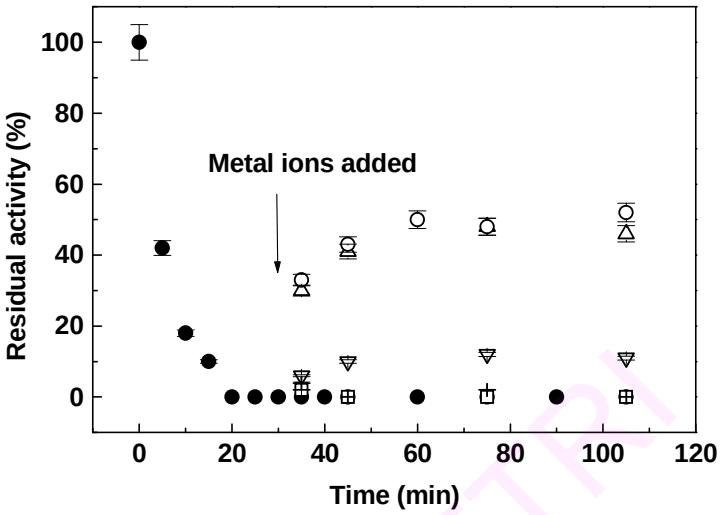


Figure 47. The effect of metal ions in the renaturation of sorghum amylase

Sorghum amylase was incubated in presence of EDTA at pH 7.0 and 27°C. After complete inactivation of amylase, at the point indicated by arrow, metal ions were added to follow renaturation.

-●- Amylase (in absence of added Ca^{2+}) inactivation with EDTA

-○- Activity recovery by addition of Ca^{2+}

-Δ- Activity recovery by addition of Ba^{2+}

$^{-\nabla-}$ Activity recovery by addition of Mg^{2+}

-□- Activity recovery by addition of Zn^{2+}

$^{-+-}$ Activity recovery by addition of Sr^{2+}

Thiol reactivity during denaturation and renaturation

In the native state, 1.2 moles of cysteine residues were accessible to DTNB. When α-amylase is treated with 1 mM EDTA, the number of cysteine residues assayed are 2.9, suggesting that the buried thiol groups in α-amylase are exposed and accessible to DTNB. Number of available thiols to DTNB is reduced by the addition of Ca^{2+} to the EDTA treated amylase.

Sorghum amylase retains ~ 70 % of activity when its cysteine residues are blocked by iodoacetamide, in absence of EDTA. This was considered as initial activity (100%). Thiol groups, when blocked with iodoacetamide (0.5 mM) in presence of 1 mM EDTA became unavailable to DTNB**.** A small amount of thiol group (0.1 mole/mole of protein) was estimated for the iodoacetamide-treated amylase after incubation with 1 mM EDTA. The enzymatic activity regained after addition of Ca^{2+} is found to be insignificant (~ 8%) for the enzyme whose cysteine residues are blocked in presence of 1 mM EDTA (Figure 48).

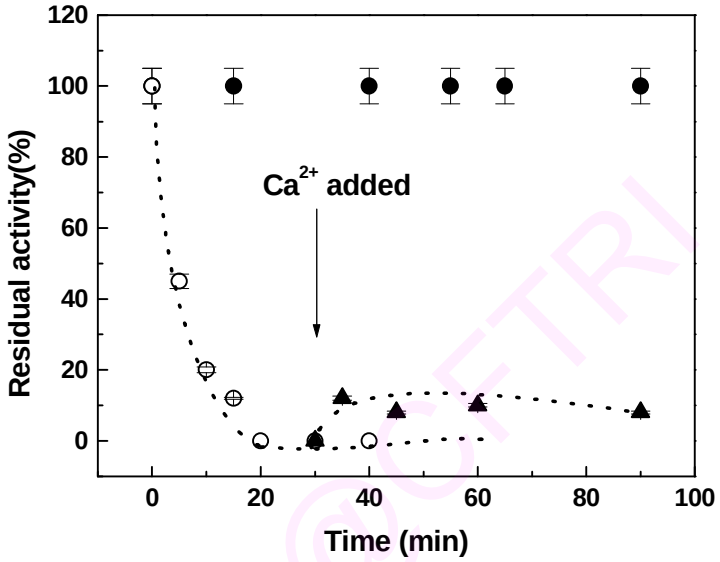


Figure 48. Essentiality of cysteine residues for the catalytic activity of sorghum amylase.

Sorghum amylase (~0.4 μM) was incubated with iodoacetamide in presence of EDTA (1 mM) at pH 7.0 and 27°C. After complete inactivation of amylase, at the point indicated by arrow, $CaCl_2$ was added to follow renaturation.

-●- Amylase (in absence of added Ca^{2+}) activity

-○- Amylase incubation with Iodoacetamide and EDTA

-▲- Activity recovery by addition of Ca^{2+}

DISCUSSION

Recent advances in protein engineering have often been directed at obtaining more stable enzymes suitable for industrial use under demanding conditions. The understanding of how exactly stability is achieved through metal binding may very likely aid the efforts to design new improved enzymes. The present investigation has been attempted to clarify whether calcium plays a role in the stability of sorghum α-amylase and to study the effect of calcium on the structure and stability of the enzyme.

Thermal stability of sorghum α-amylase

Non-covalent interactions involved in stability are electrostatic interactions, hydrogen bonding and hydrophobic interactions (Table 12). Covalent mechanisms that trigger the instability of enzyme include oxidation of cysteine, often leading to the formation of aggregates, can be prevented by the addition of 15 mM DTT during heating. Fungal and cereal α-amylases are less stable compared to bacterial amylases. Amylases are unstable at higher temperatures either due to the oxidation of cysteine residues or disruption of the disulphide bridges.

Bacterial α-amylases from *Bacillus licheniformis* and *Bacillus amyloliquefaciens* (Tm: 86°C or 102°C) lack cysteine residues and disulphide bridges (Fitter, 2005), which prevent them from undergoing oxidation and reduction processes. Presence of free cysteines and disulphide bridges in fungal

alpha amylases and free cysteine residues in cereal α-amylases (lack disulfide bridges) make them vulnerable to oxidation and reduction processes.

The effect of Ca^{2+} ions, on the thermal stability of sorghum amylase, was elucidated by performing thermal unfolding by CD in the presence of EDTA as well as added calcium. Addition of 1 mM EDTA resulted in considerable reduction in thermal stability, whereas addition of 1 mM Ca^{2+} enhanced the thermal stability of sorghum amylase (Figure 31). Similar observations were found in other amylases also (Hasim et al., 2005; Nielsen et al., 2003). Calcium could confer additional stability probably through the formation of additional salt bridges between the domains of the enzyme (Boel et al., 1990).

It has been reported that the irreversible thermal unfolding nature exhibited by amylases involve a two-step process, with the first step of reversible unfolding, followed by an irreversible conformational change (Duy and Fitter, 2005). Calcium ions have a role in the thermal inactivation mechanisms of the α-amylases. It is proposed that the irreversible denaturation at high temperatures follows the reversible dissociation of calcium ions from the native enzyme (Tanaka and Hashino, 2002). It is observed that some secondary structural elements are retained in the thermally unfolded sorghum α-amylase . In general, thermally unfolded proteins remain much more compact compared to proteins unfolded by chemical denaturants (Strucksberg et al., 2007).

Table 12. Interactions involved in the stability of α-amylase against thermal unfolding

Enzyme + reagent	Half life of thermal inactivation (min)	Interactions involved
α-amylase	14	
α-amylase + NaCl	7.9	Electrostatic
Acetylated amylase	7.8	H-bonding
α-amylase+ Ethylene glycol	34.1	Hydrophobic
α-amylase+ glycerol	48.1	Hydrophobic
α-amylase+ Cu^{2+}	7	Thiol oxidation
α-amylase+ DTT	18	**Prevention of thiol oxidation**

The Ca^{2+} content of sorghum α-amylase

The requirement for calcium to maintain structural integrity is a characteristic feature of α-amylases. Removal of calcium leads to decreased thermostability and enzymatic activity (Violet and Meunier, 1989) and increased susceptibility to proteolytic degradation (Bush et al., 1989). α-Amylase, isolated from various sources, has been shown to contain a few atoms of firmly bound calcium which are involved in the maintenance of the catalytically active conformation of the amylase molecule (Vihinen and Mantsala, 1989; Vallee et al., 1959). Calcium content of sorghum α-amylase is determined to be 0.8 mole of Ca^{2+}/ mole of protein. This intrinsic calcium can not be removed from the enzyme even after extensive dialysis against buffer. However, it can be removed by dialyzing the enzyme against Hepes buffer (pH 7.0, 50 mM) containing 1 mM EDTA, but with a total loss of enzymatic activity, signifying the role of calcium. Inhibition of amylases from other various sources, by metal chelating agents has been reported (Bush et al., 1989). Enzymatic activity of sorghum amylase can be recovered up to 50%, for the EDTA inhibited amylase by the addition of 5 mM Ca^{2+}. The role of Ca^{2+} in the catalysis process is emphasized by its ability to neutralize the action of EDTA and to restore amylolytic activity. Barley amylase is irreversibly inactivated when treated with EDTA, while the addition of calcium did not yield active enzyme (Bush et al., 1989). The removal of calcium alone does not cause the irreversible denaturation of the protein molecule. Human salivary and *Basillus subtilis* α-amylase, incubated in 10 mM EDTA, can

be reactivated by the addition of 20 mM Ca^{2+}(Vallee et al., 1959). Improper conditions during the exposure to EDTA leading to the disruption of the secondary and tertiary structure of the metal free protein or degradation to a proteolytic activity results in the irreversible loss of activity (Stein and Fisher, 1958).

The structural role of Ca^{2+}

The loss of sorghum amylase activity, upon removal of calcium, suggests that Ca^{2+} is important for maintenance of the structure of the active site. The reduction in activity of sorghum amylase, corresponding to the simultaneous calcium depletion, may not be attributed to changes in primary structure, since the molecular weight of the Ca^{2+} removed form is the same as that of the native enzyme (Figure 46). Changes in the structure of amylase, followed by calcium removal, are indicated not only by altered enzymatic activity but also by increased susceptibility to proteolytic degradation (Figure 46) and change in the CD (Figure 45) and fluorescence spectrum (Figure 45). These data indicate that Ca^{2+} is responsible for maintaining structural features as well as the activity of the sorghum amylase. It is suggested by Buisson *et al*., that in PPA, Ca^{2+} holds β-sheet regions together (one in domain A and one in domain B), thereby creating and stabilizing the active site (Buisson et al, 1987).

Metal ion induced renaturation

Sorghum α-amylase can be taken as a conjugated protein (metallo enzyme) which contains Ca^{2+} as a cofactor. It is clarified from the refolding

studies that Ca^{2+} is necessary for the correct refolding of the unfolded enzyme. It is also observed in other amylases that Ca^{2+} is crucial for the proper refolding of unfolded enzyme (Takagi and Isemura, 1965; Nazmi et al., 2006). Reportedly, calcium either stabilizes the native protein form through a binding process or acts as folding cofactor by lowering the energy barrier that blocks the conversion of the transition form to its native state (Haddaoui et al., 1997).

Activity recovery of the EDTA inactivated sorghum α-amylase in presence of barium was similar to that of calcium. Metal ions, such as Mg^{2+}, Zn^{2+}, Sr^{2+}, are not effective refolding aids. *Bacillus subtilis* exocellular α-amylase could be reversibly refolded by calcium or barium or strontium but not by magnesium (Haddaoui et al., 1997). Failure to recover activity in presence of certain metal ions might be due to non-specific electrostatic screening effect exhibited by these metal ions on the surface of the protein (Hasim et al., 2005).

Role of thiol groups

The masking of SH groups by Ca^{2+} seems to be a characteristic feature of thiol containing α-amylases (Pommier et al., 1974). The complete blocking of thiol groups, after unmasking, results in total loss of activity of PPA and Taka amylase A. Although the thiol groups do not seem to be a part of active site, their modification has resulted in the loss of activity in both the amylases (Toda et al., 1968). Similar results have also been observed in sorghum α-amylase. α-Amylase fails to regain its activity when cysteine residues are blocked with iodoacetamide, in presence of EDTA, suggesting that cysteine residues in

sorghum α- amylase are essential for biological activity. Since major part of thiol groups are exposed in the presence of EDTA and their modification causes alteration of activity, it can be concluded that thiol groups help to stabilize the native amylase form by chelating with Ca^{2+}. Results of our experiments suggests that, Ca^{2+} appears to be essential for proper refolding, conformational stability and therefore for activity.

SUMMARY AND CONCLUSIONS

SUMMARY AND CONCLUSIONS

The salient features of the present investigation are as follows

- α-Amylase activity is present in trace amounts in ungerminated sorghum but increases greatly during germination (~10 times)
- Amylase from malted sorghum, found to be the thermostable with a T_m of 70 ± 0.5 °C compared to barley (*T*m = 57 ± 0.6 °C) and ragi amylase (*T*m = 67 ± 0.3°C). Malted sorghum could become a commercially viable source of α-amylase for viscosity reduction and to increase the nutrient density of supplementary foods. The thermal stability of the enzyme could be an added advantage.
- Bacterial (from *Bacillus licheniformis*) and plant α-amylases did not bind to the alginate under experimental conditions. However, it was possible to entrap the fungal α-amylase from *Asperzillus oryzae*. The binding of enzymes to alginate is reported to be critically dependent on the pH.
    - Entrapment of α-amylase from *A. oryzae* in calcium alginate beads is simple and easy to scale-up.
    - Alginate bound α-amylase was eluted with buffer containing 0.5M NaCl and 0.2mM $CaCl_2$, this way the protein was purified to homogeneity and ascertained by SDS-PAGE.
    - There was no change in the substrate affinity for the entrapped enzyme compared to free enzyme. The V_{max} for the free enzyme

was 1764 U mg^{-1} and this was reduced to 140 U mg^{-1} for entrapped enzyme. This indicates that entrapment of the enzyme results in decreased mobility of the protein molecule thereby affecting product turnover.

- ❖ The enzyme had better thermal stability when entrapped in the beads. Entrapment affects the enzyme characteristics like change in the pH and temperature optimum of the enzyme.
- ❖ The enzyme entrapped in alginate beads could be reused and retained ~70% activity at the end of six cycles.

➢ As sorghum α-amylase failed to bind alginate matrix, it was subjected to conventional chromatography for purification of the protein. Sorghum α-amylase was purified to homogeneity with a fold purity of 25 times with a yield of 17%.

- ❖ The apparent Michaelis constant (K_m) and V_{max} for sorghum α-amylase were determined for gelatinized starch to be 1.55% and 6535 μmol/min/mg of protein, respectively.
- ❖ The enzyme was active over a wide pH range with a pH optimum between 4.5 and 5.0, similar to other cereal amylases and a temperature optimum of 60 ± 0.5 °C.
- ❖ Products of enzymatic hydrolysis of maltodextrin were maltose, glucose and maltotriose.

- ❖ The enzyme exhibited intrinsic fluorescence when excited at 280 nm. The enzyme exhibited emission maximum at 346 nm indicating that the tryptophan residues were in a exposed environment.
- ❖ CD data analysis suggested that the enzyme has 19% helix, 60% β structure and aperiodic structure 21%.

- ➢ The unfolding of sorghum α-amylase, by GuHCl and urea, exhibited a superimposable profile for activity, secondary structure (molar ellipticity in the far UV region) and tertiary (fluorescence) structure suggesting that the unfolding was a two-state process ($F \leftrightarrow U$)
  - ❖ Sorghum α-amylase was stable against the unfolding action of urea but not against GuHCl at both the pH (4.8 and 7.0) studied. Denaturant concentrations at half-completion of the transition were as follows:
  - ❖ pH 4.8 - $[urea]_{1/2}$ = 6.8 M; $[GuHCl]_{1/2}$ = 2.3 M
  - ❖ pH 7.0 - $[GuHCl]_{1/2}$ = 3.4 M; the protein does not unfold in urea at pH 7.0
- ➢ The denaturant concentration for half-completion of the reaction at pH 4.8, in presence of NaCl, was $[urea + 1\ M\ NaCl]_{1/2,}$ = 3.9 compared to $[urea]_{1/2}$ = 6.8 M in absence of NaCl. At pH 7.0, $[urea + 1M\ NaCl]_{1/2}$ = 6.4 M suggesting the dependence of conformational stability of sorghum α-amylase on the ionic strength of the aqueous solution, specifying the role of electrostatic interactions.

- The refolding of sorghum α-amylase at pH 7.0, in presence of DTT, indicated the pivotal role of free thiol groups in the refolding process.
- The enzyme retains its native conformation in the pH range 4.8 - 8. Beyond this pH range (below 3.0 or above pH 10.0), it is unfolded with loss of both structure and activity. The mid-point of transition for the pH induced unfolded states ($pH_{1/2}$) is 4.1 and 8.9.
- The conformational stability indicators such as the change in excess heat capacity (ΔC_p), unfolding enthalpy (H_g) and temperature at $\Delta G = 0$ (T_g) are:
    - pH 4.8 - ΔC_p = 17.9 ± 0.7 kJ mol^{-1} K^{-1}, H_g = 501.2 ± 18.2 kJ mol^{-1} and T_g = 337.3 ± 6.9 and
    - pH 7.0 - ΔC_p = 14.3 ± 0.5 kJ mol^{-1} K^{-1}, H_g = 509.3 ± 21.7 kJ mol^{-1} and T_g = 345.4 ± 4.8
- Multiple sequence alignment of cereal α-amylases has revealed a high degree of homology, wherein; cysteine residues are highly conserved at positions 136, 156 and 351. Cysteine residues corresponding to C-136 and C-156 are present in irregular fold of domain 'B'. The other conserved residue equivalent to C-351 is located in the central 'A' domain.
- The 3D construct of sorghum α-amylase suggests that the cysteine residues, exposed at lower concentrations of GuHCl, must be from domain B. The cysteine residue present in central A domain is buried and may not be accessible to DTNB at lower concentrations of GuHCl. Unfolding of sorghum α-amylase studied by CD, fluorescence and activity

measurements, has shown a single transition, indicating that the domains interact strongly during the cooperative unfolding of all the domains.

- The unfolding of sorghum α-amylase, which starts from domain B located near the N-terminal end, continues towards the C- terminal.
- The $T_{m,}$ the mid point of thermal inactivation of α-amylase decreased to 66.3 ± 0.3, 58.1 ± 0.2, and 48.1 ± 0.5 °C in the presence of 0.1, 0.5, and 1 M NaCl, respectively. Sucrose was a stabilizer of α-amylase. The T_m in the presence of 1 M sucrose shifted from 69.6 ± 0.3 to 77.3 ± 0.3 °C.
- Mechanism of the thermoinactivation can be characterized by the simple model: $N \leftrightarrow U \rightarrow I$
- Non-covalent interactions contributing for the thermal stability of sorghum α-amylase are electrostatic interactions, hydrogen bonding and hydrophobic interactions.
- Covalent mechanisms that contribute to the inactivation of enzyme include oxidation of cysteine, which often leads to the formation of aggregates. This can be prevented by the addition of 15 mM DTT during thermal unfolding.
- Calcium content of sorghum α-amylase is 0.8 mole of Ca^{2+}/ mole of protein.
- Removal of calcium leads to decreased thermo stability and enzymatic activity, and increased susceptibility to proteolytic degradation.

- Addition of 1 mM EDTA results in reduction in thermal stability, and addition of 1 mM Ca^{2+} enhanced the thermal stability of sorghum amylase.
- The loss of sorghum amylase activity upon removal of calcium suggested that Ca^{2+} was important for maintenance of the structure of the active site.
- Activity recovery of the EDTA inactivated sorghum α-amylase in presence of barium, is similar to that of calcium. Metal ions such as Mg^{2+}, Zn^{2+}, Sr^{2+} were not effective.
- α- Amylase failed to regain its activity when cysteine residues were blocked with iodoacetamide in presence of EDTA, suggesting that cysteine residues in sorghum α- amylase are essential for activity.

These studies on structure, function and stability of sorghum α-amylase could lead to the development of enzymes with improved stability and help in understanding the folding and refolding of a multi – domain protein.

BIBLIOGRAPHY

BIBLIOGRAPHY

1. Acevedo, E. and Cardmelli, L. Regulation of α-amylase isoenzymes expression in *Araucaria araucona* by giberelic acid abscisic acids. Phytochemistry. 1997, 44, 1401-1405.
2. Adebiyi, A. O, Adebiyi, A. P and Olaniyi, E.O. Nutritional composition of *sorghum* bicolor starch hydrolyzed with amylase from *Rhizopus* sp. African Journal of Biotech. 2005, 4 (10), 1089-1094.
3. Adelaide Beleia, E. and Varriano-Marston. Pearl millet amylases. I. Properties of partially purified α-amylase. Cereal Chem. 1981, 58 (*5*), 433-437.
4. Aiyer, V.P. Amylases and their applications. African Journal of Biotech. 2005, 4 (13), 1525-1529.
5. Aksoy, S., Tumturk, H. and Hasirci, N. Stability of alpha-amylase immobilized on poly(methyl methacrylate–acrylic acid) microspheres. J Biotechnol. 1998, 60, 37–46.
6. Andrew, J. S. and Bailey, C. H. The amylases of normal and germinated wheat flours. Cereal Chem. 1934, 11, 551-556.
7. Ann MacGregor, E., Janecek, S. and Svensson, B. Relationship of sequence and structure to specificity in the α-amylase family of enzymes. Biochim.Biophys.Acta. 2001, 1546, 1 – 20.

8. Babson, A. L., Tenney, S. A. and Megraw, R. E. New Amylase Substrate and Assay Procedure. Clinical Chem. 1970, 16, 39-43.

9. Baker, J. L. and Hulton, H. F. E. Amylases of the cereal grains-rye. J. Chem. Soc. 1921, 119, 805-809.

10. Bernfeld, P., Colowick, S. P. and Kalpan, N. O. Amylases α and β. Methods Enzymol. 1962, 1, 149-158.

11. Bhuyan, A. K. Protein stabilization by urea and guanidine hydrochloride. Biochem. 2002, 41, 13386 – 13394.

12. Bidlingmeyer, B .A., Cohen, S. A. and Tarvin, T. L. Rapid analysis of amino acids using pre-column derivatization. J.Chromatogr. 1984, 336, 93 -104.

13. Blum, H., Beier, H and Gross, H. J. Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. Electrophoresis. 1987, 8, 93–7.

14. Boel, E., Brady, L., Brzozowwski, A.M., Derewanda, Z., Dodson, G.G., Jensen, V.J., Petersen, S.B., Swift, H., Thim, L. and Woldike, H.F. Calcium binding in α-amylases: An X-ray diffraction study at 2.1-A° resolution of two enzymes from *Asperzillus*. Biochem. 1990, 29, 6244–6249.

15. Borchert, T.V., Lassen, S.F., Svendsen, A. And Frantzen, H. B. Oxidation stable amylases for detergents, in: S.B. Petersen, B. Svensson, S. Pedersen (Eds.), Carbohydrate Bioengineering, Elsevier Science, Amsterdam, 1995, 175-179.

16. Botes, D. P., Joubert, F. J. and Novellie, L. Kaffircorn malting and brewing studies XVII. Purification and properties of sorghum malt α-amylase. J. Sci. Food Agric. 1967, 18, 409-415.

17. Brady, R.L., Brzozowski, A.M., Derewenda, Z.S., Dodson, E.J. and Dodson G.G. Solution of the structure of *Aspergillus niger* acid α-amylase by combined molecular replacement and multiple isomorphous replacement methods, Acta Cryst. B .1991, 47, 527-535

18. Brayer, G.D., Luo, Y. and Withers S.G. The structure of human pancreatic α-amylase at 1.8 A° resolution and comparisons with related enzymes, Protein Sci. 1995, 4, 1730-1742.

19. Brena B.M., Pazos C., Franco-Fraguas, L. andBatista-Viera F. Chromatographic methods for amylases. Journal of Chromatography B: Biomedical Sciences and Applications. 1996, 684,217-237.

20. Brosnan, M. P., Kelly, C. T. and Fogarthy, W. M. Investigation of the mechanisms of irreversible thermoinactivation of *Bacillus sterothermophilus* α-amylase. Eur. J. Biochem. 1992, 2*03*, 225-231.

21. Buckow, R., Weiss, U., Heinz, V. and Knorr D. Stability and catalytic activity of alpha-amylase from barley malt at different pressure-temperature conditions. Biotechnol Bioeng. 2007, 97(1), 1-11

22. Buisson, G., Duée, E., Haser, R. and Payan F. Three dimensional structure of porcine pancreatic alpha-amylase at 2.9 A resolution. Role of calcium in structure and activity. EMBO J. 1987, 20, 3909–3916.

23. Bush, D. S., Sticher, L., van Huystee, R.; Wagner, D. and Jones, R. L. The calcium requirement for stability and enzymatic activity of two isoforms of barley aleurone α-amylase. J. Biol. Chem. 1989, 264, 19392-19398.

24. Combet, C., Jambon, M., Deléage, G. and Geourjon, C. Geno3D an automated protein modelling Web server. Bioinformatic. 2002, 18, 213-214

25. Dandekar, T., Schuster, S., Snel, B., Huynen, M. and Bork, P. Pathway alignment: application to the comparative analysis of glycolytic enzymes, Biochem. J. 1999 , 343, 115-124.

26. Dauter, Z., Dauter, M., Brzozowski, A.M., Christensen, S., Borchert, T.V., Beier, L., Wilson, K.S. and Davies G.J. X-ray structure of Novamyl, the five-domain 'maltogenic' α-amylase from *Bacillus stearothermophilus*: maltose and acarbose complexes at 1.7 A° resolution, Biochem. 1999, 38, 8385-8392.

27. Davies, G., Sinnott, M.L. and Withers, S.G. Glycosyl transfer, in: M. Sinnott (Ed.), Comprehensive Biological Catalysis, Academic Press, New York, 1998, 119-208.

28. De Cordt, S., Vanhoof, K., Hendrickx, M., Maesmans, G. and Tobback P. Thermostability of soluble and immobilized α-amylase from *Bacillus licheniformis*. Biotechnol Bioeng. 1992, 40, 396–402.

29. Declerck, N ., Machius, M., Joyet, P., Wiegand,G., Huber, R. and Gaillardin, C . Hyperthermostabilization of *Bacillus licheniformis* α-amylase and modulation of its stability over a 50°C temperature range. Protein Eng. 2003, 16, No. 4, 287-293.

30. Declerck, N., Joyet, P., Gaillardin, C. and Masson, J.M. Use of amber suppressors to investigate the thermo-stability of *Bacillus licheniformis* α-amylase. Amino acid replacements at 6 histidine residues reveal a critical position at His-133, J. Biol.

31. Declerck, N., Joyet, P., Trosset J.Y., Garnier, J. and Gaillardin, C. Hyperthermostable mutants of *Bacillus licheniformis* α-amylase: multiple amino acid replacements and molecular modelling, Protein Eng. 8 (1995) 1029-1037.

32. Declerck, N., Machius, M., Chambert, R., Wiegand, G., Huber, R. and Gaillardin, C. Hyperthermostable mutants of *Bacillus licheniformis* α-amylase: thermodynamic studies and structural interpretation, Protein Eng. 1997, 10, 541-549.

33. Dey, G., Singh, B. and Banerjee, R. Immobilization of α-amylase produced by *Bacillus circulans* GRS 313. Brazil Arch Biol Technol. 2003, 167–76.

34. Duy, C. and Fitter, J. Thermostability of irreversible unfolding alpha-amylases analyzed by unfolding kinetics. J. Biol. Chem. 2005, 280, 37360-37365.

35. Emne'us, J. and Gorton L. Effects on the hydrolysis of native starch and glycogen by a thermostable α-amylase after immobilization on solid supports. Anal Chim Acta. 1990, 234, 97–106.

36. Feller, G., Payan, F., Theys, F., Qian, M., Haser, R. and Gerday, C. Stability and structural analysis of α-amylase from the Antarctic psychrophile *Alteromonas haloplanctis*. Eur. J. Biochem. 1994, 222, 441-447

37. Fields, R. The measurement of amino groups in proteins and peptides. Biochem.J. 197, 1124, 581 – 590.

38. Fischer, E. H. and Stein, E. A. α-Amylases. In: The Enzymes (Boyer, P. D. Ed.) 1960, 4, 313 – 340, Academic Press, New York.

39. Fitter, J. and Pohlmeier, S. H. Structural stability and unfolding properties of thermostable bacterial α-amylases: A comparitive study of homologous enzymes. Biochem. 2004, 43, 9589 – 9599.

40. Fitter, J. Structural and dynamical features contributing to thermostability in α-amylases. Cell.Mol.Life Sci. 2005, 62, 1925-1937

41. Fitter, J., Herrmann, R., Dencher, N. A., Blume, A. and Hauss, T. Activity and stability of a thermostable α-amylase compared to its mesophilic homologue: Mechanisms of thermal adaptations. Biochem. 2001, 40, 10723 – 10731.

42. Fraenkel, H. C. Methods for investigating the essential groups. Methods Enzymol. 1957, 4, 247-269.

43. Friedberg, F. On the primary structure of amylases. FEBS Lett.1983, 152, 139-140

44. Gaboriaud, C., Bissery, V., Benchetrit, T. and Momon, J. P. Hydrophobic cluster analysis: an efficient new way to compare and analyse amino acid sequences. FEBS Lett. 1987, 224, 149-155.

45. Gouda, M. D., Singh, S. A., Rao, A. G. A., Thakur, M. S. and Karanth, N. G. Thermal inactivation of glucose oxidase- mechanism and stabilization using additives. J. Biol. Chem. 2003, 278, 24324 – 24333.

46. Greenwood, C. T. and MacGregor, A. W. The isolation of α-amylase from barley and malted barley, and a study of the properties and action patterns of the enzymes. J. Inst. Brew. 1965, 71, 405 - 417.

47. Greenwood, C. T., MacGregor, A. W., & Milne, E. A. Action pattern of broad bean alpha-amylase. Arch. Biochem.Biophys. 1965, 112, 466–470.

48. Griko, Y., Sreerama, N., Osumi-Davis, P., Woody, R.W. and Woody AY., Thermal and urea-induced unfolding in T7 RNA polymerase: calorimetry, circular dichroism and fluorescence study. Protein Sci. 2001 , 10, 845-53.

49. Gupta, M.N. Thermostabilization of proteins. Biotech. Appl. Biochem, 1991, 14, 1-11.

50. Gupta, M.N., Guoqiang, D. and Mattiason B. Purification of endo-polygalacturonase by affinity precipitation using alginate. Biotechnol Appl Biochem. 1993, 18, 321–7.

51. Guzman-Maldonado, H. and Paredes-Lopez, O. Amylolytic enzymes and products derived from starch: a review, Crit. Rev. Food Sci. Nutr. 1995, 35373-403.

52. Haddaoui, E.A., Leloup, L., Petit-Glatron, M.F. and R. Chambert, Characterization of a stable intermediate trapped during reversible refolding of *Bacillus subtilis* α-amylase. Eur. J. Biochem. 1997, 249, 505–509.

53. Hagenimana, V., Vezina, L. P. and Simard, R. E. Distribution of amylases within sweet potato (Ipomoea batatas L.) root tissue. J. Agri. Food. Chem. 1992, 40,1777 – 1783.

54. Hasegawa, A. and Imahori, K. Studies on α-amylase from a thermophilic bacterium. J. Biochem. 1976, 79, 469-477.

55. Hasim, S.O., Kaul, R.H., Anderson, M., Mulaa, F.J. and Bo Mattiasson. Differential scanning calorimetric studies of a Bacillus halodurans α-amylase. Biochim.Biophys.Acta. 2005, 1723, 184 – 191.

56. Henrissat, B. A classification of glycosyl hydrolases based on amino acid sequence similarities. Biochem J. 1991, 280, 309-316

57. Henrissat, B. and Bairoch, A. New families in the classification of glycosyl hydrolases based on amino acid sequence similarities .Biochem. J. 1993, 293, 781-788.

58. Hiromi, K., Ohnishi, M. and Tanaka, A. Subsite structure and ligand binding mechanism of glucoamylase. Mol. Cell. Biochem. 1983, 51, 79-95.

59. Holm, L., Koivula, A. K., Lehtovaara, P. M., Hemminki, A. and Knowles, J. K. C. Random mutagenesis used to probe the structure and function of Bacillus stearothermophilus alpha-amylase. Prot. Eng. 1990, 3, 181-191.

60. Huynen, M.A., Dandekar, T. and Bork, P. Variation and evolution of the citric-acid cycle: a genomic perspective, Trends Microbiol. 1999, 7 281-291.

61. Igarashi, K. Thermostabilization by proline substitution in an alkaline, liquefying K-amylase from *Bacillus* sp. Strain KSM-1378, Biosci. Biotechnol. Biochem. 1999, 63,1535-1540.

62. Ishikawa, K., Matsui, I., Kobayashi, S., Nakatani, H. and Honda, K. Substrate recognition at the binding site in mammalian pancreatic alpha-amylases. Biochem. 1993, 32, 6259-6265.

63. Jambunathan, R. Singh, U and Subramanian, V. Grain quality of sorghum, pearl millet, pigeon-pea, and chick-pea. In: Food and Nutrition Books; Interfaces between agriculture, nutrition and food science (Achaya K.T Ed.) 1984, The united nations university press, Tokyo 150, Japan.

64. James, J. and Simpson BK. Application of enzymes in food processing. Crit Rev Food Sci Nutr. 1996, 36(5), 437–63.

65. Janecek, S. New conserved amino acid region of α-amylases in the third loop of their $(\alpha/\beta)_8$-barrel domains, Biochem. J. 1992, 288, 1069-1070.

66. Janecek, S., Svensson, B. and Henrissat, B. Domain evolution in the α-amylase family, J. Mol. Evol. 1997, 45, 322-331.

67. Jansen, A. P. and Wydeveld, P. G. A. B. (*p*-Nitrophenyl)Maltoside as a Substrate for the Assay of Amylase. Nature, 1958, 182, 525 – 526.

68. Jensen, M. T., Gottschalk, T. E. and Svensson, B. Conformational stability of barley alpha-amylase isozymes 1 and 2.Role of charged groups and isozyme 2 specific salt-bridges. J.Cereal.Sci. 2003, 38, 289-300.

69. Joyet, P., Declerck, N. and Gaillardin, C. Hyperthermostable variants of a highly thermostable α-amylase, Biotechnology. 1992, 10, 1579-1583.

70. Kadziola, A., Abe, J., Svensson, B. and Haser, R. Crystal and molecular structure of barley α-amylase, J. Mol. Biol. 1994, 239104-121.

71. Kadziola, A., Sogaard, M., Svensson, B. and Haser, R. Molecular structure of a barley α-amylase-inhibitor complex: implications for starch binding and catalysis, J. Mol. Biol. 1998, 278,205-217.

72. Karuppiah, N. and Sharma, A. Cyclodextrins as protein folding aids. Biochem. Biophys. Res. Commun. 1995, 211, 60 – 66.

73. Kennedy, J.F. Enzyme technology. In: Kennedy JF, Carbral JMS, editors. Biotechnology, vol. 7a. Weinbein, Germany: VCH Publ. Verlagsgesellschaft mbh; 1978.

74. Khajeh, K., Ranjbar, B., Manesh, H.N., Habibi, A.E and Gorgani, M.N. Chemical modification of bacterial α-amylases: changes in tertiary structures and the effect of additional calcium. Biochim.Biophys.Acta. 2001, 1548, 229 – 237

75. Khodarahmi, R. and Yazdanparast, R. Refolding of chemically denatured α-amylase in dilution additive mode. Biochim.Biophys.Acta. 2004, 1674, 175 – 181.

76. Khosro, K., Shokri, M. M., Asghari, S.M., Fatemeh, M., Ghasemi, A., Sadeghi, M., Ranjbar, B., Hosseinkhani, S., Gharavi S.and Naderi-Manesh, H. Acidic and proteolytic digestion of α-amylases from *Bacillus licheniformis* and *Bacillus amyloliquefaciens* : Stability and flexibility analysis. Enz. Microb. Tech. 2006, 38, 422-428.

77. Killenberg-Jabs, M., Kern, G., Hubner, G. and Golbik, R. Folding and stability of different oligomeric states of thiamin diphosphate dependent homomeric pyruvate. Biophys.J. 2002, 96, 259 – 271.

78. Kim, J., Mao. J. and Gunner, M. R. Are Acidic and Basic Groups in Buried Proteins predicted to be ionized?. J. Mol.Biol. 2005, 348, 1283 – 1298.

79. Knowles, J. R. Tinkering with enzymes: what are we learning?. Science, 236, Issue 4806, 1252-1258.

80. Kondo, H., Nakatani, H., Matsuno, R. and Hiromi, K. Product distribution in amylase-catalyzed hydrolysis of amylose: comparison of experimental results with theoretical predictions. J. Biochem. 1980, 87, 1053-1070

81. Kumar, R. S. S., Singh, S. A.and Rao A. G. A. Thermal stability of α-amylase from malted jowar (*Sorghum bicolor*). J. Agri. Food. Chem. 2005, 53,6883 – 6888.

82. Kumar, R., Prabhu, N. P., Yadaiah, M. and Bhuyan, A. K. Protein stiffening and entropic stabilization in the subdenaturing limit of guanidine hydrochloride. Biophys.J. 2004, 87, 2656 – 2662.

83. Kuriki, T. and Imanaka, T. Nucleotide sequence of the neopullulanase gene from Bacillus stearothermophilus, J. Gen. Microbiol. 1989, 135, 1521-1528

84. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 1970, *277*, 680-685.

85. Larson, S.B., Greenwood, A., Cascio, D., Day, J. and McPherson, A. Refined molecular structure of pig pancreatic α-amylase at 2.1 A°resolution, J. Mol. Biol. 1994, 235, 1560-1584.

86. Lawson, C.L., van Montfort, R., Strokopytov, B., Rozeboom, H.J., Kalk, K.H., de Vries, G.E., Penninga, D., Dijkhuizen, L. and Dijkstra, B.W. Nucleotide sequence and X-ray structure of cyclodextrin glycosyltransferase from *Bacillus circulans* strain 251 in a maltose-dependent crystal form, J. Mol. Biol. 1994, 236,590-600.

87. Lemesle-Varloot, L., Henrissat, B., Gaboriaud, C., Bissery, V., Morgat, A. and Mornon, J. P. Hydrophobic cluster analysis: procedures to derive structural and functional information from 2-D-representation of protein sequences. Biochimie 1990, 72, 555-574.

88. Linne, E., Garg, N., Kaul, R. and Mattiasson B. Evaluation of alginate as a ligand carrier in affinity precipitation. Biotechnol Appl Biochem. 1992, 16, 48–56.

89. Ly, H.D. and Withers, S.G. Mutagenesis of glycosidases, Annu. Rev. Biochem. 1999, 68 , 487-522.

90. MacGregor, A. W. α-amylase from malted barley-physical properties and action pattern on amylose. Cereal Chem. 1978, 55 (5), 754-765.

91. MacGregor, E.A. Alpha-amylase structure and activity. J Protein Chem. 1988, 7(4), 399–415.

92. MacGregor, E.A. and MacGregor, A.W. A model for the action of cereal alpha amylases on amylose. Carbohydrate Res. 1985, 142, 223-236.

93. Machius, M., Declerck, N., Huber, R. and G. Wiegand. Activation of *Bacillus licheniformis* α-amylase through a disorder-order transition of the substrate-binding site mediated by a calcium-sodium-calcium metal triad. Structure, 1998, 6, 281-292.

94. Machius, M., Wiegand, G. and Huber, R. Crystal Structure of calcium-depleted *Bacillus licheniformis* α-amylase at 2.2 Å Resolution. J. mol. Biol. 1995, 246, 545-559.

95. Maldonado, H.G. and Lopez O.P. Amylolytic enzymes and products derived from starch: a review, Crit Rev Food Sci Nutr. 1995, 35(5):373-403.

96. Marchylo, B., Kruger, J. E. and Irvine, G. N. α-amylase from immature hard red spring wheat. I. Purification and some chemical and physical properties. Cereal Chem. 1976, 53 (2), 157-173.

97. Matsuura, Y., Kusunoki, M., Harada, W. and Kakudo, M. Structure and possible catalytic residues of Taka amylase A, J. Biochem. 1984, 95, 697-702

98. Monera, O. D., Kay, C. M. and Hodges, R. S. Protein denaturation with guanidine hydrochloride or urea provides a different estimate of stability depending on the contributions of electrostatic interactions. Protein Sci. 1994, 3, 1984 – 1991.

99. Muralikrishna. and Nirmala. Cereal α-amylases—an overview. Carbohydrate Polymers. 2005, 60, 163–173.

100. Mutisya, M., Satish, P., Sun, C.,Anderson, L., Ahlandsberg, S., Baguna, Y., Palmquist, S., Odhiambo, B., Aman, P. and Jansson, C. Starch branching enzymes in sorghum (*Sorghum bicolor*) and barley (*Hordeum* V*ulgare*): comparative analysis of enzyme structure and gene expression. J. Plant Physiol. 2003, 160, 921- 930.

101. Nakajima, R., Imanaka, T. and Aiba, S. Comparison of amino-acid-sequences of 11 different alpha-amylases .Appl. Microbiol. Biotechnol. 1986, 23, 355-360

102. Nazmi, A. R., Reinisch., T and Hinz, H. J. Ca-binding to *Bacillus licheniformis* α-amylase (BLA). Arch. Biochem.Biophys. 2006, 453, 18-25

103. Nielsen, A. D., Pusey, M. L., Fuglsang, C. C. and Westh, P. A proposed mechanism for the thermal denaturation of a recombinant *Bacillus halmapalus* α-amylase – the effect of calcium ions. Biochim.Biophys.Acta. 2003, 1652, 52 – 63.

104. Nielsen, J.E. and Borchert, T.V. Protein engineering of bacterial α-amylases, Biochim.Biophys.Acta . 2000, 1543, 253-274.

105. Nirmala, M. Amylases from malted ragi (*Eleusine coracana*, Indaf-15) and studies on their role in the degradation of cereal flours and starches. Ph.D. thesis, University of Mysore, Mysore, India, 2001; p 60.

106. Nirmala, M. and Muralikrishna, G. Three α-amylases from malted finger millet (ragi, *Eleusine coracona*, Indaf-15)-purification and partial characterization. Phytochemistry 2003, 62, 21-30.

107. Pace, C. N. and Scholtz, M. J. Measuring conformational stability of a protein. In Protein Structure : A Practical Approach (Creighton, T. E. ed.), 1987, 299 – 321, Oxford University Press Inc., New York.

108. Pace, C. N. Conformational stability of globular proteins.Trends. Biochem. Sci. 1990, 15, 14 – 17.

109. Pace, C. N., Laurents, D. V. and Thomson, J. A. pH dependence of the urea and guanidine hydrochloride denaturation of Ribonuclease A and Ribonuclease T1. Biochem. 1990, 29, 2564 – 2572.

110. Pandey A, Nigam P, Soccol CR, Soccol VT, Singh D. and Mohan R. Advances in microbial amylases. Biotechnol Appl Biochem . 2000, 31, 135–52.

111. Pommier G, Cozzone P, Marchis-Mouren G. The sulfhydryl groups of porcine pancreatic alpha-amylase: unmasking, reactivity and function. Biochim Biophys Acta. 1974, 350(1):71-83.

112. Qian, M., Haser, R. And Payan, F. Structure and molecular model refinement of pig pancreatic α-amylase at 2.1A° resolution, J. Mol. Biol. 1993, 231, 785-799

113. Ramasubbu, N., Paloth, V., Luo, Y., Brayer, G.D. and Levine, M.J. Structure of human salivary α-amylase at 1.6 A° resolution: implications for its role in the oral cavity, Acta Cryst. D52, 1996, 435-446.

114. Raviyan, P., Juming, T. and Barbara, A.R. Thermal stability of α-amylase *Aspergillus oryzae* entrapped in polyacrylamide gel. J Agric Food Chem. 2003, 51, 5462–6.

115. Razvi, A. and Scholtz, J.M. Lessons in stability from thermophilic proteins. Protein Sci. 2006,15(7),1569-78.

116. Richardson TH, Tan X, Frey G, CallenW, Cabell M and Lam D. A novel high performance enzyme for starch liquefaction. Discovery and optimization of a low pH, thermostable α-amylase. 2002, J. Biol. Chem., Vol. 277, Issue 29, 26501-26507.

117. Riddles, P. W., Blakeley, R. L. and Zerner B. Reassessment of Ellman's reagent. Methods Enzymol. 1983, 91, 49–60.

118. Rinderkneet, H., Wilding, P. and Haverback, B.J. A new method for the determination of alpha-amylase. Experientia. 1967 , 23(10), 805-807

119. Robert, X., Haser, R., Gottschalk, T. E., Svensson, B.and Aghajari, N. The structure of barley a-amylase isozyme 1 reveals a novel role of domain C in substrate recognition and binding: a pair of sugar tongs. Structure. 2003, 11, 973–984.

120. Robert, X., Haser, R., Svensson, B. and Aghajari. N. Comparison of crystal structures of barley α-amylase 1 and 2: implications for isozyme differences in stability and activity. Biologia, Bratislava, 57/Suppl. 2002, 11, 59-70,

121. Robyt, J. F., and Whelan, W. J. In: J. A. Radley (Ed.), Starch and its derivatives. Chapunear and Hall condess, fourth edn. 1968, 430–497.

122. Robyt, J.F. and French, D. The action pattern of porcine pancreatic α-amylase in relationship to the substrate binding site of the enzyme, J. Biol. Chem. 1970, 245 3917-3927.

123. Rodaway, S. J. Composition of α-amylase secreted by aleurone layers of grains of H2imalaya barley. Phytochem. 1978, 17, 385-359.

124. Rogers, J. C. Two barley alpha-amylase gene families are regulated differently in aleurone cells. J. biol. Chem. 1985, 260, 3731-3738

125. Saboury, A. A. and Karbassi, F. Thermodynamic studies on the interaction of calcium ions with alpha-amylase. Thermochimica Acta. 2000, 362, 121-129.

126. Sadana, A. and Beelaram, A, M. Efficiency and economics of bioseparation: some case studies. Bioseparation. 1994, 4, 221–5.

127. Sardar, M. and Gupta, M.N. Alginate beads as an affinity material for alpha amylases. Bioseparation. 1998, 7, 159–65.

128. Savchenko. A., Vieille. C., Kang. S, and Zeikus J. G. (*Pyrococcus furiosus* α-amylase is stabilized by calcium and zinc. Biochem. 2002, 41 (19), 6193 - 6201

129. Seigner, C., Prodanov, E. and Marchis-Mouren, G. The determination of subsite binding energies of porcine pancreatic alpha-amylase by comparing hydrolytic activity towards substrates. Biochim Biophys Acta. 1987, 913, Issue 2, 200-209.

130. Sharma, A., Sharma, S. and Gupta MN. Purification of wheat germ amylase by precipitation. Protein Expres Purif 2000;18:111–4.

131. Sinha, S., Mitra, N., Kumar, G., Bajaj, K. and Surolia, A. Unfolding studies of soybean agglutinin and concanavalin A tetramers: A comparative account. Biophys.J. 2005, 88, 1300 – 1310.

132. Sivaramakrishnan, S., Gangadharan, D., Madhavan, K., Nampoothiri, Carlos Ricardo Soccol and Pandey, A. α-amylases from microbial sources

– an overview on recent developments. Food Technol. Biotechnol. 2006, 44 (2), 173–184.

133. Søgaard, M., Kadziola, A., Haser. R., and Svensson, B . Site-directed mutagenesis of histidine 93, aspartic acid 180, glutamic acid 205, histidine 290, and aspartic acid 291 at the active site and tryptophan 279 at the raw starch binding site in barley alpha-amylase 1. J Biol Chem. 1993, 268(30), 22480-4

134. Sommers, W, Visser, J, Rombouts, F. M. and van't Riet K. Developments in downstream processing of (poly)saccharide converting enzymes. J Biotechnol 1989, 11, 199–222.

135. Song, S. W., Hwang, K. Y., Chang, C. and Suh, S. W. () In *Enzymes for Carbohydrate Engineering,* 1996, 167-170 (eds. K. H. Park, J. F. Robyt and Y.-D. Choi), Elsevier, Amsterdam.

136. Stein, E.A and Fischer, E.H. The resistance of alpha-amylases towards proteolytic attack. J Biol Chem. 1958, 232(2), 867–879.

137. Strucksberg, K. H., Rosenkranz, T. and Fitter J. Reversible and irreversible unfolding of multi-domain proteins. Biochim.Biophys.Acta. 2007, 1774, 1591-603.

138. Sudharshan, E. and Rao, A. G. A. Involvement of cysteine residues and domain interactions in the reversible unfolding of lipoxygenase-1. J. Biol. Chem. 1999, 274, 35351-35358.

139. Suzuki, Y., Ito, N., Yuuki, T., Yamagata, H. and Udaka, S. Amino acid residues stabilizing a *Bacillus* α-amylase against irreversible thermoinactivation, J. Biol. Chem. 1989, 264, 18933-18938.

140. Svensson, B. Protein engineering in the alpha-amylase family: catalytic mechanism, substrate specificity, and stability. Plant Mol Biol. 1994, 25(2), 141-57

141. Swift, H.J., Brady, L., Derewenda, Z.S., Dodson, E.J., Dodson ,G.G., Turkenburg, J.P. and Wilkinson, A.J. Structure and molecular model refinement of Aspergillus oryzae (TAKA) alpha-amylase: an application of the simulated-annealing method. Acta Crystallogr B. 1991, 47, 535–544.

142. Takagi, T. and Isemura, T. Necessity of calcium for the renaturation of reduced Taka-amylase A. J Biochem. 1965, 57, 89-95.

143. Takase, K. Site-directed mutagenesis reveals critical importance of the catalytic site in the binding of alpha-amylase by wheat proteinaceous inhibitor Biochem. 1994, 33, 7925-7930.

144. Tanaka, A. and Hashino, E. Calcium binding parameters of *Bacillus amyloliquefacians* α-amylase determined by inactivation kinetics. Biochem. J. 2002, 364, 635-639.

145. Teotia, S. and Gupta, M. N. Purification of alpha-amylases using magnetic alginate beads. Appl Biochem Biotechnol. 2001, 90, 211–20.

146. Teotia, S. and Gupta, M.N. Magnetite–alginate beads for purification of some starch-degrading enzymes. Mol Biotechnol. 2002, 20, 231–7.

147. Teotia, S., Khare, S.K. and Gupta, M.N. An efficient purification process for sweet potato beta-amylase by affinity precipitation with alginate. Enzyme Microb Technol. 2001, 28, 792–5.

148. Teotia, S., Lata, R., Khare, S.K. and Gupta MN. One-step purification of glucoamylase by affinity precipitation with alginate. J Mol Recognit 2001;14: 295–9.

149. Tkachuk, R. and Kruger, J. E. Wheat α-amylases II. Physical characterization. Cereal Chem. 1974, 51, 508-529

150. Toda, H., Kato, I. and Narita, K. Correlation of the Masked sulfhydryl group with the essential calcium in Taka-amylase A. J. Biochem, 1968, 63, 295-301.

151. Tomazic, S. J. and Klibanov, A. M. Why is one *Bacillus* α-amylase more resistant against irreversible thermoinactivation than another?. J. Biol. Chem. 1988, 263, 3092-3096.

152. Torgerson, E.M., Brewer, L.C. and Thoma, J.A. Subsite mapping of enzymes. Use of subsite map to simulate complete time course of hydrolysis of a polymeric substrate. Arch Biochem Biophys. 1979, 196, 13-22.

153. Uitdehaag, J.C.M., Mosi, R., Kalk, K.H., van der Veen, B.A., Dijkhuizen, L., Withers, S.G. and B.W. Dijkstra. X-ray structures along the reaction pathway of cyclodextrin glycosyltransferase elucidate catalysis in the α-amylase family, Nat. Struct. Biol. 1999, 6, 432-436.

154. Ulhrich, R. and Schellenberger, A. Studies on the thermal inactivation of immobilized enzymes. Biotechnol Bioeng. 1986, 28, 511–22.

155. Vallee, B.L., Stein, E.A., Sumerwell, W.N. and Fischer. Metal content of amylases of various origins. J. Biol. Chem. 1959, 234, 2901-2905.

156. Vallée, F., Kadziola, A., Bourne, Y., Juy, M., Rodenburg, K. W., Svensson, B. and Richard Haser. Barley α-amylase bound to its endogenous protein inhibitor BASI: crystal structure of the complex at 1.9 å resolution. Structure. 1998, 6, 649-659.

157. Vecchio, P. D., Granziano, G., Granata, V., Barone, G., Mandrich, L., Rossi, M. and Manco, G. (2002) Denaturing action of urea and guanidine hydrochloride towards two thermophilic estarases. Biochem. J. 367, 857 – 863.

158. Vecchio, P. D., Granziano, G., Granata, V., Farias, T., Barone, G., Mandrich, L., Rossi, M. and Manco, G. (2004) Denaturant-induced unfolding of the Acetly-Esterase from *Eschericia coli*. Biochem. 43, 14637 – 14643.

159. Vihinen, M. and Mäntsälä, P. Conserved residues of liquefying α-amylases are concentrated in the vicinity of active site. Biochem. Biophys. Res. Commun. 1990, 166, 61 – 65.

160. Vihinen, M. and Mantsala, P. Microbial amylolytic enzymes. Crit. Rev. Biochem. Mol. Biol. 1989, 24, 329-418.

161. Vincent G.H. Eijsink., Alexandra Bjørk., Sigrid Gaseidnes., Reidun Sirevag., Bjørnar Synstad., Bertus van den Burg and Gert Vriend. Rational engineering of enzyme stability. Journal of Biotechnology. 2004, 113, 105–120.

162. Violet, M. and Meunier, J.C. Kinetic study of the irreversible thermal denaturation of Bacillus licheniformis alpha amylase. Biochem. J. 1989, 263, 665-670.

163. Whitaker, J. R. and Granum P. E. An absolute method for protein determination based on difference in absorbance at 235 and 280 nm. Anal. Biochem. 1980, 109, 156 –159.

164. Whitaker, J.R. and Granum, P.E. An absolute method for protein determination based on difference in absorbance at 235 and 280 nm. Anal Biochem. 1980, 109, 156–9.

165. Wong, D. W. S. and Robertson, G. H. α-amylases. In Handbook of Enzymology; Whitaker, J. R., Voragen, A. G. J., Wong, D. W. S., Eds., Dekker: New York, 2003; 707-718.

166. Xiao, Z., Storms, R and Tsang, A. A quantitative starch–iodine method for measuring alpha-amylase and glucoamylase activities. Anal. Biochem. 2006, 351, Issue 1, 146-148.

167. Yang, J. T., Wu, C. S. C. and Martinez, H. M. Calculation of protein conformation from circular dichroism. Methods Enzymol. 1986, 130, 208-269.

168. Yang, M., Galizzi, A. and Henner, D. Nucleotide sequence of the amylase gene from *Bacillus subtilis*. Nucleic Acids Res. 1983, 11, 237-249.

169. Yuuki, T., Nomura, T., Tezuka, H., Tsuboi, A., Yamagata, H., Tsukagoshi, N. and Udaka, S. Complete nucleotide sequence of a gene coding for heat- and pH-stable alpha-amylase of *Bacillus licheniformis*: comparison of the amino acid sequences of three bacterial liquefying alpha-amylases deduced from the DNA sequences. J. Biochem. 1985, 98(5), 1147–1156.