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Cereal α-amylases—an overview

G. Muralikrishna[a,*], M. Nirmala[b]

[a]Department of Biochemistry and Nutrition, CFTRI, Cheluvamba Mansion, Mysore, Karnataka 570 020, India
[b]Department of Biochemistry and Molecular Biology, Indiana-Purdue University, Indiana Polis, IN, USA

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Abstract

This review article covers various facets of cereal α-amylase research, i.e. definition, history, types, sources, classification based on their mode of action, assay methods, molecular basis of α-amylase induction during malting; isolation, fractionation, purification procedures, purity criteria, kinetic properties of cereal amylases and their activators, stabilizers and inhibitors, α-amylase and its active site, mechanism of action, primary, secondary and tertiary structures. In this article emphasis is also given to recently characterized finger millet α-amylases. The future perspectives of the cereal α-amylases are also mentioned.

Keywords: Cereal α-amylases; Structure; Properties

1. Introduction

Amylases are a class of hydrolases widely distributed in microbes, plants and animals. They can specifically cleave the *O*-glycosidic bonds in starch. Starch is a storage polysaccharide present in seeds, tubers, etc. of various plants. Starch consists of two components, a linear glucose polymer, amylose, which contains α-1,4 linkages and a branched polymer, amylopectin in which linear chains of α-1,4 glucose residues are interlinked by α-1,6 linkages (Figs. 1 and 2). Starch depolymerization by amylases is the basis for several industrial processes such as the preparation of glucose syrups, bread making and brewing. Amylases also play a significant role in seed germination and maturation and are instrumental in starch digestion in animals resulting in the formation of sugars, which are subsequently used for various metabolic activities (Leloup, Colona, & Buléon, 1994).

The use of amylases in starch hydrolysis dates back to the 9th century AD when malt was used to convert arrowroot starch to sweetener. In 1833, precipitated malt extract was observed to produce a sugar and at that time the mysterious material responsible for the reaction was termed as 'diastase' from the French word meaning 'separation' and then onwards diastase became the generic term for amylases (Hebeda & Teague, 1993). Trivial enzyme names were later derived by adding the suffix 'ase' to the root of the word denoting the substrate or action of the particular enzyme. Development and commercialization of α-amylases from fungal and bacterial sources occurred in the late 19th and early 20th centuries, respectively. By 1930s, these enzymes were used commercially in a variety of applications such as brewing, textiles, paper and corn syrup industries.

The effectiveness of bacterial α-amylases as an antistaling agent in baked foods was realized in the middle of the 20th century (Miller, Johnson, & Palmer, 1953). A significant increase in amylase production and utilization occurred in the early 1960s when *Bacillus subtilis* α-amylase and *Aspergillus niger* glucoamylase were used to replace acid catalysis in the production of dextrose from starch. Development of genetic engineering tools in 1970s successfully paved the way for the production of cloned amylases (*Bacillus stearothermophillus*) in 1980s and 1990s for industrial applications (Brumm, Hebede, & Teegue, 1991; Zemen & McCrea, 1985). Sequencing and crystallographic studies of various amylases were also completed in 1980s (Lin, Chyan, & Hsu, 1998). Efforts have been on the way to produce raw starch degrading enzymes for

* Corresponding author. Tel.: +91 821 2514876; fax: +91 821 517233.
E-mail address: gmk@cscftri.ren.nic.in (G. Muralikrishna).

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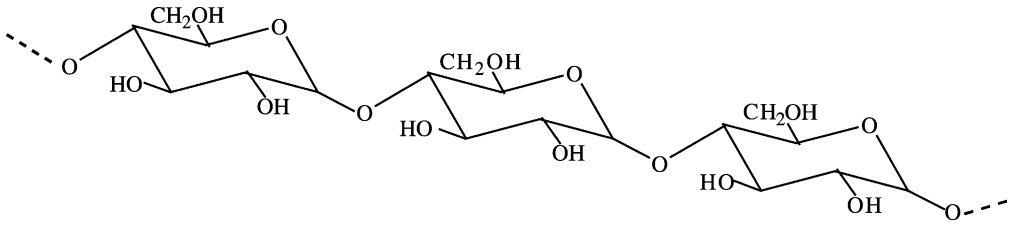

Fig. 1. Structure of amylose.

the past 15 years on industrial scale (Nakajima, Imanaka, & Aiba, 1986).

1.1. Types of starch degrading enzymes

Starch degrading enzymes can be classified into three main groups based on their mode of action: (1) endoamylases, (2) exoamylases, (3) debranching enzymes.

1.2. Sources of amylases

Microbes, digestive juices of animals and plants are the main sources of amylases (Table 1).

1.3. Classification of amylases based on their mode of action

1.3.1. Endoamylases

Endoamylases, also known as 'liquefying' enzymes are termed as α-amylases, which cleave α-1,4 glycosidic bonds in amylose, amylopectin and related polysaccharides such as glycogen. The products of hydrolysis, which are oligosaccharides of varying chain lengths, have the α-configuration at the C_1 of the reducing glucose unit, hence the name α-amylase. As the name suggests, endoamylases hydrolyze the bonds located in the inner regions of the substrate resulting in rapid decrease of the viscosity of the starch solution as well as decrease in iodine staining power (Hill & MacGregor, 1988).

1.3.2. Exoamylases

Exoamylases, also known as 'saccharifying' enzymes cleave α-1,4 glucosidic bonds in amylose, amylopectin and glycogen from the non-reducing end by successive removal of maltose/glucose in a stepwise manner. The products of hydrolysis have the β-configuration at the C_1 of the reducing glucose unit due to inversion of the product. In contrast to the action of endoamylases, this results in a slow decrease in the viscosity and iodine staining power of starch. Cereal and bacterial β-amylases and fungal glucoamylases come under this category (Banks & Greenwood, 1975).

1.3.3. Debranching enzymes

The branch points containing α-1,6 glycosidic linkages are resistant to attack by α- and β-amylases resulting in α/β limit dextrins, respectively. Pullulanase is produced by *Aureobasidium pullulans* and is capable of specifically attacking α-1,6 linkages (Abdulla & French, 1970). Glucoamylase can also attack α-1,6 linkages but the reaction proceeds slowly compared to pullulanase action.

2. Assay method for amylases

A number of analytical techniques are available in the literature for the determination of amylase activity. The most quantitative procedure generally used involves

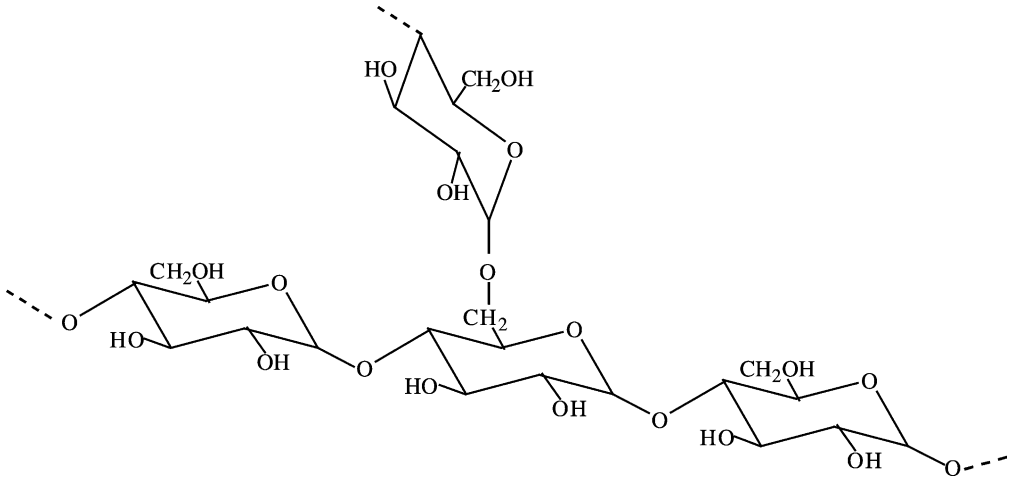

Fig. 2. Structure of amylopectin.

Table 1
Sources of amylases

Plant α-amylase	Plant β-amylase	Bacterial α-amylase	Fungal α-amylase	Gluco-amylase	Mammalian α-amylase
Barely and malted barely	Barely and malted barely	*Bacillus amyloliquefaciens*	*Aspergillus oryzae*	*Aspergillus niger*	Saliva
Wheat and malted wheat	Soybean sweet potato	*Bacillus subtilis*	*Aspergillus candidus*	*Rhizopus delemar*	Pancreas
Malted sorghum pearl millet maize	Wheat	*Bacillus coagulans*		*Rhizopus niveus*	
Malted finger millet (Ragi)		*Pseudomonas stutzen*			

the measurement of new reducing groups formed upon the amylolytic hydrolysis of starch. The unit enzyme activity is represented as the micromoles of products formed or substrate transformed per minute under defined conditions (Greenwood & MacGregor, 1965).

The colorimetric measurement of the released reducing groups can be carried out primarily by the use of the following reagents (a) alkaline copper (Nelson, 1944), (b) alkaline ferricyanide (Robyt, Ackerman, & Keng, 1972) and (c) alkaline 3,5, dinitrosalicylate (Bernfeld, 1955; Robyt & Whelan, 1968). Among these methods, DNS method is the most commonly used because of its reliability and simplicity. The copper and ferricyanide procedures give equimolar reducing values for equimolar reducing ends of maltooligosaccharides (Robyt et al., 1972; Robyt & Whelan, 1972). The ferricyanide reagent involves measurement of the decrease in loss of the ferricyanide ion concentration. Of late neocuprine method (Dygert, Li, Florida, & Thoma, 1965) is also used for the reducing sugar estimation in amylase assay.

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 (vanDyk & Caldwell, 1956). This procedure reflects the endoclevage of starch and can be used routinely to assay α-amylases.

Measurement of the decrease in the viscosity of the starch solution has also been used to measure α-amylase activity (Greenwood, MacGregor, & Milne, 1965). This procedure measures only endoactivity, so it can be used to detect α-amylase.

Chromogenic substrates were developed for α-amylase assays especially for clinical samples. The dye is covalently linked to starch or one of its constituents (amylose and amylopectin) to give an insoluble material (azure derivative) (Rinderkneet et al., 1967). When these substrates are acted on by α-amylase, fragments containing dye is solubilized, the remaining insoluble substrate is removed by centrifugation and absorbance of the supernatant is taken as measure of amylase activity. *p*-Nitrophenyl derivative of oligosaccharide also has been used to detect both α- and β-amylase activities. The amount of *p*-nitrophenol released correlates with the amylase activity. Even though this method is very apt and accurate to differentiate between α- and β-amylase activities, it is not used for routine analysis due to its high cost.

3. Cereal α-amylases

Cereal α-amylases play a very important role in the starch metabolism in developing as well as germinating cereals. These highly expressed enzymes are getting synthesized under the influence of plant growth hormones such as gibberellic acid (GA_3) and they exist in multiple forms (MacGregor, 1977a,b; Mitchell, 1972).

Generally, the number of isoenzymes identified depends on the cultivars studied and the sensitivity of the resolving method used (Hill & MacGregor, 1988; Khan, Verbeek, Waters, & Onckelen, 1973; Kruger, 1972; Marchylo, LaCroix, & Kruger, 1980). Essentially, cereal amylases are separable into two groups based on their chemical, physical and immunochemical properties (Kruger & Marchylo, 1985). Group-I has a pI value close to 5.8 and is calcium independent. It appears very quickly at the onset of germination in the presence of gibberellic acid. Group-II is characterized by a pI close to 4.5 and represents 60% of the total α-amylase activity and this group of enzymes requires calcium for their activity. Enzymes of this group are synthesized during germination, induced by gibberellic acid (MacGregor, 1983; Mundy & Munk, 1985).

The presence of α-amylase activity during barley, wheat and oat seed maturation (Meredith & Jenkins, 1973), as well as during seed germination (Hill & MacGregor, 1988) has been extensively examined. It is approximately 30% of the total protein synthesized during germination. The site of amylase synthesis is reported to be either in aleurone layer (Bewly & Black, 1985) or scutellum (Okamato et al., 1980). In this regard, the idea of whole pattern of endosperm modification, including starch degradation, being under the control of hydrolytic enzymes from the aleurone may has to be readdressed to unequivocally determine the site of amylase synthesis.

3.1. Molecular basis for the α-amylase biosynthesis and regulation

The molecular mechanism of α-amylase synthesis and regulation has been the major area of research in cereal science for the past several years especially with respect to barley (Jacobsen, 1977; Paleg, 1960a,b). Gibberellic acid was found to enhance the synthesis of mRNA specific for α-amylase and it may increase the efficiency of translation (Higgins, Zwar, & Jacobsen, 1976). On the other hand, abscissic acid inhibits the α-amylase synthesis at the stage of transcription and translation (Chrispeels & Varner, 1966; Jacobsen, Higgins, & Zwas, 1979). Gibberellic acid was identified to be responsible for the stimulation of α-amylase in the endosperm. In presence of Ca^{2+} thermal stability of amylases was found to be increased. Recently, cell culture studies have proved that cytoplasmic and even exogenously added Ca^{2+} ions play an important role in the induction and secretion of amylases from aleurone or scutellar tissues. This indicates the involvement of Ca^{2+} in the signal transduction at the cellular level.

3.2. Isolation and purification of α-amylases

Cereal α-amylases usually occurs in soluble forms and their extraction is also simple. Isolation of amylases from cereal malts are generally carried out using buffers such as sodium phosphate, sodium acetate and Tris–HCl and also with, calcium chloride and water. The pH of the buffer should be above 4.5 in order to prevent their inactivation at lower pHs (Hill & MacGregor, 1988). Phosphate buffer was found to be better extractant than acetate and tris buffers with respect to the isolation of ragi malt amylases (Nirmala & Muralikrishna, 2003a). Seventy-two-hour ragi malt gave the maximum amylase activity compared to other malting conditions (Nirmala, Subba Rao, & Muralikrishna, 2000). A process for the isolation of amylase rich fraction from malted cereals was developed (Patent—Muralikrishna & Nirmala, 2002).

3.2.1. Heat treatment of the extract

The contaminating thermo labile proteins/enzymes are often removed by heat inactivation of the crude extract. Generally, it is carried out ~70 °C. Selective inactivation of either α- or β-amylases in the presence of one another involves decreasing the pH of the enzyme extract below 4.0 (α) or heat treatment at 70 °C (β) at neutral pH, respectively. This step has been employed in the purification of α-amylases to separate heat labile β-amylases. However, heat inactivation of amylases is complex and the results are difficult to interpret because of the extent of inactivation depends not only on the temperature and time of heating but also on the pH and protein content of the extract (Greenwood & MacGregor, 1965).

3.2.2. Fractional precipitation

The large volumes of enzyme extracts can be concentrated by fractional precipitation using salts such as ammonium sulphate or organic solvents such as ethanol or acetone. This step has to be carried out at lower temperature to minimize the inactivation of the enzyme at high salt or organic solvent concentration. This has been used for the fractional precipitation of amylases from cereal malts (Greenwood & MacGregor, 1965). Acetone precipitation (20–75%) of phosphate buffer extraction of ragi malt gave maximum activity (90%) (Nirmala & Muralkikrishna, 2003a).

3.2.3. Purification of cereal amylases

Several amylases have been purified and characterized from different cereals using conventional as well as classical methods. The main hurdle in purifying cereal amylases is their occurrence in multiple forms as isoenzymes.

Ion exchange chromatography is a tool for the separation of proteins on the basis of charge. DEAE–cellulose matrix has been used to purify most of the cereal amylases (Marchylo, Kruger, & Irvine, 1976; Warchalewski & Tkachuk, 1978), whereas in few cases CM-cellulose has also been employed (MacGregor, 1977a,b). DEAE-sephacel was found to be very efficient ion exchanger for purifying the ragi malt isoenzymes α-1, α-2 and α-3 (Nirmala & Muralikrishna, 2003a).

Affinity chromatography is one of the best tools for the selective purification of enzymes. Substrate, substrate analogs, inhibitor, metal ligands, antibodies were covalently linked to insoluble Sepharose matrix and selective elution of the loaded enzyme result in highly pure enzyme (Tkachuk, 1975). Sephacryl-S-200 was found to be appropriate gel matrix in separating two closely related α-amylases of ragi malt (Nirmala & Muralikrishna, 2003a). Starch adsorption chromatography was used to purify amylase from sorghum malt (Beleia & Varriano Marston, 1981), whereas affinity matrices such as cyclohepta amylose sepharose (Silvanovich & Hill, 1976), soluble starch in the presence of ammonium sulphate (Kobayashi, Sasaki, & Kobayashi, 1997), glycogen-Sepharose, (Silvanovich & Hill, 1977) were also employed in the purification of cereal amylases.

Chromatofocusing is a technique that separates proteins on the basis of isoelectric point. This technique has been used mainly to separate isoenzymes wherein they differ in their pI value to the level of 0.05 units. This has been used to resolve barley amylase isoenzymes (Marchylo & MacGregor, 1983) and wheat α-amylases (Kruger & Marchylo, 1985). However, use of this method is very limited because of the high cost of the polybuffer, which has to be used to elute the proteins.

3.3. Criteria of purity of enzymes

There are several methods to ascertain the purity of the enzymes which mainly dependent on the charge, molecular

mass, biological activity and other biophysical properties of the proteins. General methods used to ascertain the purity of a protein are polyacrylamide gel electrophoresis (PAGE), gel permeation chromatography (GPC), activity staining and capillary electrophoresis.

3.4. Determination of type of amylases

Both α- and β-amylases are present in the cereals. There are several methods to identify α- and β-amylases. Physical properties such as stability at high temperature and extreme pHs are usually used to differentiate between these amylases. The most reliable method to differentiate α- and β-amylases is by product characterization. α-Amylases release high proportion of oligosaccharides along with α-D-maltose, whereas β-amylase releases only β-D-maltose as the product. These products can be separated and identified by paper chromatography, HPLC and also by optical rotation (Miller et al., 1953).

3.5. Properties of α-amylases

3.5.1. pH optima and pH stability of cereal α-amylases

The pH optima values of cereal amylases are in the range of 4.5–5.5. The different isoenzymes from the same cereal may or may not have the same pH optima. For the determination of pH optima values of α-amylases, different buffers are used to maintain the maximum buffering capacity. Buffers such as phathalate, glycine, acetate, phosphate, Tris, borate buffers have been used for this purpose. pH stability is one of the important criteria in enzyme studies. This is very important in storing the pure enzymes for long periods. α-Amylases undergo irreversible inactivation in the extreme pHs (Bertoft, Andtfolk, & Kulp, 1984). The finger millet α-amylases were found to be more or less completely inactivated below pH 4.0 but found to be stable in alkaline pH (Nirmala & Muralikrishna, 2003a,c).

3.5.2. Temperature optima and temperature stability

Temperature is one of the most important parameters that affect the rate of enzyme hydrolysis. The temperature optima of known cereal/finger millet amylases is in between 40 and 55 °C (Nirmala & Murlaikrishna, 2003a; Nirmala & Murlaikrishna, 2003c; Tomazic & Klibanov, 1987). Above this temperature range, most of the cereal amylases undergo inactivation. Generally, α-amylases are more thermostable compared to β-amylases. Comparative properties of cereal α-amylases were provided in Table 2.

3.5.3. Amino acid composition

Determination of amino acid composition of a given enzyme is the primary step in understanding the structure–function relationship and other physical properties. Amino acid composition of several amylases has been studied. Generally, the protein/enzyme is hydrolyzed completely by 6 N HCl (Moore & Stein, 1963), and the released amino acids are identified by either precolumn (Bildingmeyer, Cohen, & Tarvin, 1984), or post-column derivatization (Spackman, Stein, & Moore, 1958). Precolumn derivatization using phenyl isothiocyanate is more common. The derivatized amino acids can be identified by reverse phase HPLC.

The amino acid composition of α-1b, α-2 and α-3 of ragi malt isoenzymes indicated very high amount of glycine (α-1 29%, α-2 25% and α-3 25%), which is substantially higher than the reported values for cereal α-amylases. The glutamic acid/glutamine and aspartic acid/aspargine ratios were found to be in the range of 4–8%. The percentage of hydrophobic amino acids was relatively high in α-1b compared to α-2 and α-3. Sulphur containing amino acids (methionine and cysteine) were found to be very less especially in α-3. The quantity of aromatic amino acids (Tyrosine and Phenyl alanine) ranged from 3 to 4% (Nirmala & Muralikrishna, 2003a).

Amino acid composition has also been determined for a few cereal amylases. In case of sorghum α-amylase, aspartic acid content (13%) was found to be more than glycine (11%) (Botes, Joubert, & Novellie, 1967). Amino acid analysis of carboxy methylated α-amylases from wheat has shown the absence of sulphydryl groups. Wheat α-amylase isoforms indicated quantitative similarity except in the content of arginine (Tkachuk & Kruger, 1974). Barely aleurone layer α-amylases showed a high content of glycine followed by asportic acid, whereas the content of glutanic acid was considerably less. However, in barley aleurone

Table 2
Comparative account of the properties of cereal α-amylases

Source		pH	Temp. (°C)	Mol. wt. (kDa) by SDS-PAGE	pI
Immature wheat	α-1	–	–	–	4.65
	α-2	3.6–5.75	–	52–54	4.84
	α-3	–	–	–	5.11
Malted wheat	α-1	5.5	–	41.5–42.5	6.16
	α-2	5.7	–	–	6.2
	α-3	5.5	–	–	6.05
Immature barley		5.5	–	46	4.83
Malted barley	α-1	5.5	55	52	5.1
	α-2	–	–	–	6.2–6.4
Pearl millet	α-1	4.4–4.8	55	31	4.8
	α-2	–	–	46	5.2
	α-3	–	–	53	6.2
Malted sorghum		4.6	–	47–51	–
Malted triticale		4.9–5.0	54–56	–	–
Malted rye		4.7–4.8	–	–	–
Rice		–	–	48	4.6
Malted finger millet	α-1_b	5.0	45	47	–
	α-2	5.5	50	47	–
	α-3	5.0	40	47	–

Source. Kruger and Lineback (1987) and Nirmala and Muralikrishna (2003a).

Table 3
Effect of metal ions on purified ragi amylases activity (represented as % activity)

Salts (5 mM)	α-1	α-2	α-3
Control	100	100	100
$CaCl_2$	115	108	125
$BaCl_2$	106	102	114
$CoCl_2$	100	98	95.8
$MgCl_2$	95	100	98
$CdCl_2$	57	86	62
$ZnCl_2$	30	26	20
$CuCl_2$	11.8	11.3	6.3
$AlCl_3$	0	0	0
$FeCl_2$	0	0	0
$HgCl_2$	0	0	0

layer α-amylase histidine content was found to be very high compared to other cereal α-amylases (Roadway, 1978). It can be concluded that amino acid composition of α-amylases vary from cereal to cereal and also in different parts of the same cereals.

3.5.4. Effect of metalions

Various metal ions such as Ca^{2+}, Ba^{2+}, Co^{2+}, Mg^{2+}, Zn^{2+}, Cu^{2+}, Al^{3+}, Fe^{2+} and Hg^{2+} at 5 mM concentration were tested for purified ragi amylases activation/inhibition effect and the results are given in Table 3. Ca^{2+} and Ba^{2+} were found to have both activating and stabilizing effect as indicated by increased activity where as Co^{2+} and Mg^{2+} were having negligible effect on activity. However, the inactivating effect of Ca^{2+}, Zn^{2+} and Cu^{2+} was found to be partial. Metals such as Al^{3+}, Fe^{2+} and Hg^{2+} completely inactivated all the three α-amylases (Nirmala & Muralikrishna, 2003a). The inactivation by these metals may be due to their binding to either catalytic residues or replacing the Ca^{+2} from the substrate binding site of the enzyme. The cereal amylases are also inactivated by molebdate at a concentration of 10 mM whereas cyanide and chloride did not have any visible effect in barley α-amylase (Greenwood & Milne, 1968).

Enhancement of amylase activity of Ca^{2+} ions is based on its ability to interact with negatively charged amino acid residues such as aspartic and glutamic acids, which resulted in stabilization as well as maintenance of enzyme conformation. In addition calcium is known to have a role in substrate binding (Sprinz, 1999). It has also been documented that binding of Ca^{2+} to amylase is preferred over other cations such as Mg^{2+} (Bush, Sticher, Huystee, Wagner, & Jones, 1989). Replacement of Ca^{2+} by Sr^{2+}, Na^{2+} and Ba^{2+} resulted in partial activation of barley α-amylases; however, it is not comparable to the effect of Ca^{2+} on amylase activity.

3.5.5. Effect of activators/stabilizers

α-Amylases are known to be metalloenzymes as observed by several researchers. It has been shown that these enzymes contain covalently bound Ca^{2+}, which act as an allosteric activator. In addition to Ca^{2+}, other divalent cations such as Ba^{2+}, Mg^{2+} and Sr^{2+} can also act as activators for the α-amylases. Recent studies on barley α-amylase show that these cations, especially Ca^{2+} help in maintaining the three-dimensional structure of amylases (Bush et al., 1989). Purified finger millet amylases for inactivated above 70 °C and $CaCl_2$ (5–7.5 mM) was enhancing their thermal stability and found to be an activator (Nirmala & Muralikrishna, 2003c).

3.5.6. Inhibitors

All the metal chelators are strong inhibitors of amylases as they are metalloenzymes. In cereal amylases Ca^{2+} is loosely bound to enzyme and can be removed by treating with metal chelators such as EDTA, EGTA, etc. (Bush et al., 1989). Cabodiimide (50 mM) and metal ions such as Al^{3+}, Fe^{2+} and Hg^{2+} (5 mM) were inhibited the ragi amylases completely at 45°. Citric and oxalic acid inhibited there enzymes completely at 10 and 12.5 mM concentration, respectively. α-1b, from ragi malt was more susceptible to these organic acids compared to α-2 and α-3. The inhibition was enhanced at high temperatures. In addition to this, heavy metal ions such as Hg^{2+}, Pb^{2+}, Al^{3+} were also reported to be inhibitors for amylases. EDTA was one of the most important inhibitor of α-amylases since they depend on the calcium for their activity, which is chelated by EDTA and make them inactive. Ragi amylases were inhibited by EDTA at micromolar concentrations and inhibition was temperature dependent, higher at elevated temperature (45°). However, in the presence of substrate the inhibition was minimal (10%) (Nirmala & Muralikrishna, 2003c). The active site residues can be identified by using group specific reagents. Glutamic/aspartic acid residues specific reagent such as carbodiimide is a strong inhibitor to amylases suggesting that these amino acids are the active site residues.

4. Structure of α-amylases

4.1. Primary structure

α-Amylases are small proteins with a molecular weight of 20–55 kDa. They are calcium-containing enzymes with a single polypeptide chain. Nakajima et al. (1986) clearly pointed out the existence of four highly conserved regions, especially in the catalytic and substrate binding regions in 11 different α-amylases. The residues such as Asp-206, Glu-230 and Asp-297 were identified as the catalytic residues in Taka amylase-A, *B. subtilis*, *B. stearothermophillus* and barley.

In barley seeds two α-amylase groups, i.e. low pI (amy-1) and the high pI (amy-2) forms encoded by two multigene families, are synthesized during germination. Complete amino acid sequence of the enzymes has been deduced from cDNA sequences encoding a member of each family and N-terminal sequence of isolated proteins (Rogers & Mulliman, 1985). Amy-1 has been

C-terminally trimmed due to the action of several endogenous malt carboxypeptidases. The sequence of the processed form of amy-1 contain approximately 407 amino acid residues and that of Amy-2 have 403 amino acid residues with 75% positional residue identity.

4.2. Secondary structure

The α-amylases are generally composed of three distinct domains. A large 'A' domain with a typical barrel shaped $(\beta/\alpha)_8$ super structure. A 'B' domain inserted between the third β sheet and the following α helix of the $(\beta/\alpha)_8$ super structure. This domain is firmly attached to the 'A' domain by disulphide bond. A 'C' domain with a β sheet structure linked to the A domain by a simple polypeptide chain. A representation of secondary structure of barely α-amylase is given in Fig. 3. This 'C' domain may carry a carbohydrate chain depending on the origin of the enzyme (Buisson, Duee, Haser, & Payen, 1987).

Some α-amylases, especially those of pancreatic and plant origin, are metal dependant with respect to calcium (Ca^{2+}). This is situated between the A and B domains and may act in stabilizing the three-dimensional structure and as allosteric activator. The major difference in sequence and number of amino acids among the cereal α-amylases occur in these regularly folded loops of β-strand.

4.3. Tertiary structure

X-ray crystal structures have been described in the case of amylases from *Aspergillus oryzae* (Taka amylase 3.0 and 2.1 Å) porcine pancreatic amylase (2.9 Å) *A. niger* (2.1 Å) and barley malt α-amylase isoform Amy-2 (68 Å × 53 Å × 36 Å). Ribbon diagram of barley Amy-2 is given in Fig. 4.

The three-dimensional structure of barley malt isoform Amy-2 was determined by multiple isoforms replacement using three heavy atom derivatives and solvent flattering (Kadziola, Abe, Svensson, & Haser, 1994).

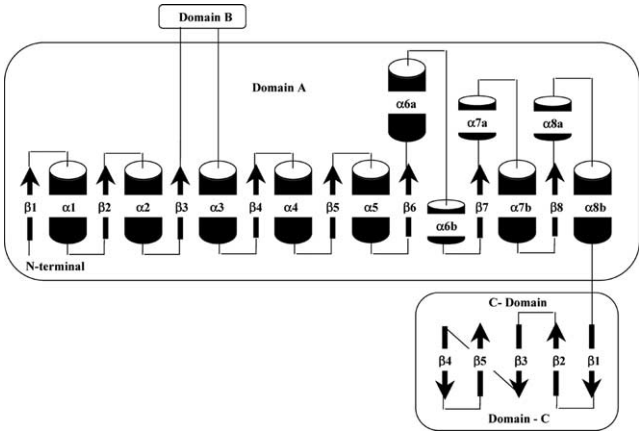

Fig. 3. A representation of domains and of the secondary structure of barley α-amylase, α-helices are shown as cylinders and β-strands as arrows (Kadziola et al., 1994, reprinted with permission).

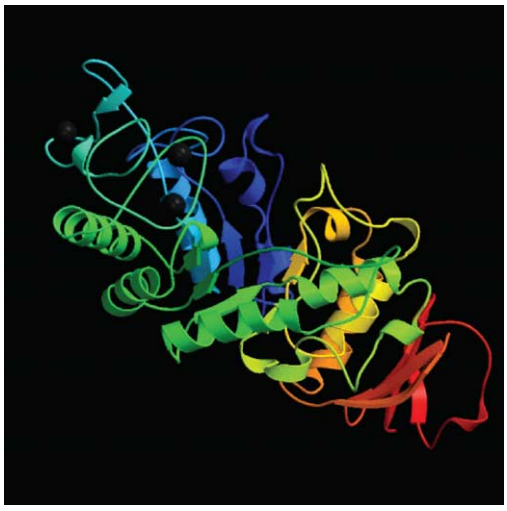

Fig. 4. Ribbon diagram of barley α-amylase (Kadziola et al., 1994, reprinted with permission).

4.4. Active site of α-amylase

The active site of the α-amylase is situated in a long cleft of about 3 nm, located between the carboxyl end of the A domain and the B domain. A model for the organization of active site and subsites, each capable of binding glucose has been proposed (Fig. 5) (MacGregor, MacGregor, Macri, & Morgan, 1994). The active site of different α-amylases are thereby made up of 5–11 subsites (A–K). The catalytic site is situated between subsites F and G. The reducing end of the α-glucose chain is located towards K subsite. Difference in the specificity of α-amylase, i.e. in the detailed way in which they hydrolyze a polysaccharide has been explained in terms of subsites at the active site.

Each subsite interacts with one glucose unit of the substrate. The catalytic site of the enzyme is located between subsites F and G. Subsites F and G will be occupied by Asp-206 or Glu-230 or Asp-297. Interaction of the enzyme with the primary hydroxyl of a glucose ring unmodified at C-6 is an important requirement for binding at that subsite.

5. α-Amylase catalytic mechanism

Catalytic mechanism of α-amylase was well characterized with respect to Taka amylase and found that active site has been localized in the cleft of the $(\beta/\alpha)_8$ barrel domain with Asp 206, Glu 230, Asp 297 residues playing the catalytic role whereas His 122 and His 296 might bind to glucosyl residues of substrate (Buisson et al., 1987; Matsura, Kunusok, Harada, & Kakado, 1984). The active site is divided into two parts (a) the binding site made up of a number of subsites and (b) the catalytic site made up of 2–3 groups that are proton donors (electrophiles) and proton acceptors (nucleophiles). The number of subsites and their arrangement in conjunction with the catalytic groups

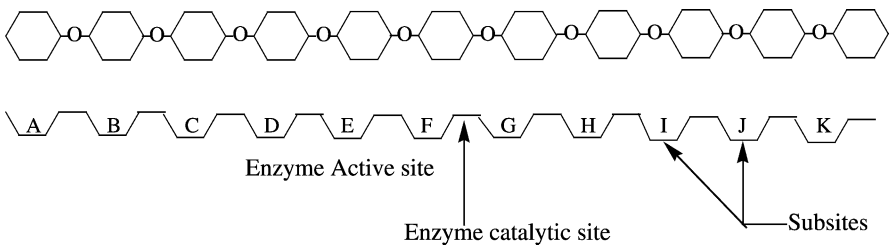

Fig. 5. Schematic representation of active site and subsites of α-amylases reprinted with permission from Elsevier, McGregor, E. A. et al., 1994, Carbohydrate Research, 257, 249–268.

determine the type of the products formed. The retention of the configuration occurring during α-amylase action suggests that a double displacement mechanism involving a covalent intermediate. Such a mechanism is shown in Fig. 6. It was proposed that transglycosylation which involves formation of both α-1,4 and 1,6 bonds could be catalyzed by the same mechanism (Kuriki & Imanaka, 1999).

6. Action pattern of α-amylases

6.1. Action pattern on starch granule in vivo

Starch granule hydrolysis, an important biological event in germinating seeds is a very slow process and often results in poorly hydrolyzed substrates. Information on in vivo hydrolysis of starch is very limited. Digestion of native granule proceeds in two stages; initial rapid hydrolysis followed by a slower constant hydrolysis. It involves diffusion to the solid surface, followed by adsorption and final catalysis. The porosity and accessibility of the substrate surface essentially control the number of absorption sites on the native starch granule (Beck & Ziegler, 1989; MacGregor, 1993).

In any starch preparation all the granules are not equally susceptible to enzymatic degradation, and depend upon how the amylases adsorb to a granule. Despite the specific mode of attack, starch hydrolysis occurs granule by granule and finally hydrolyzed completely. Granules of different maturity and sizes will vary in the amount of loose material at their surface. Minor constituents such as surface proteins and lipids also influence enzyme adsorption and the hydrolysis of the starch granules. The binding site is essential for granule hydrolysis. In general enzymes either erode the starch granule by exocorrosion or by endocorrosion (selected points on the surface). Some regions of the granules are more readily digested than others (Gallant, Bouchet, Bulelon, & Perez, 1992).

Hydrolysis patterns are controlled by the structure of starch granules. SEM studies on wheat and barley starch granules revealed that the initial α-amylase attack was on the starch granule forming discrete tunnels in to the granule and finally digest the central portion (Dronzek et al., 1972; Evers & McDermott, 1970; Lineback & Panpipom, 1977).

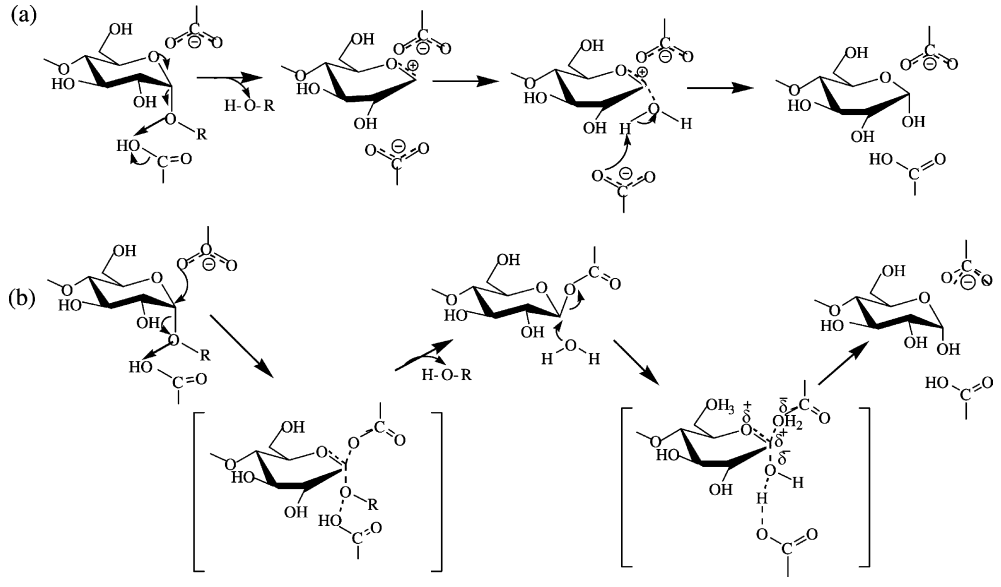

Fig. 6. Possible catalytic mechanisms of α-amylase. (a) S_N1 reaction via carbonium ion intermediate. (b) S_N2 reaction via formation of glycosyl–enzyme complex through a covalent bond reprinted with permission from the Society of Bitotechnology of Japan, Kuriki T., and Imanaka, T., 1999, Journal of Bioscience and Bioengineering, 87, 557–565.

SEM studies on native and germinated ragi starches showed the pattern of granular hydrolysis, which is dependant on the size and shapes; big granules are more susceptible than smaller granules. And malting did not affect the relative viscosity of starches isolated from ragi malts (Nirmala & Muralikrishna, 2002).

6.2. Degradation of starch in vitro

α-Amylases are endoenzymes, which hydrolyze α-1,4 linkages in starch randomly releasing oligosaccharides with a DP of 2–12 and their quantity may vary with respect to substrate concentration as well as the amount of enzyme used.

The action pattern of α-amylase on starch constituents, i.e. amylose and amylopectin is shown in Fig. 7.

Finger millet α-amylases were able to hydrolyse cereal flours and starches very effectively and ragi starch was found to be the most easily digestable compared to rice, wheat and maize starches (Nirmala & Muralikrishna, 2003b). Out of the three finger millet isoenzymes α-3 was found to be the most efficient compared to α-2 and α-1b. This was also substantiated by calculating the Km values of α-1b, α-2 and α-3 of finger millet (Nirmala & Muralikrishna, 2003a). Maltotetraose was the major oligosaccharide produced by these enzymes from cereal flours, starches and germinated ragi starches after 15 min of hydrolysis. Higher oligosaccharides (DP8 and above) have under gone further degradation after 120 min hydrolysis (Nirmala & Muralikrishna, 2003b).

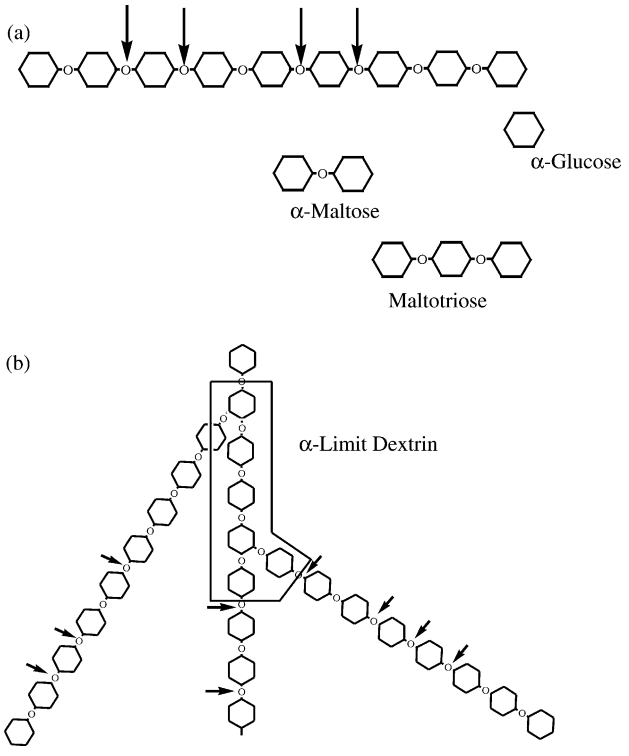

Fig. 7. α-Amylase action pattern on (a) amylose; (b) amylopectin.

Two mechanisms were proposed for the action of α-amylase on amylose in solution, i.e. multiple attack and multichain attack (Banks & Greenwood, 1977; French, 1981; Mazur, 1984). In the multiple attack (French, 1981), the encounter between enzyme and substrate is a chance factor and all the bonds are equally liable for hydrolysis. After hydrolysis, only one molecule is released, the other retained by enzyme and it slides along the active site and undergoes another hydrolysis. The multiple attacks lead to the formation of small saccharides during early stages of amylose hydrolysis whereas multichain attack does not. These two possibilities have been thoroughly discussed by Banks and Greenwood (1977) and Thoma (1976).

In the multichain attack mechanism, the encounter between enzyme and the substrate leads to a single hydrolysis, both molecules being released after the catalytic event. All the bonds are not equally susceptible to enzymatic hydrolysis, the ends of the chain being particularly resistant.

7. Future perspectives

Even though amylases were isolated from several sources lot of basic information pertaining to the induction, expression and regulation of α-amylases, sequence and X-ray crystallographic studies are lacking with respect to many cereal/millet amylases. Only barley α-amylase was characterized completely with respect to the above aspects. The glycoprotein nature of the induced α-amylases of the cereals was not documented unequivocally. The interaction of cereal polysaccharides such as arabinoxylans/β-1,3/1,4/D-glucans in stabilization of cereal amylases is also found warranted as they are closely associated with α-amylases during the germination of the cereals. These polysaccharides are presumed to have hydrophobic interaction with α-amylases which helps in stabilizing these enzymes during brewing process at elevated temperatures. Site directed mutagenesis experiments will reveal the unequivocal role of acidic/aromatic amino acids in many of the cereal/millet amylases.

The unexplored millet/minor millets α-amylases can be tested for their efficacy in brewing industries in the place of barley amylase which is found to be limiting cereal in several of the Asian countries. The relative efficiency of the many of cereal α-amylases (isoenzymes) with respect to the various starch degradation and characterization of the oligosaccharides as well as the unattacked dextrins is also lacking. This information will be very useful for the appropriate use of different cereal malts in the brewing industry as a source α-amylases as well as starches which can be used as adjuncts.

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