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REGULATION OF BIOSYNTHESIS OF  $\alpha$ -AMYLASE AND GLUCOAMYLASE  
ENZYMES IN Aspergillus awamori

by

Resham S. Bhella

A thesis presented to the University of Ottawa in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biochemistry.

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## ABSTRACT

The  $\alpha$ -amylase and glucoamylase enzymes of Aspergillus awamori were observed to be present as extracellular enzymes. The purified  $\alpha$ -amylase and glucoamylase enzyme proteins were found to be single polypeptides of molecular weights 54,000 and 94,000 respectively. Isoelectric point of  $\alpha$ -amylase was around pH 4.2. The enzyme was found to be most active around 50°C and pH 4.8-5.0.  $K_m$  for starch hydrolysis by  $\alpha$ -amylase was  $1\text{gL}^{-1}$  (pH 5.3, 37°C) and maltose was found to be an uncompetitive inhibitor of starch hydrolysis by this enzyme, with a  $K_I$  value of  $20.05\text{gL}^{-1}$ . The glucoamylase enzyme had isoelectric point around pH 3.4. The glucoamylase enzyme exhibited maximum activity around 55-60°C and at a pH range of 4.8-5.0.  $K_m$  for starch hydrolysis by glucoamylase was  $0.3\text{gL}^{-1}$  (pH 5.3, 37°C). Although glucoamylase, purified from the culture medium had a molecular weight of 94,000, this enzyme was shown to be synthesized de novo in two forms with molecular weights of 100,000 and 82,000. The 94,000 molecular weight form of glucoamylase appeared to be produced by deglycosylation of the 100,000 molecular weight form.

Both  $\alpha$ -amylase and glucoamylase enzymes were shown to be synthesized in response to lack or exhaustion of glucose from the culture medium and it was concluded that these enzymes are non-inducible and regulated solely by catabolite repression. Cyclic adenosine-3', 5'-monophosphate (cAMP) when added exogenously to the culture media repressed the synthesis of both of these enzymes

in a manner similar to glucose. The repression of  $\alpha$ -amylase and glucoamylase synthesis by exogenous cAMP was about 50% of that by glucose. In addition, intracellular cAMP levels were found to be in a positive correlation with the glucose concentration in the culture medium. The catabolite repression of these enzymes was suggested to be mediated by cAMP.

Although glucose repressed the glucoamylase protein synthesis by about four fold, there were only two fold differences in its mRNA levels during the conditions of repression and de-repression. Like glucose, cAMP also seemed to repress the glucoamylase mRNA levels by about two fold. It was thus concluded that the glucoamylase gene expression is regulated at the levels of both transcription and translation.

Unlike glucoamylase,  $\alpha$ -amylase specific mRNA levels as measured by in vitro translation assays were essentially similar under all conditions of the enzyme synthesis. It was concluded that  $\alpha$ -amylase gene expression is regulated at the translational level. Most likely, higher efficiency of  $\alpha$ -amylase mRNA translation in vivo during de-repression of enzyme synthesis results in higher levels of enzyme protein under these conditions.

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## ABBREVIATIONS

BSA	Bovine serum albumin
cAMP	Cyclic adenosine-3',5'-monophosphate
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediamine tetra acetic acid
PMSF	Phenyl methyl sulfonyl fluoride
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
1XSSC	0.15M Sodium chloride 0.015M Sodium citrate
TCA	Trichloro-acetic acid
Tris	Tris(hydroxymethyl) amino methane

## CHAPTER I

### INTRODUCTION

Starch is one of the most abundant energy sources available. Consequently most of the organisms both in plant and animal kingdoms, have the capability of utilizing starch as an energy source. All of these organisms produce one or more enzymes which hydrolyze the starch into simple and metabolizable sugars. Hydrolysis of starch by such enzymes is also one of the oldest industrial applications of the enzymes. Because of the ease of cultivation of molds and microorganisms, starch hydrolyzing or amylolytic enzymes used in starch processing industries are usually derived from these sources. The fungus Aspergillus was not only the first organism used for the commercial production of amylolytic enzymes, it still continues to be the major source of these enzymes for use in industry (Barfoed, 1976).

Most species of Aspergillus produce  $\alpha$ -amylase, glucoamylase and  $\alpha$ -glucosidase enzymes as parts of their amylolytic systems. However, various species do differ in the production of relative proportions of these enzymes (Fogarty and Kelly, 1980) and that forms the basis of preference for certain species for industrial production of certain enzymes: e.g. A. niger is the organism of choice for glucoamylase production and A. oryzae is usually used for the production of  $\alpha$ -amylase (Barfoed, 1976). Although these enzymes have been extensively characterized from the point of view of their catalytic activities as generally is the case for most industrially significant enzymes, very few investigations have

been directed towards the elucidation of mechanisms regulating the gene expression of these enzymes. Elucidation of the mechanisms controlling gene expression in fungi, is not only important in terms of understanding gene expression in lower eukaryotes but also is of considerable significance if one is to improve these organisms for exploitation in industry.

The synthesis of hydrolytic enzymes in microorganisms, in general, is governed by induction, catabolite repression or both. In fact it was the study of these phenomena regarding lactose utilization by Escherichia coli which led to the postulation of the operon concept of gene expression by Jacob and Monod (1961). Since that time many additional operons have been found to exist in prokaryotes (for review see The Operon, 1980, edited by J.H. Miller and W.S. Reznikoff) and the molecular basis of their regulation has been elucidated to a considerable extent. In contrast the mechanisms underlying gene regulation in eukaryotes are just beginning to be understood. It seems that operons do not exist in eukaryotes, although certain related genes may be expressed in coordination. Recent reports dealing with regulation of genes involved in amino acids biosynthesis (Hinnenbusch, 1986), galactose utilization (Guarente et al., 1982) in Saccharomyces, quinic acid utilization in Neurospora (Giles et al., 1985) and acetamide utilization in Aspergillus (Hynes et al., 1985) indicate that modulation of gene expression in fungi is much more complex than in prokaryotes and involves multicircuitary regulatory systems.

Despite the apparent differences in gene organization in prokaryotes and eukaryotes, carbon catabolite repression or glucose repression is an important regulatory system in organisms belonging to both of these groups. Although the catabolite repression has been subject of extensive research over the last three decades, this phenomenon continues to be poorly understood. According to an hypothesis originally formulated by Magasanik (1961), growth conditions that lead to an excess catabolism over anabolism would reduce the synthesis of catabolic enzymes. The rationale behind this hypothesis is that the catabolites which are formed from glucose or any other readily utilizable carbon source, accumulate in the cells and repress the enzymes whose activity would further increase the already large intracellular pool of these compounds. It is believed that phosphoenol pyruvate, a metabolite resulting from the catabolism of glucose or other hexose sugars, plays some role in the mediation of catabolite repression at least in prokaryotes. Phosphoenol pyruvate is known to play a major role in the transport of many sugars in bacteria through a phosphoenol pyruvate - phosphotransferase system (Kornberg, 1981). It is believed that in the absence of the sugars, phosphoenol pyruvate is available to phosphorylate membrane bound adenylate cyclase. The phosphorylated adenylate cyclase produces cAMP from ATP. The high intracellular cAMP concentration results in the release of enzymes from catabolite repression. The regulation of adenylate cyclase is then key to the mediation of catabolite repression. However, the observations

(Erlagaeva et al., 1977; Shulgina et al., 1979) that certain mutants of Escherichia coli that grow on glucose by phosphotransferase - phosphoenol pyruvate phosphorylation mechanism, have high intracellular cAMP concentration in the presence of glucose, contradict the proposed mechanism of adenylate cyclase regulation.

Whatever the mechanism of regulation of intracellular cAMP concentration, both genetic as well as molecular analysis of catabolite sensitive operons in prokaryotes suggest strongly for a role of cAMP in the mediation of catabolite repression (Ullman and Danchin, 1983). It is generally believed that cAMP binds to a receptor protein (CRP, sometimes referred to as catabolite activator protein or CAP) and this cAMP-CRP complex acts as a positive regulator by binding to the promoter sequences of catabolite repression sensitive operons. The observations over the last few years, however, cast doubt about this generally accepted simplistic mechanism of modulation of catabolite repression in prokaryotes. The observations worth consideration are as follows: The mutants which have high intracellular cAMP levels show persistent catabolite repression which cannot be relieved by exogenous cAMP thus indicating that the catabolite repression cannot be accounted for by cAMP deficiency (Wanner et al., 1978). Mutants lacking cAMP-CRP complex express catabolite sensitive operons at the levels comparable to wild type strains but still are sensitive to catabolite repression (Dessein et al., 1978; Guidi-Rontani et al., 1980) suggesting cAMP-CRP independent regulation of catabolite repression. These observations as well as

the facts that exogenous cAMP can lift the repression in wild type E. coli cells and cAMP-CRP complex binds and stimulates the transcription of catabolite sensitive operons (Ullman and Danchin, 1983), suggest that cAMP-CRP complex is probably required for efficient expression of catabolite sensitive operons but its direct involvement in the mediation of catabolite repression is questionable.

Recently, identification of a cellular factor referred to as catabolite modulator factor (CMF), concentration and action of which parallel the effects of glucose has lead Ullman (1985) to postulate another hypothesis for mediation of catabolite repression in prokaryotes. According to this hypothesis, glucose or some of its metabolites somehow increase the cellular concentration of CMF during catabolite repression. CMF is thought to be a negative regulator of transcription of sensitive operons and is also inhibitor of adenylate cyclase. In the absence of glucose or during the conditions of release from catabolite repression CMF concentration is low and hence the transcription of catabolite sensitive operons is uninhibited. Further enhancement of expression of such operons occurs by cAMP-CRP complex as CMF regulated inhibition of cAMP generating adenylate cyclase is also removed under these conditions. Basically then, according to this hypothesis cAMP is not the cause of catabolite repression rather a consequence.

In fungal organisms, the catabolite repression has been studied to a considerable extent only in yeasts. In these

organisms, it appears that hexokinase II plays a key role in the initial trigger of glucose repression. Mutants in the structural gene for hexokinase II are defective in glucose repression (Entian and Mecke, 1982) and the glucose repression in these mutants can be restored by transformation of the cells with hexokinase II gene but not by the genes of hexokinase I or glucokinase (Entian et al., 1984). These findings indicate that evocation of glucose repression by hexokinase II is independent of possible defect in hexose phosphorylation. In fact it has been shown that the hexokinase II mutants that are defective in glucose repression have the hexokinase II catalytic activity. The hexokinase II gene in these mutants is affected outside the coding region for its catalytic activity (Entian et al., 1985). In the light of these findings, it is thought that hexokinase II enzyme in yeasts is a bifunctional enzyme with a catalytic domain for glucose phosphorylation and a regulatory domain responsible for triggering glucose repression. Although there is sufficient evidence for the essential or key role of hexokinase II in triggering glucose repression including the fact that the glucose increases the synthesis of this enzyme during repression (Entian, 1986), the molecular mechanisms by which the hexokinase II brings about the repression of catabolite sensitive enzymes is unknown. According to a hypothesis by Entian et al., (1985) hexokinase II changes its conformation during phosphorylation of glucose. A conformation change of this enzyme results in phosphorylation-dephosphorylation of some unknown regulatory protein which in return brings about the repression in some

yet to be defined manner.

The role of cAMP, if any in the mediation of catabolite repression in fungal organisms has not been investigated to the same extent as in prokaryotes. However, there is strong evidence that cAMP is involved in the processes regulating growth, development and differentiation in the fungal organisms (Matsumoto et al., 1983; Williams et al., 1980; Zonneveld, 1980). It has also been reported to be involved in the modulation of synthesis of  $\alpha$ -glucosidase and invertase in yeasts (Schlanderer and Dellweg, 1974; Van Wijk and Konijm, 1971),  $\beta$ -glucosidase in Mucor (Borgia and Sypherd, 1977) and protease and trehalase enzymes in Aspergillus nidulans (Zonneveld, 1980). But unlike prokaryotes where extensive genetic and molecular analysis is now available to evaluate the role of cAMP in the catabolite repression, hard evidence regarding the role of cAMP in the mediation of catabolite repression in fungi is lacking. The genetic as well as molecular level analysis of several catabolite repressible genes of fungal organisms indicate that modulation of gene expression in fungi is more complex than observed so far in prokaryotes. The gene expression in fungal organisms in general is regulated by multicircuitary regulatory systems. For instance the SUC2 gene which encodes for invertase enzyme in Saccharomyces is regulated by at least six other genes referred to as SSN6, SNF1-SNF6 (Sarokin and Carlson, 1985; Abrams et al., 1986). The products of these regulatory genes modulate the SUC2 gene expression by interacting with each other as well as with the upstream regions of SUC2 gene. Similarly genes

encoding for galactose utilization enzymes in yeast have been shown to be regulated by the interaction of the products of at least two regulatory genes (Giniger et al., 1985; Guarente et al., 1982). Analysis of expression of quinic acid utilization genes in Neurospora (Giles et al., 1985) and acetamidase gene in Aspergillus (Hynes et al., 1985) indicates that modulation of gene expression in filamentous fungi occurs in a manner similar to yeasts. The utilization of quinic acid by Neurospora as a sole source of carbon under carbon deficiency conditions of growth requires the expression of four genes referred to as Qa genes. Based on the mutant analysis studies (Giles et al., 1985), it seems that a product of qaIF gene is a positive regulator of the four Qa genes. This regulatory qaIF gene itself is negatively regulated by a product of qaIS gene. In Aspergillus amdS gene encodes for an acetamide enzyme which enables this fungus to utilize acetamide as sole source of carbon and/or nitrogen under the conditions of starvation. This enzyme is inducible by  $\omega$ -amino acids such as  $\beta$ -alanine and  $\gamma$ -aminobutyric acid as well as by acetate. Both genetic and molecular level studies suggest that different inducers bring about their effects through different regulatory gene products (Hynes et al., 1985). Thus amdR gene is required for the induction of amdS gene expression by  $\omega$ -amino acids and amdA as well as facB genes are required for the induction by acetate. The products of these regulatory genes are believed to bind to different and distinct sequence motifs present at the 5' end of the amdS gene. Catabolite repression of the amdS

gene expression involves the product of another regulatory gene referred to as cre A. The cre A gene product brings about the repression of acetamidase probably by suppressing either synthesis or the activities of the positive regulatory proteins. Considering the complex multicircuitary nature of modulation of gene expression in fungal organisms, it is unlikely that cAMP, if involved in the mediation of catabolite repression in these organisms, would be doing so through a mechanism operative in prokaryotes.

Despite considerable advances in our understanding of modulation of gene expression in fungal organisms, very little is known about the mechanisms underlying the regulation of amylolytic enzymes in filamentous fungi.

Our present knowledge of  $\alpha$ -amylase and glucoamylase enzymes of Aspergilli in terms of isolation, characterization and regulation is briefly outlined below.

#### 1.1.0 $\alpha$ -AMYLASE

##### 1.1.1 Isolation and General Properties

$\alpha$ -Amylase ( $\alpha$ -1,4-glucan 4-glucanohydrolase, EC3.2.1.1, endoamylase) is an enzyme that degrades starch by cleaving internal  $\alpha$ -1,4-glucosidic bonds.  $\alpha$ -1,6-Glucosidic bonds are not attacked by this enzyme. The primary products of  $\alpha$ -amylase hydrolysis of starch are malto-oligosaccharides of varying lengths depending upon the source of the enzyme (Robyt and Whelan, 1968; Umecki and Yamamoto, 1975). Thus, the enzyme is different from  $\beta$ -

amylase, an exohydrolase producing maltose, and glucoamylase, an exohydrolase producing glucose.  $\alpha$ -Amylase occurs universally in prokaryotes and eukaryotes of both plant and animal origin. However for industrial use, this enzyme is usually obtained from bacterial or fungal sources, especially various species of Bacillus and Aspergillus being the organisms of choice.

Isolation and properties of  $\alpha$ -amylases from various species of Bacillus have been reviewed by Kindle (1983). In general, these bacterial enzymes hydrolyze starch to dextrans with an average chain length of 6-10 glucose units. Amylases from Bacilli are extremely heat stable with optimal temperature for activity in the 50° to 80°C range. As to the optimal pH values, enzymes from different sources are active over a wide range from pH 2.0 to pH 10. Similarly their molecular weights range from 45,000 to 70,000 daltons.

So far as Aspergillus species are concerned,  $\alpha$ -amylases have been purified from A. niger (Arai et al. 1968; Chong and Tsujisaka, 1976; Minolda and Yamada, 1963) and A. oryzae (Akabor et al., 1964; Fischer and DeMontmollin, 1951; McKelvy and Lee, 1969; Morita and Wadano, 1971). Fungal  $\alpha$ -amylases are usually glycoproteins containing 5-10% carbohydrates (Anai and Ikenaka, 1966; McKelvy and Lee, 1969). Their molecular weights are around 55,000 daltons. These enzymes show optimal activity around 50°C and at pH values around 5.0. Fungal  $\alpha$ -amylases differ from bacterial counterparts in the end products of starch hydrolysis in that these enzymes rapidly hydrolyze starch to maltose and

maltotriose with slow generation of glucose. Final products of the starch hydrolysis are usually maltose and glucose (Barfoed, 1976; Spencer-Martins and van Uden, 1979).

#### 1.1.2 Regulation of $\alpha$ -Amylase Biosynthesis

A considerable effort has been made to elucidate the control mechanisms involved in the biosynthesis of  $\alpha$ -amylases in Bacilli. In contrast, our knowledge in terms of the regulation of biosynthesis of these enzymes in fungal organisms is very limited. Synthesis of  $\alpha$ -amylase enzymes in general, is governed by induction, catabolite repression or both. All of these mechanisms seem to be operative in the regulation of amylases in Bacilli. Thus, regulation of  $\alpha$ -amylase synthesis in B. stearothermophilus seems to be controlled by both induction and catabolite repression (Welker and Campbell, 1963). On the other hand, the synthesis of amylases in B. amyloliquefaciens (Coleman and Grant, 1966), B. licheniformis (Saito and Yamamoto, 1975; Thirunavukkarasu and Priest, 1980) and B. subtilis (Coleman, 1967; Heineken and O'Connor, 1972) has been reported to be regulated, only by catabolite repression. Catabolite repression of  $\alpha$ -amylase synthesis occurs at the translational level in B. subtilis (Semets et al., 1973) and at both transcriptional and translational level in B. amyloliquefaciens (Gould et al., 1975).

In Aspergilli, the regulation of  $\alpha$ -amylases has only been studied to some extent in A. oryzae. The enzyme has been shown to be inducible in response to starch or maltose in the culture

medium (Angelova et al., 1980; Erratt et al., 1984; Yakubi et al., 1977). In addition to induction by  $\alpha$ -1,4-glucosidic containing polymers,  $\alpha$ -amylase in A. niger also seems to be sensitive to catabolite repression (Angelova et al., 1980). However, a study of  $\alpha$ -amylase production by this organism, reported by Vallier et al., (1977) leads to the conclusion that the enzyme synthesis is solely regulated by catabolite repression. Similarly there are conflicting reports about  $\alpha$ -amylase gene expression at the molecular level in A. oryzae. Based on the studies of in vivo and in vitro  $\alpha$ -amylase synthesis, Erratt et al. (1984) have suggested that control of synthesis occurs at the transcriptional level. But according to a report by Angelova et al. (1983), the enzyme synthesis is regulated at the level of mRNA translation. Regulation of  $\alpha$ -amylase biosynthesis in A. oryzae, has also been reported to occur at both transcriptional and translational level (Yurkevich et al., 1984).

So far as A. awamori is concerned,  $\alpha$ -amylase has not been extensively characterized and essentially nothing is known regarding the regulation of its gene expression.

### 1.2.0 GLUCOAMYLASE

#### 1.2.1 Isolation and General Properties

Glucoamylase ( $\alpha$ -1,4-D-glucan glucohydrolase, EC3.2.1.3) is an exo-acting enzyme that yields  $\beta$ -D-glucose from the non-reducing chain-ends of amylose, amylopectin and other related oligo- or polysaccharides, by hydrolysing  $\alpha$ -1,4 and  $\alpha$ -1,6 linkages. Glucoamylases occur almost exclusively in fungi and have been isolated

from Aspergillus awamori (Hayashida, 1975; Ryzhakova and Feniksova, 1972; Yamasaki et al., 1977), A. niger (Lineback and Aira, 1972; Pazur et al., 1971; Svensson et al., 1982), A. oryzae (Miah and Ueda, 1977), Mucor rouxianus (Tsuboi et al., 1974) and Rhizopus formosaensis (Lai et al., 1974). Most of these organisms produce more than one type of glucoamylase differing in either physical or physicochemical properties. Thus A. niger produces two types of glucoamylases referred to as G1 and G11 with apparent molecular weights of 65,000 and 55,000 respectively as estimated by gel filtration or 85,000 and 75,000 respectively when determined by electrophoretic mobilities (Svensson et al., 1982). Both forms of the enzyme reportedly are glycoproteins with 18-20% carbohydrate content and have identical NH<sub>2</sub>-terminal amino acid sequences but differ in the COOH-terminal region (Svensson et al., 1982). However, according to a report by Pazur et al., (1971) two types of glucoamylases produced by A. niger have molecular weights of 112,000 and 99,000 respectively. Both forms have identical NH<sub>2</sub>-terminal amino acid sequences but unlike the finding of Svensson et al. (1982), these two types of enzyme have different carbohydrate content.

A. awamori has been reported to produce three forms of glucoamylases (Hayashida, 1975) referred to as type 1, 1' and 11 with molecular weights of 90,000, 83,000 and 62,000 respectively. Recently glucoamylase genes have been cloned and sequenced from both A. awamori (Nunberg et al., 1984) and A. niger (Boel et al., 1984a). The glucoamylase genes from both organisms have identical

sequences and are present as single copy in both of these organisms. The glucoamylase genes from these fungi contain four intervening sequences which thus are not found in the mature mRNA. While at least in A. niger a fifth intron towards the 3'-end of mRNA is retained in G1 type mature mRNA and processed out in G11 type mature mRNA, thus resulting in G1 and G11 types of glucoamylases with identical NH<sub>2</sub>-terminal sequences but differing in COOH-terminal region (Boel et al., 1984b). Although, the A. awamori glucoamylase gene is identical in every respect to the A. niger gene, there is yet no direct evidence that differential processing of mRNA leads to two or more types of mature mRNA and two or more types of glucoamylases. According to Yoshino and Hayashida (1978) A. awamori synthesizes a single high molecular weight glucoamylase and various proteases and glycosidases synthesized by the fungus, degrade the native enzyme stepwise to generate the multiple forms often observed in this organism.

With regards to the properties of glucoamylases, in general, high molecular weight species (G1 or type 1) seem to be highly active in raw starch hydrolysis where as low molecular weight ones have very weak activity towards raw starch (Hayashida, 1975; Svensson et al., 1982; Ueda and Kano, 1975). Besides the differences in molecular weights and raw starch digestibility, all forms of glucoamylases have similar physico-chemical properties. The pH optima of glucoamylases are generally in the range of 4.5-5.0 (Lineback and Baumann, 1970; Finch and Leonard, 1978; Miah and Ueda, 1977; Yamasaki et al., 1977). The temperature optima of most

glucoamylases are in the range of 40°-60°C (Miah and Ueda, 1977; Tsuboi et al., 1974). In general, these enzymes are stable up to 40°-45°C (Miah and Veda, 1977, Morita et al., 1966; Yamasaki et al., 1977). The stability of these enzymes at high temperatures has been attributed to their glycoprotein nature (Yoshino and Hayishida, 1978; Pazur et al., 1970). Rates of hydrolysis of various glucose polymers by glucoamylases depend upon the kind of bonds and chain lengths of the polymers.  $\alpha$ -1,6-Glucosidic bonds are hydrolysed more slowly than  $\alpha$ -1,4 linkages (Reily, 1979; Yamasaki et al., 1977). The glucoamylases from A. awamori and Rhizopus are most effective in hydrolysing the  $\alpha$ -1,6 glucosidic bonds (Reily, 1979; Ueda, 1981). Rates of hydrolysis of substrates with  $\alpha$ -1,4 linkages continue to increase with chain length, at least up to 6-7 units of glucose. Thus, rates of hydrolysis of maltotriose and amylose by glucoamylases are about 1.5-5 and 4-10 times respectively the rate of maltose hydrolysis (Smiley et al., 1971; Yamasaki et al., 1977).

### 1.2.2 Regulation of Glucoamylase Biosynthesis

Because of their significance in the starch processing industries, considerable attention has been given to studying the effect of the composition of the growth medium on the production of glucoamylases by various fungal organisms. Yet, the mode of regulation of biosynthesis of these enzymes continues to be vaguely understood. A. awamori has been reported to produce glucoamylase when grown on medium containing either glucose or

starch as a carbon source (Hayashida, 1975). However, the glucoamylase synthesis reportedly occurs during the early period of growth on starch and during the late period of growth on glucose; thus implying that induction by polysaccharides is not necessary for the enzyme biosynthesis. In A. niger, grown on a wide variety of carbohydrates, the maximum production of glucoamylase seems to occur at the end of growth or during the stationary period (Barton et al., 1969, 1972). Moreover, growth of this fungus on starch or maltose as a carbon source, results in the production of higher levels of glucoamylase compared to growth on monosaccharides (Barton et al., 1972). In terms of the regulation of the biosynthesis of glucoamylases by Aspergilli, these studies taken together, seem to indicate that the enzyme synthesis may be regulated by catabolite repression. Whether any inducer or inducers are required for the biosynthesis of glucoamylases by these organisms, needs to be clarified. Although considerable efforts have been made to analyse the structure of glucoamylase gene in Aspergilli (Boel et al., 1984; Nunberg et al., 1984), our knowledge regarding the molecular basis of its expression is completely lacking.

#### Scope of the Thesis

The purpose of the present study was to clarify and shed some additional light on the cellular and molecular mechanisms underlying the regulation of gene expression of  $\alpha$ -amylase and glucoamylase enzymes of A. awamori. To this end, both of these enzymes

were purified and characterized. Only the characterization of  $\alpha$ -amylase is presented in detail as the isolation and properties of glucoamylase enzyme have previously been reported by several investigators.

In terms of regulation at the cellular level, studies dealt with the role of induction, catabolite repression as well as cAMP in the biosynthesis of the  $\alpha$ -amylase and glucoamylase enzymes by A. awamori. The biosynthesis of the enzymes was followed by measuring the enzyme activities as well as in vivo protein labelling studies.

The molecular level of regulation of these enzymes was studied at the level of mRNA. The quantitation of  $\alpha$ -amylase mRNA was based on the measurement of its in vitro template activity i.e. functional mRNA analysis. The glucoamylase gene expression was studied both by its functional as well as actual mRNA concentration analysis. The functional analysis as for  $\alpha$ -amylase was based on in vitro translation assays and the actual mRNA concentration was analysed by hybridization studies (Northern and dot blot analysis) using glucoamylase specific gene probe.

CHAPTER-II

MATERIALS AND METHODS

2.1 Organism and culture conditions

Aspergillus awamori (ATCC 22342) was maintained on agar slants composed of 3% (w/v) glucose in the following basal medium: 5g NaNO<sub>3</sub>, 1g KH<sub>2</sub>PO<sub>4</sub>, 0.5g KCl, 0.5g MgSO<sub>4</sub>.7H<sub>2</sub>O, 0.1g FeSO<sub>4</sub>.7H<sub>2</sub>O and 1 litre tap water; adjusted to pH 5.6. For the growth and enzyme production studies the following media were used:

Glucose media - contained 2% (w/v) glucose plus the basal medium.

Starch media - contained 2% (w/v) soluble starch plus the basal medium.

Starvation media - contained the basal medium without any carbon source added to it.

For the enzyme purifications, the fungus was cultured in Fernback flasks containing 500 ml of starch medium. In my experience, spores inoculated directly into starch medium grew very poorly. To get around this problem, spores (10<sup>8</sup>/ml) were pre-germinated for 12 hours in the glucose medium. Culture flasks were then inoculated to 2% with the germinated spore suspension. Incubations were carried out at 30°C for 5 days on a rotary shaker at 150 rev/min.

For the time course studies of production of enzymes, the organism was cultured in 250 ml flasks containing 50 ml of appropriate medium. Incubation for various periods was carried out at 30°C on a rotary shaker at 150 rev/min.

## 2.2 Purification of $\alpha$ -amylase and glucoamylase enzymes

Both of these enzymes were purified from the culture medium using the following procedure:

### Step 1 - Ethanol precipitation

To the culture filtrate (500 ml), ethanol previously chilled to -20°C was added dropwise at 4°C with gentle and continuous stirring to the final concentration of 75% (v/v) and the solution was left at -20°C for 24 hours. The resultant precipitate was collected by decantation and centrifugation.

### Step 2 - Sephacryl-200 column chromatography

Ethanol precipitated protein (14 mg) was dissolved in 0.05M sodium acetate buffer, pH 5.3, and applied to a Sephacryl-200 column (2.5 x 75 cm) equilibrated previously with 0.05M acetate buffer, pH 5.3, containing 0.05M KCl. Elution was carried out with the same buffer. The fractions (5 ml) were collected using a Pharmacia F-3000 fraction collector equipped with a U.V. light absorbance monitor. The fractions containing amylolytic enzyme activities were pooled and concentrated by membrane ultrafiltration using an Amicon ultrafiltration cell. The concentrated solutions were dialyzed against 0.01M sodium acetate buffer, pH 5.3, for 6 hours.

Step 3 - Anion-exchange chromatography on Dowex AGI-X4 resin

The enzyme protein fractions obtained from Step 2 were subjected to anion-exchange chromatography. Protein samples were applied to a Dowex anion-exchange resin column (2.5 x 5 cm) previously equilibrated with 0.01M acetate buffer, pH 5.3. The column was washed with 3-4 bed volumes of the same buffer and the enzyme proteins were then eluted with stepwise salt gradients consisting of 0.05M, 0.1M, 0.2M, 0.3M and 0.4M NaCl in the same buffer. The eluates containing enzyme activities were again concentrated by membrane ultrafiltration, dialyzed against 0.05M acetate buffer, pH 5.3 and stored at 4°C.

2.3 Assay of  $\alpha$ -amylase activity

$\alpha$ -Amylase activity was assayed using one of the following two methods. One assay for  $\alpha$ -amylase was based on the reduction in blue color resulting from enzyme hydrolysis of starch. Each reaction mixture consisted of about 30  $\mu$ g enzyme protein in 3 ml of 0.05M acetate buffer, pH 5.3, containing 0.6% soluble starch. After 10 min of incubation at 37°C, 0.2 ml of the reaction mixture was added to 1 ml of an iodine solution (1% KI + 0.3% I<sub>2</sub>). The resulting solution was then diluted to 5 ml with distilled water and the color intensity was read at 550 nm with a Bausch and Lomb Spectronic-20 spectrophotometer.

One unit of  $\alpha$ -amylase was defined as the quantity of protein producing a change in O.D.<sub>550</sub> of 0.1 under the conditions described above.

The second method of  $\alpha$ -amylase activity assay was based on the production of reducing sugars upon starch hydrolysis by  $\alpha$ -amylase. This method was routinely used for measuring the  $\alpha$ -amylase activity in the culture media. The enzyme activity in 0.5 ml aliquots of dialyzed culture filtrates was assayed in 3 ml reaction volume containing 0.05M acetate buffer, pH 5.3 and 0.6% soluble starch. After incubating the reaction mixture for 10 min at 37°C, the amount of reducing sugars was measured by the dinitro-salicylic acid assay method of Fisher and Stein (1961).

One unit of  $\alpha$ -amylase was defined as the amount of enzyme required to produce 1  $\mu$ mole maltose equivalent of reducing sugars per min under the conditions described above.

#### 2.4 Assay of glucoamylase enzyme activity

The glucoamylase activity was determined by measuring the production of glucose from soluble starch. The reaction mixture consisted of either 30  $\mu$ g enzyme protein or 0.5 ml of culture filtrates, 0.6% soluble starch and 0.05M acetate buffer, pH 5.3, in a total volume of 3 ml. After 10 min of incubation at 37°C, 0.5 ml aliquots were withdrawn and the amount of glucose was determined according to the glucose oxidase-peroxidase method of Papadopoulos and Hess (1960) as modified by Dahlqvist (1961) using the glucose determination kit from Sigma.

One unit of glucoamylase was defined as the amount of enzyme required to produce 1  $\mu$ mole of glucose per min under the standard assay conditions.

## 2.5 Assay of $\alpha$ -glucosidase enzyme activity

The  $\alpha$ -glucosidase activity was determined exactly as for glucoamylase except that maltose was used as a substrate in place of soluble starch.

## 2.6 Estimation of protein

Protein was estimated by the method of Bradford (1976) using reagents from Biorad.

## 2.7 Growth measurements

For growth measurements, the culture flasks were removed at various intervals and mycelial mass was collected by filtration on Whatman-1 filter papers. Excess fluid was removed by squeezing the mycelial patties between Whatman-3MM filter papers. Growth was defined as wet weight of mycelial mass per flask.

## 2.8 Glucose determination

The levels of glucose in the culture medium were determined at various intervals by glucose oxidase-peroxidase method as described above (section 2.4).

## 2.9 cAMP extraction and determination

For the measurements of intracellular concentration of cAMP, mycelial mass was collected by filtration at various intervals, frozen in liquid nitrogen and stored at  $-70^{\circ}\text{C}$ . After all the samples had been collected, cAMP concentration in each was determined using the following procedure. The cell mass was immersed in 6% TCA (1g/10 ml) and homogenized with the use of a Braun homoge-

nizer. Cell debris was removed by centrifugation (15,000 x g, 10 min at 4°C) and the supernatant was extracted three times with equal volume of water-saturated ether. The aqueous phase obtained after ether extraction was evaporated to dryness and re-dissolved in 3 ml of 0.05M sodium acetate buffer, pH 6.2. The cAMP content in each sample was then determined without further purification by the radioimmuno assay system of Steiner et al (1972) using a cAMP determination kit purchased from New England Nuclear. Efficiency of cAMP extraction was routinely determined by including  $4 \times 10^3$  CPM of [<sup>3</sup>H]-cAMP during the extraction procedure. Recovery of the [<sup>3</sup>H]-cAMP was usually 75 to 80%.

#### 2.10 In vivo protein labelling

To study the rate or patterns of enzyme synthesis, fungal cells were labelled in 10 ml basal medium containing 100 µCi of [<sup>35</sup>S]-met (>1000 Ci/mmol) and various carbohydrates and other effectors as indicated. After incubating for the indicated time at 30°C, mycelia were separated by filtration and the filtrates were used for the studies of extracellular proteins. The mycelial mass thus collected was thoroughly washed with 0.05M NaCl solution and was homogenized in 10 ml solution containing 50 mM Tris-Cl, pH 6.8, 2 mM EDTA and 5 mM PMSF. Cell debris was removed by centrifugation (15,000 x g, 10 min at 4°C). The supernatant thus collected was used for intracellular protein analysis studies. All the samples were stored at -70°C.

### 2.11 Analysis of the labelled proteins

Proteins from both intracellular and extracellular samples (0.1 - 0.2 ml) were precipitated by adding 500 µg/ml BSA and TCA to the final concentration of 10% (v/v). The precipitates were washed three times with chilled (-20°C) acetone and finally dissolved in 30-50 µl of SDS-PAGE sample buffer (0.062M Tris-Cl, pH 6.8, 2% SDS, 10% glycerol and 0.2% bromophenol blue) containing 5% 2-mercaptoethanol by heating in a boiling water bath for 4-5 min. Proteins were then analyzed by SDS-PAGE.

### 2.12 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed according to the method of Laemmli (1970) with the use of a 10% acrylamide separating gel and 4% acrylamide stacking gel.

### 2.13 Fluorography

Fluorography was performed by the modification of a method by Laskey and Mills (1975). After electrophoresis protein gels were fixed in 10% TCA solution for 1-2 hours. Gels were then submerged in Enhance solution (New England Nuclear) for 1 hour. After Enhance treatment, gels were washed for 1 hour in water and dried with a gel dryer. The gels were then fluorographed by exposing to Kodak XAR-5 X-ray films at -70°C for the indicated time periods.

#### 2.14 Estimation of molecular weight of labelled protein bands in fluorographs

To determine the molecular weight of the labelled protein bands visualized by fluorography, during electrophoresis of the proteins, molecular weight standards were run in parallel. After electrophoresis, the gels were stained for 1 hour with 0.25% (w/v) Coomassie blue-R dye in methanol - acetic acid-water solution (4:1:5, by volume) and destained in the same solution without the dye. The gels were then fluorographed as described above. The molecular weight of appropriate protein bands in the fluorographs was estimated by their relative mobilities compared to the standards. The molecular weight standards used were: myosin, 200 000;  $\beta$ -galactosidase, 116 000; phosphorylase b, 94 000; BSA, 67 000; ovalbumin, 43 000; carbonic anhydrase, 30 000; trypsin inhibitor, 20 000.

#### 2.15 Antibody production

Antibodies were raised against purified enzyme protein preparations in white New Zealand male rabbits. The rabbits were injected subcutaneously with 100-200  $\mu$ g of proteins emulsified in Freund's complete adjuvant. After three weeks of initial injections, rabbits were boosted with 100  $\mu$ g of antigens in Freund's incomplete adjuvant. One week after the booster injections, blood was collected and the IgG fraction was isolated by  $(\text{NH}_4)_2\text{SO}_4$  precipitation followed by ion-exchange chromatography on DEAE-cellulose essentially as described by Mayer and Walker (1978).

## 2.16 Immunoprecipitations

To samples of in vivo synthesized proteins as well as reaction mixtures of reticulocyte cell free translations, was added 1 volume of a buffer solution containing 0.5M Tris-Cl, pH 7.5, 1% (v/v) Triton-X-100 and 1 mg/ml BSA. Appropriate amounts of antisera raised against different antigens were then added and allowed to react for 16 hours at 4°C. In the case of in vivo protein synthesis samples, immunoprecipitations were collected directly by centrifugation (10,000 x g, 10 min at 4°C) and washed 3-4 times with 0.15M Tris-Cl, pH 7.5, containing 0.5% (w/v) SDS and 2 mM EDTA. Protein-A Sepharose suspension (2 µg protein-A/µg antiserum) was added to in vitro protein synthesis samples. After 30 minutes of incubation at room temperature, protein-A Sepharose bound immunoprecipitates were pelleted by centrifugation and washed as above. Finally, immunoprecipitates were dissolved by heating for 5 min in SDS-sample buffer containing 10% 2-mercaptoethanol and analyzed by SDS-PAGE.

## 2.17 RNA isolation

Total cellular RNA was isolated from A. awamori mycelia by modification of the guanidinium thiocyanate procedure of Chirgwin et al (1979). Mycelia were ground to powder under liquid nitrogen with a mortar and pestle. The resulting cell powder was suspended (1 g/10 ml) in 0.1M Tris-Cl, pH 7.4 buffer containing 0.15M LiCl, 10 mM EDTA, 2 mM DTT, 2% N-lauroylsarcosine and 1 mg/ml heparin. The suspension was homogenized for 1 min with a Braun homogenizer

and cell debris was removed by centrifugation (15,000 x g, 10 min at 4°C). The supernatant thus collected was extracted with equal volume of phenol-chloroform (1:1) solution and the aqueous phase was collected. Total RNA from the aqueous phase was precipitated by adding sodium acetate solution to 0.25M followed by 2.5 volumes of ethanol and chilling the mixture at -20°C for 2 hours. The RNA precipitate was collected by centrifugation (10,000 x g, 10 min at 4°C) and washed with ethanol. The RNA pellet was then dissolved in a solution containing 6M guanidinium thiocyanate, 10 mM Tris-Cl, pH 7.4, 2 mM DTT and 0.5% N-lauroylsarcosine. RNA was pelleted through a cushion of 5.7M CsCl as described by Glisin et al (1974). RNA pellet was dissolved in sterile distilled water and RNA was precipitated twice with ethanol. Finally RNA was dissolved in sterile distilled water at a concentration of 2-5 mg/ml and stored at -70°C.

#### 2.18 Selection of Poly(A)<sup>+</sup> RNA

For synthesis of cDNA and preparation of screening probes, polyA<sup>+</sup> containing RNA was separated from the total cellular RNA. Usually 5 mg of total RNA in 0.5 ml of sterile distilled water was denatured by heating to 65°C for 10 minutes followed by quick chilling in ice-bath. RNA solution was then made to contain 10 mM Tris-Cl (pH 7.4), 0.5M LiCl, 5mM EDTA and 0.2% SDS. This RNA solution was loaded onto oligo(dT)-cellulose column (dT-cellulose to the height of about 1 cm in 5 ml disposable plastic syringe) previously equilibrated with a buffer solution containing 20 mM

Tris-Cl, pH 7.4, 0.5M LiCl, 5 mM EDTA and 0.2% SDS. The column was washed with 8 column volumes of the above buffer solution to elute the unbound RNA. PolyA<sup>+</sup> RNA was then eluted with 2-3 volumes of a solution containing 10 mM Tris-Cl pH 7.4 and 1 mM EDTA and precipitated with ethanol. Usually two cycles of oligo-dT chromatography were used for PolyA<sup>+</sup> RNA preparation.

### 2.19 cDNA synthesis

1st-strand synthesis was carried out by the modification of a method described by Maniatus et al., (1982). The reaction mixture consisted of the following components in a total volume of 50  $\mu$ l.

- 10  $\mu$ g PolyA<sup>+</sup> RNA (from the starch medium cells)
- 50 mM Tris-Cl, pH 8.3
- 10 mM MgCl<sub>2</sub>
- 140 mM KCl
- 100  $\mu$ g/ml oligo(dT) 12-18
- 10 mM DTT
- 20 units RNasin
- 1 mM each dNTPs
- 25  $\mu$ Ci [ $\alpha$ -<sup>32</sup>P]dCTP
- 2 mg/ml Actinomycin-D
- 100 units Reverse Transcriptase

The reaction was carried out for 1 hour at 43°C. After 1 hour reaction was stopped by adding EDTA to 20 mM. The reaction mixture was extracted with equal volume of phenol-chloroform (1:1) solution. Aqueous phase was separated and ammonium sulfate

solution was added to 2M. RNA-cDNA hybrid was then precipitated from this solution by adding 2.5 volumes of ethanol and chilling the solution on dry ice for 20 minutes. The precipitate was collected by centrifugation, washed with ethanol, dried under vacuum and dissolved in 10  $\mu$ l of sterile distilled water.

2nd - strand was synthesized essentially as described by Gubler and Hoffman (1983).

#### 2.20 Cloning of cDNA

Poly(dC) tailing of cDNA, annealing to poly(dG) tailed plasmid pBR322 and transformation of E. coli HB101 cells were carried out essentially as described by Maniatis et al., (1982).

#### 2.21 Preparation of cDNA probes

To screen the cDNA libraries by differential hybridization signals, probes were prepared by synthesizing 1st-strand cDNAs from the polyA<sup>+</sup> RNA isolated from glucose and starch media cells. The 1st-strand synthesis was carried out as described above except dCTP concentration in the reaction mixture was reduced to 0.5 mM and labelled dCTP concentration was increased to 2  $\mu$ Ci/ $\mu$ l. RNA from the RNA-cDNA hybrids was removed by hydrolysis with 0.3M NaOH for 1 hour at 65°C. Excess NaOH in the solution was neutralized by adding Tris-Cl, pH 7.0 and NaCl to the final concentration of 0.5M and 1.5M respectively.

## 2.22 In vitro translation

In vitro translation of RNA was carried out in the rabbit reticulocyte cell free translation system essentially as recommended by the supplier of the translation kit. A typical translation reaction mixture contained in a total volume of 30  $\mu$ l: 5  $\mu$ g of total RNA, 1  $\mu$ Ci/ $\mu$ l [ $^{35}$ S]-met and 70% (v/v) reticulocyte lysate mixture.  $K^+$  and  $Mg^{++}$  ions concentrations in the reaction mixture were adjusted to 180 mM and 1.2 mM respectively. These concentrations were determined to be optimal for A. awamori RNA translation in rabbit reticulocytes (Appendix 1). The amount of RNA (5  $\mu$ g) used for translation was determined to be non-saturating under these in vitro translation assay conditions (Appendix 1). The reaction mixture was incubated for 1 hour at 30°C. The reaction was stopped by hydrolyzing the RNA with ribonuclease-A (50  $\mu$ g/ml) for 20 min at 35°C.

## 2.23 RNA gel electrophoresis

Total cellular RNA was separated by agarose gel electrophoresis under denaturing conditions following a procedure of Thomas (1980). RNA (10-15  $\mu$ g) was denatured by incubating for 1 hour at 50°C with 1M glyoxal, 50% (v/v) DMSO and 10 mM sodium phosphate, pH 7.0, in a total volume of 10  $\mu$ l. The reaction mixture was then cooled on ice and 2.5  $\mu$ l of sample buffer (50% (v/v) glycerol, 10 mM sodium phosphate, pH 7.0 and 0.25% (w/v) bromophenol blue) was added. The samples were then electrophoresed on horizontal 1.2% agarose gels in 10 mM phosphate buffer, pH 7.0,

using a BRL agarose gel electrophoresis chamber. Routinely, Hind III restriction fragments of lambda DNA treated as above were co-electrophoresed to serve as size markers.

#### 2.24 Transfer of RNA from gels to nitrocellulose filters (Northern Blotting)

Glyoxylated RNA from the agarose gels was transferred to nitrocellulose papers essentially as described by Thomas (1980). After transfer was complete (12-16 hours), the blots were dried and baked for 2.5 hours at 80°C under vacuum.

#### 2.25 Preparation of RNA dot blots

RNA samples in 5X SSC were spotted directly onto nylon membranes. After air drying the spots, RNA was crosslinked to the nylon membrane by exposing the blots to UV light for 3 min.

#### 2.26 Blot Hybridizations

Both Northern and dot blots were prehybridized for 6 hours at 42°C in the following solution:

50% (v/v) Formamide

5X SSC, pH 7.0

50 mM Sodium phosphate, pH 7.0

0.01M EDTA

0.5% SDS

250 µg/ml denatured salmon sperm DNA

0.02% BSA

0.02% Ficoll

0.02% polyvinylpyrrolidone

After 6 hours of prehybridization, blots were removed and hybridized for 24-36 hours to the radio-labelled probe in the above buffer at 42°C.

The blots were then washed with 3 changes of 2X SSC containing 0.1% SDS and 0.05% sodium pyrophosphate for 10 min each at room temperature. The final washing was done for 20 min at 62°C in 0.1X SSC solution. The blots were damp dried, wrapped in Saran Wrap and exposed to XAR-5 X-ray films at -70°C, using Kodak intensifying screens.

#### 2.27 Preparation of glucoamylase gene probe

The probe used for analysing glucoamylase specific mRNA sequences was a 3.4 kb glucoamylase specific genomic DNA insert in plasmid pGARI (Nunberg et al., 1984) cloned in E. coli strain MM294. For the isolation of plasmid DNA, the cell growth and plasmid amplification were done essentially as described by Clewell and Helinsky (1972). Plasmid DNA was then isolated by the alkaline lysis method of Birnboim and Doly (1979) and purified by equilibrium centrifugation in cesium chloride essentially as described by Maniatis et al. (1982). The plasmid DNA was restricted with EcoRI enzyme and the 3.4 kb glucoamylase specific insert was isolated by agarose gel electrophoresis and electroelution essentially as described by Maniatis et al. (1982).

### 2.28 Radio-labelling of probe

The glucoamylase specific genomic insert obtained as above was radiolabelled with ( $\alpha$ - $^{32}\text{P}$ )-dCTP by the Nick Translation procedure of Rigby *et al.* (1977) using a Nick Translation kit from Amersham. Usually, 0.5  $\mu\text{g}$  DNA was labelled to the specific activity of  $0.5 - 1.0 \times 10^8$  CPM/ $\mu\text{g}$  DNA.

### 2.29 In vivo labelling of ribonucleic acids

The fungal cells were cultured in various media in the presence of [ $^3\text{H}$ ]-uracil (50  $\mu\text{Ci/ml}$ ). After one hour of incubation, further incorporation of label into ribonucleic acids was stopped by the addition of 1000-fold excess of unlabelled uracil and the cells were cultured for the indicated time. The labelled cells collected at various times were washed extensively with sterile water and total cellular RNA was prepared as described above.

### 2.30 Hybridization of in vivo labelled RNA to immobilized glucoamylase specific DNA

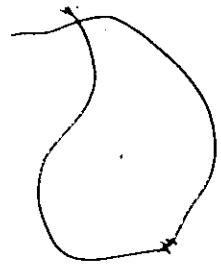
The glucoamylase specific genomic DNA (EcoRI fragment of plasmid pGAR1) was denatured by adding NaOH to 0.1M and incubating the solution for 10 minutes at 65°C. The solution was chilled on ice and neutralized by adding one volume of a solution containing 1.0M Tris-Cl, pH 7.0 and 3.0M NaCl. Denatured DNA solution (5  $\mu\text{g}$  DNA) was spotted on nitrocellulose filters (4  $\text{mm}^2$ ). After air drying, the filters were baked for 2 hours at 80°C under vacuum. The filters containing immobilized DNA were pre-hybridized for 4 hours at 42°C in a solution containing 20 mM PIPES (piperazine-N,

N-bis-2-ethanesulfonic acid, pH 6.4), 60% (v/v) formamide, 0.02% bovine serum albumin, 0.02% polyvinyl-pyrrolidone, 0.02% Ficoll, 0.02% SDS, 25 µg/ml poly(dA) and 100 µg/ml E. coli tRNA. The filters were then hybridized to the labelled RNA in the above solution with the replacement of tRNA with the labelled fungal RNA. Hybridization solution for each filter contained 0.2 mg labelled RNA ( $>2.0 \times 10^6$  CPM) in a total volume of 0.2 ml. Hybridizations were carried out for 48 hours at 42°C. After hybridization the filters were washed for 10 minutes at 65°C in a solution containing 10 mM Tris-Cl, pH 7.4, 0.15M NaCl and 2 mM EDTA. Unhybridized RNA still present on the filters was hydrolysed by treating the filters with ribonuclease (100 µg/ml in 10 mM Tris-Cl, pH 7.4 and 0.15M NaCl) for 20 minutes at 37°C. The filters were washed twice as described above dried and assayed for radioactivity. The counts of all the filters were corrected for non-specific radioactivity by subtracting the counts obtained from the control filters. The control filters contained pBR322 DNA instead of glucoamylase specific DNA in the hybridization experiment.

### 2.31 Materials

Various carbohydrates, nucleotides and glucose determination kit were obtained from Sigma. cAMP assay kit and Enhance were purchased from New England Nuclear. Gel electrophoresis material was from Biorad. Protein-A Sepharose and EcoRI restriction enzyme were obtained from Pharmacia. Rabbit reticulocyte in vitro trans-

lation kit, Nick Translation kit, [ $^{35}\text{S}$ ]-met (>1000 Ci/mmol) and NCS tissue solubilizer were acquired from Amersham. All the other chemicals used in this work were of reagent grade and obtained from commercial sources.



CHAPTER III

RESULTS

3.1.0 Purification and Properties of  $\alpha$ -Amylase and Glucoamylase

Enzymes

3.1.1 Purification

To characterize the extracellular  $\alpha$ -amylase and glucoamylase enzymes of A. awamori, these enzymes were purified from the culture medium. Proteins in the culture medium were precipitated with ethanol without any significant loss of amylolytic enzyme activities. The ethanol precipitated crude enzyme extract when passed through the Sephacryl-200 column showed one minor and one major protein peak (peaks I and II respectively in Fig. 1). The major protein peak contained both  $\alpha$ -amylase and glucoamylase activities. These enzymes were further purified from the Sephacryl-200 eluted major protein peak by anion-exchange chromatography.

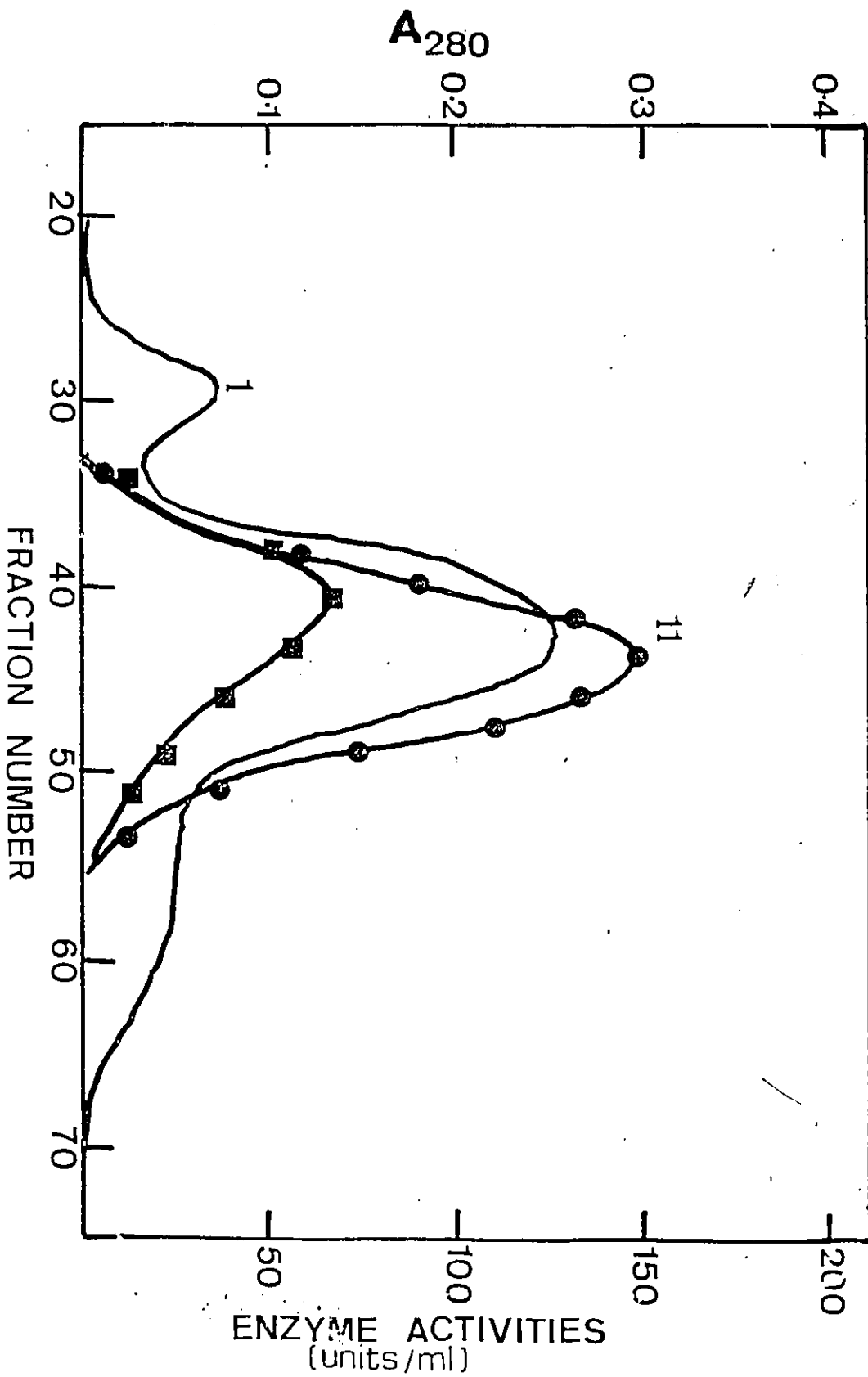
As mentioned above the attempts to separate  $\alpha$ -amylase and glucoamylase proteins by Sephacryl-200 gel filtration were unsuccessful. Both of these proteins were always found together in the Sephacryl-200 eluted protein fractions. This behaviour of these proteins necessitated the use of anion-exchange chromatography for their separations. Anion-exchange chromatography was attempted with the use of commonly used DEAE-cellulose column. Adsorption and subsequent elution of proteins from the DEAE-cellulose column

using linear or stepwise salt gradient always resulted in co-elution of both  $\alpha$ -amylase and glucoamylase proteins. In addition the elution patterns were observed to be ambiguous in that both proteins seemed to be eluted with a wide range of salt concentration (Table 1). However, anion-exchange chromatography on Dowex (AGI-X4) resin column was found to be very effective in separating  $\alpha$ -amylase and glucoamylase enzymes. The  $\alpha$ -amylase enzyme was completely eluted from the resin column with 0.05M sodium acetate buffer containing 0.1M NaCl. The glucoamylase enzyme remained adsorbed to the resin anion exchange ligands under these conditions of elution (Table 1). After complete elution of  $\alpha$ -amylase enzyme protein with 0.1M NaCl containing 0.05M sodium acetate buffer, the glucoamylase was eluted with the buffer containing 0.3M NaCl. The two step separation procedure involving Sephacryl-200 gel filtration and anion-exchange chromatography on resin column resulted in electrophoretically homogenous preparations of  $\alpha$ -amylase and glucoamylase proteins (Fig. 2). A typical purification profile of  $\alpha$ -amylase and glucoamylase proteins using this two step separation procedure is shown in Table 2.

### 3.1.2 Properties of glucoamylase enzyme

Some of the major properties of the glucoamylase enzyme are listed in Table 3 and Table 4. As mentioned in the Introduction, enzymes has already been extensively characterized by several investigators and the results reported here simply confirm the

Figure 1. A typical elution profile of the extracellular  $\alpha$ -amylase and glucoamylase enzymes of *A. awamori* from a Sephacryl-200 column (2.5 x 75 cm). About 10 ml (14 mg) of total extracellular protein extract in 0.05 M sodium acetate buffer was applied to the column and elution was carried out with the same buffer containing 0.05 M KCl. Fractions of 5 ml each were collected with a Pharmacia F-3000 fraction collector. Absorbance at 280 nm was measured with a UV light monitor and enzyme activities in each fraction were assayed as described in Materials and Methods. \_\_\_\_\_,  $A_{280}$ ; -■-■-, glucoamylase activity and -●-●-,  $\alpha$ -amylase activity.



v

TABLE 1

Comparison of Effectiveness of DEAE-Cellulose and Resin  
Anion-Exchange Chromatography for Separation of  $\alpha$ -Amylase and  
Glucoamylase Enzymes

Salt concentration in elution buffer		Relative elution of activity (%)	
		$\alpha$ -Amylase	Glucoamylase
Resin <sup>a</sup>	0.05M	20	0
	0.10M	80	0
	0.30M	0	100
DEAE cellulose <sup>b</sup>	0.05M	20	0
	0.10M	40	40
	0.30M	40	60

<sup>a</sup>Bio-Rad AGI-X4

<sup>b</sup>DEAE-cellulose chromatography was carried out exactly as  
described for resin in Materials and Methods Section (2.2).

Figure 2. Electrophoretic mobilities of  $\alpha$ -amylase and gluco-  
amylase enzymes from A. awamori.

SDS-PAGE was performed with 10% polyacrylamide gel as described in Materials and Methods section. After electrophoresis, the gel was stained for 1 h with 0.25% Coomassie blue-R dye in methanol-acetic acid - water solution (4:1:5, by volume) and destained in the same solution without the dye. Lanes: 1, gluco-  
amylase (100  $\mu$ g); 2,  $\alpha$ -amylase (100  $\mu$ g); 3, molecular weight markers (phosphorylase b, 94 000; bovine serum albumin, 67 000; ovalbumin, 43 000; carbonic anhy-  
drase, 30 000; trypsin inhibitor, 20000).

E



1

2

3

TABLE 2

A Typical Purification Profile of the Extracellular  $\alpha$ -Amylase  
and Glucoamylase Enzymes of A. gwasiori

Step	$\alpha$ -Amylase			Glucoamylase		
	Total Protein (mg)	Total Units	Specific Activity (units/mg)	Total Units	Specific Activity (units/mg)	Recovery (%)
Culture Fluid	15	11812	787	473	31.5	100
Ethanol precipitate	14.2	11216	789.9	446	31.4	93.3
Sephacryl-200 Peak II (Fr. 38-52)	12.4	10687	861.9	392	31.6	82.9
Anion-Exchange Chromatography 0.1M NaCl eluate	9.0	10125	1125	-	-	-
0.3M NaCl eluate	2.8	-	-	378	135	79.9

TABLE 3

Major Properties of Glucoamylase Enzyme of Aspergillus awamori

---

Molecular Weight	94 000
Isoelectric Point	3.4
Optimum Temperature	55-60°C
Temperature Stability	up to 50°C
Optimum pH	4.8 - 5.0
pH Stability	3.0 - 6.5
K <sub>m</sub> Starch (pH 5.3, 37°C)	0.30gL <sup>-1</sup>

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TABLE 4

Substrate Specificity of Glucoamylase Enzyme of A. swamori

Substrate	Relative rates of hydrolysis
Soluble starch	100%
Maltotriose	25%
Maltose	15%

existing knowledge. For this reason, further in-depth description of the results regarding physico-chemical properties of enzyme is not included.

### 3.1.3 Properties of $\alpha$ -amylase

Molecular weight: Based on its mobility in the SDS-polyacrylamide gel (Fig. 2), the molecular weight of  $\alpha$ -amylase was estimated to be  $54,000 \pm 2,500$ .

Isoelectric point: The isoelectric point of  $\alpha$ -amylase was found to be around pH 4.2 (Fig. 3).

pH Optimum and pH stability: The pH range for maximal enzyme activity was found to be between pH 4.8 and 5.0. The enzyme was stable to the degree of more than 80% in the pH range of 4.0 - 6.5, at least for 24 hours (Fig. 4).

Temperature optimum and heat stability: The optimum temperature for enzyme activity was observed to be around  $50^{\circ}\text{C}$  and the enzyme was stable upto  $45^{\circ}\text{C}$  at least for 1 hour, retaining 80% of the original activity (Fig. 5).

Kinetics: The  $K_m$  value for starch hydrolysis under standard assay conditions was calculated to be  $1.0 \text{ g.L}^{-1}$ . Maltose inhibited starch hydrolysis in an uncompetitive way with a  $K_I$  value of  $20.05 \text{ g.L}^{-1}$  (Fig. 6). Glucose had no effect on the starch hydrolysis by  $\alpha$ -amylase enzyme.

Figure 3. Isoelectric focusing of the extracellular  $\alpha$ -amylase of A. awamori.

Isoelectric focusing was performed using 5% acrylamide gel containing 2% ampholytes (pH range 3-10) on a Pharmacia Flat Bed FBE-3000 apparatus.  $\alpha$ -Amylase protein (20  $\mu$ g) was applied towards the anode and focusing was carried out for 4 h at 20W constant power. After termination of the focusing, the pH across the gel was determined by slicing a part of the gel and eluting the ampholytes into degassed, deionized distilled water for pH measurement. The part of the gel containing  $\alpha$ -amylase protein band was fixed for 1 h in a solution consisting of 10% TGA and 5% sulfosalicylic acid. The gel was then stained for 1 hour with 0.05% Coomassie blue-R dye in methanol-acetic-acid-water solution (4:1:5) and destained with the same solution without dye.

7

+

pH4.2

—

—

8

Figure 4. Effect of pH on the activity (●) and stability (○) of A. awamori  $\alpha$ -amylase.

The effect of pH on the activity of  $\alpha$ -amylase was determined under standard assay conditions except for change of pH, using 0.05M sodium acetate buffer (between pH 3.0 and 5.6) or 0.05M sodium phosphate buffer (between pH 5.6 and 7.5). Effect of pH on the enzyme stability was determined by incubating the enzyme solutions for 24 h at various pH values (same buffers as above). After incubation, each solution was adjusted to pH 5.3 and residual activity was measured by the standard assay procedure described in Materials and Methods.

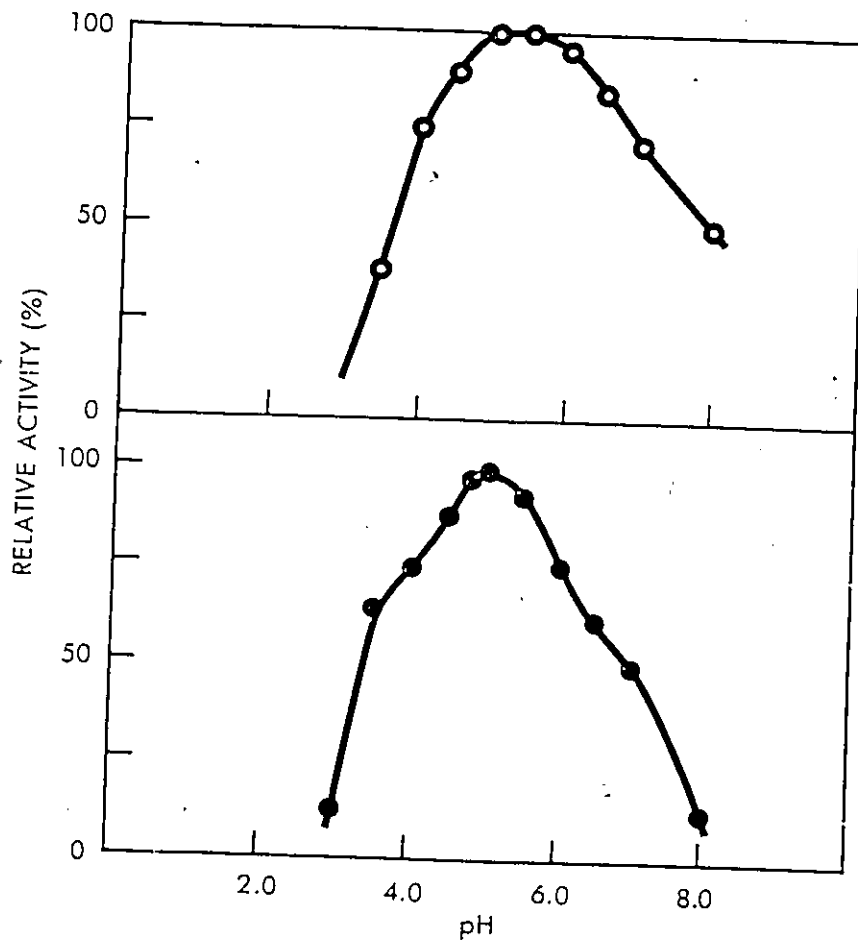


Figure 5. Effect of temperature on the activity (●) and stability (○) of A. awamori extracellular  $\alpha$ -amylase.

Activity was determined as described in Materials and Methods except for the change of temperature. Enzyme stability was determined by keeping the enzyme solutions in 0.05M sodium acetate buffer, pH 5.3, for 1 hour at various temperatures. After cooling the solutions to room temperature, remaining activity was assayed using standard conditions.

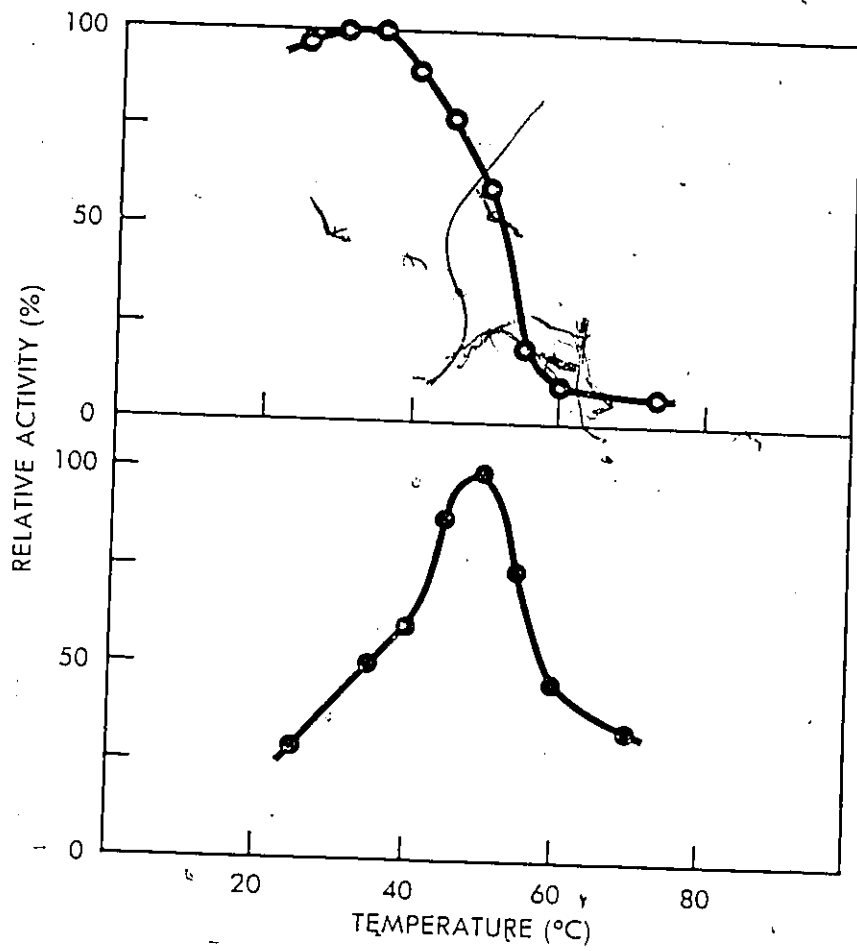
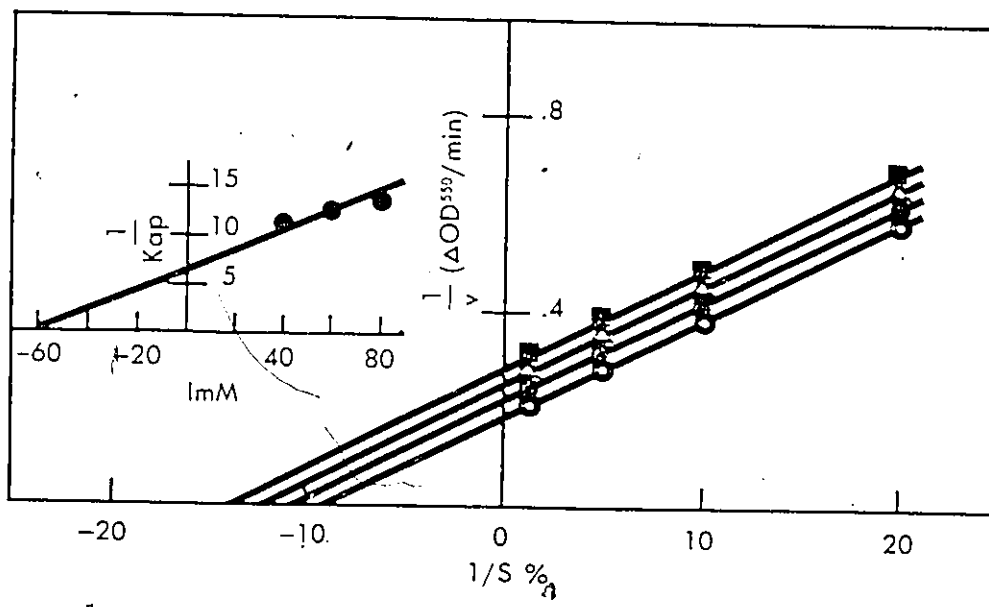


Figure 6. Lineweaver-Burk plot of A. awamori extracellular  $\alpha$ -amylase activity on soluble starch in the presence and absence of maltose.

The enzyme activity assays were performed under standard conditions as described in Materials and Methods except for the change in the substrate (soluble starch) concentration and additions of maltose as indicated. O, No inhibitor; ●, 40 mM maltose;  $\Delta$ , 60 mM maltose; ■, 80 mM maltose. Inset: secondary plots of the reciprocals of the apparent Michaelis constants ( $K_{ap}$ ) and maximal velocities ( $v$ ) versus inhibitor (I) (maltose) concentrations.



Effect of some metal ions and enzyme inhibitors: Among the metal ions tested,  $Pb^{2+}$  and  $Hg^{2+}$  were potent inhibitors. As for other fungal  $\alpha$ -amylases, A. awamori  $\alpha$ -amylase displayed no requirement of  $Ca^{2+}$  for activity (Table 5).

TABLE 5

Effect of Some Metal Ions and Enzyme Inhibitors on the Activity of

A. awamori Extracellular  $\alpha$ -Amylase

The enzyme protein (30  $\mu$ g) in 3 ml of 0.05M acetate buffer, pH 5.3, was preincubated with various metal ions and enzyme inhibitors (final concentration as indicated) for 30 min at 37°C. After preincubation, 0.2 ml of 6% starch solution was added and activity assayed as described in Materials and Methods. The results shown in this table represent the means of three replication.

Metal Ion or Inhibitor	Concentration (mM)	Relative Activity (%)
None	-	100
EDTA	5	100
	10	98.6
CaCl <sub>2</sub>	5	100
	10	100
MgSO <sub>4</sub>	5	98.2
	10	97
KCl	5	100
	10	100
Pb(CH <sub>3</sub> COO) <sub>2</sub>	1	47
	10	15.7
HgCl <sub>2</sub>	1	36
	10	10.5
Monoiodo-acetic acid	1	100
	10	98.9

### 3.2.0 Regulation of Glucoamylase Biosynthesis

#### 3.2.1 Glucoamylase production with regards to fungus growth in starch or glucose medium:

The growth (mycelial wet weight) patterns of A. awamori were similar in medium containing 2% starch (Fig. 7) or 2% glucose (Fig. 8) as carbon source, with maximum mycelial mass occurring after about 60 hours. Both culture media elaborated an increase in glucoamylase enzyme activity levels during the growth of fungus, with maximum enzyme activity levels occurring after 72 hours. Although total enzyme levels after 72 hours were only slightly higher with growth on starch (Fig. 7) than growth on glucose (Fig. 8), there was a considerable difference in the patterns of increase in the enzyme activity levels. In the case of medium containing starch, there was a sharp increase in glucoamylase levels during the first 12 hours of growth followed by an increase at low levels and another sharp increase occurring after 48 hours. With glucose as carbon source, the levels of glucoamylase enzyme increased at a low and steady rate for the first 48 hours of growth followed by a sharp increase after 48 hours. Sharp increases in enzyme levels in either starch or glucose medium corresponded to the periods when glucose levels in the culture media were very low (Fig. 7 and Fig. 8).

The changes in glucoamylase enzyme activities observed during fungal growth in glucose or starch medium whether brought about by de novo protein synthesis or some other phenomena, were investi-

Figure 7. Glucoamylase production by A. awamori with growth in starch medium.

The fungus was grown in 250 ml flasks containing 50 ml of starch medium. At various intervals flasks were removed, mycelia separated by filtration and its mass was measured as an indicator of cell growth. The culture filtrates were dialyzed against 0.05M sodium acetate buffer, pH 5.3 and the glucoamylase activity was determined using 0.5 ml aliquots as described in Materials and Methods.

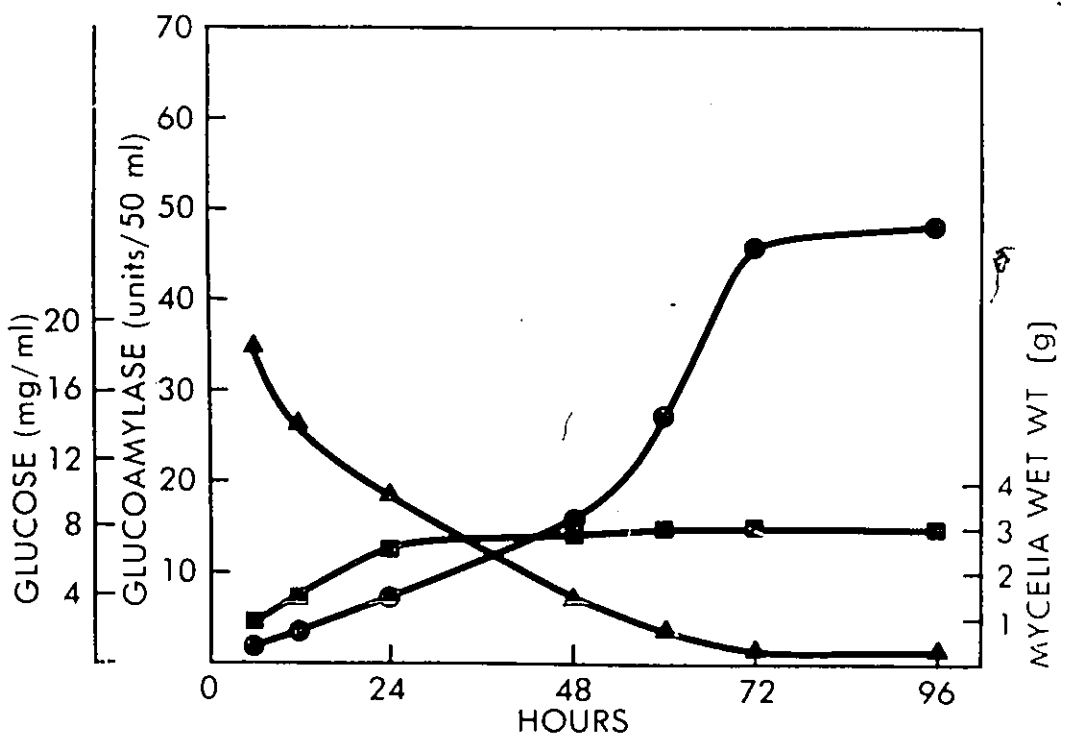
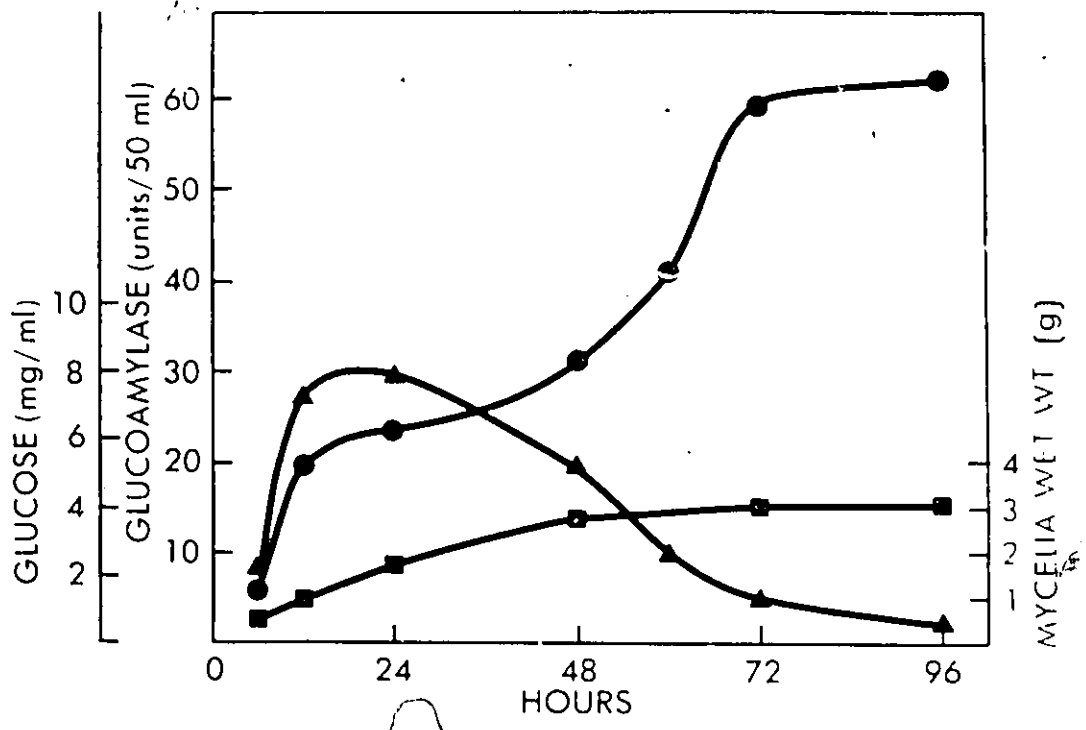
-●-●- glucoamylase activity

-■-■- cell mass

-▲-▲- glucose concentration in the culture medium.

Figure 8. Glucoamylase production by A. awamori with growth in glucose medium.

Conditions and symbols same as in Fig. 7.



gated by protein labelling studies. The fungal cells were labelled for four hours with [<sup>35</sup>S]-met at various intervals during growth in starch or glucose medium. The extracellular labelled proteins in the samples from various growth periods were analysed.

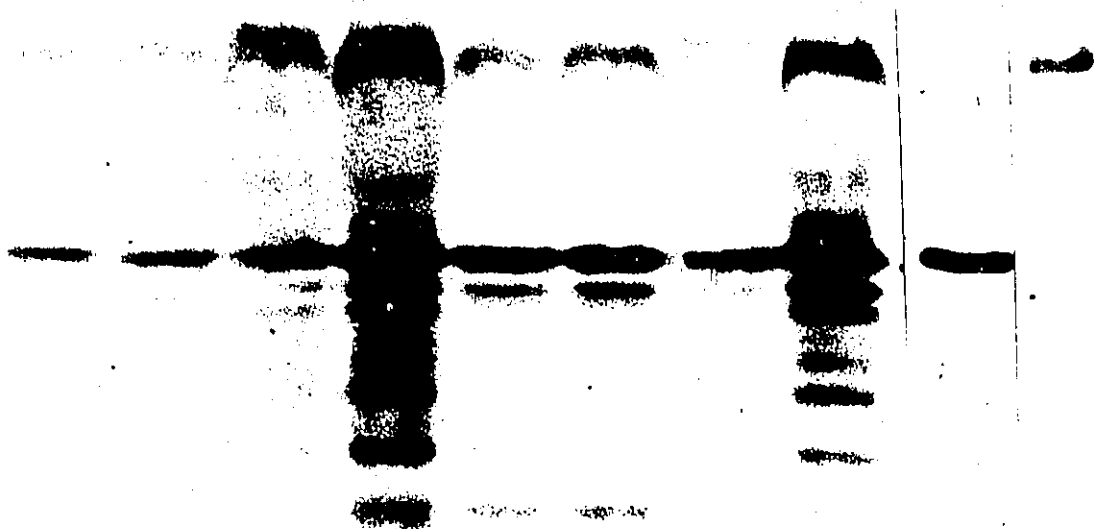
The patterns of glucoamylase protein biosynthesis as shown by in vivo labelling studies (Fig. 9) paralleled the increase in enzyme activities thus eliminating the possibilities that the observed increase in enzyme activity levels may be due to the activation of some pro-enzyme or delayed secretion of the enzyme upon glucose exhaustion from the culture media. Moreover, the rates of synthesis of enzyme as indicated by the specific activity ([<sup>35</sup>S]-met incorporated per g wet weight mycelia) were similar during the two periods of sharp increases in the starch medium and the period of single sharp increase in the glucose medium (Table 6). The identical rates of synthesis of the enzyme in the presence or absence of its substrate starch, indicated that starch is not required as an inducer of glucoamylase biosynthesis in A. awamori.

### 3.2.2 Repression of glucoamylase biosynthesis by glucose and cAMP:

To further investigate the mode of regulation of glucoamylase biosynthesis, equal amounts of mycelia obtained by growing the fungus in glucose medium for 36 hours, were shifted to various media containing 2% glucose, 2% starch, starvation medium or 2% starch + 4mM cAMP. Proteins were labelled in the presence of [<sup>35</sup>S]-met for 2 hours. As shown in Fig. 10 and Table 7, the rates of synthesis of glucoamylase were similar in the starch and star-

Figure 9. The patterns of glucoamylase biosynthesis with A. awamori growth in glucose or starch medium.

The fungus was grown in 50 ml flasks containing 10 ml of glucose or starch medium. The proteins were labelled by adding [<sup>35</sup>S]-met (10 μCi/ml) to the media 4 hours preceding the sample intervals. The extracellular labelled proteins in 100 μl aliquots of all culture filtrate samples were analysed by SDS-PAGE and fluorography as described in Materials and Methods. Lanes A-D, glucose medium samples (A, 6 h; B, 12 h; C, 24 h; D, 60 h). Lanes E-H, starch medium samples (E, 6 h; F, 12 h; G, 24 h; H, 60 h). Lane I, position of immunoprecipitated α-amylase and Lane J, position of immunoprecipitated glucoamylase. (Exposure 16 hours)



A B C D E F G H I J

TABLE 6

Relative Rates of Glucoamylase Biosynthesis During A. awamori Growth  
in Glucose or Starch Medium

<sup>a</sup>[<sup>35</sup>S]-met incorporation into extracellular glucoamylase protein/g  
cell mass (wet weight)/h.

Growth (h)	Glucose Medium	Starch Medium CPM
6	15.0 x 10 <sup>4</sup>	60.1 x 10 <sup>4</sup>
24	16.1 x 10 <sup>4</sup>	15.3 x 10 <sup>4</sup>
60	63.6 x 10 <sup>4</sup>	61.1 x 10 <sup>4</sup>

<sup>a</sup>Protein bands corresponding to glucoamylase in gel of Fig. 9 were excised, dissolved in NCS tissue solubilizer and counted by liquid scintillations. The counts were corrected by taking into consideration the recovery of activity from the immunoprecipitated glucoamylase protein run in parallel with the samples.

Figure 10. Repression of glucoamylase biosynthesis by glucose and cAMP.

Equal amounts (0.4 g) of pregrown (36 h in glucose medium) mycelia were shifted to 10 ml of indicated media and proteins were labelled with [ $^{35}\text{S}$ ]-met (10  $\mu\text{Ci/ml}$ ) for 2 hours. Both intra- and extracellular protein samples were prepared as described in Materials and Methods. 100  $\mu\text{l}$  aliquots of all samples were analysed by SDS-PAGE followed by fluorography. Lanes A-D in the fluorograph are intracellular samples (A, glucose; B, starch and cAMP; C, starch; D, starvation medium). Lanes E-H represent extracellular samples (E, glucose; F, starch; G, starch and cAMP; H, starvation medium). The position of glucoamylase and  $\alpha$ -amylase protein bands are marked by arrows. (Exposure 60 hours).

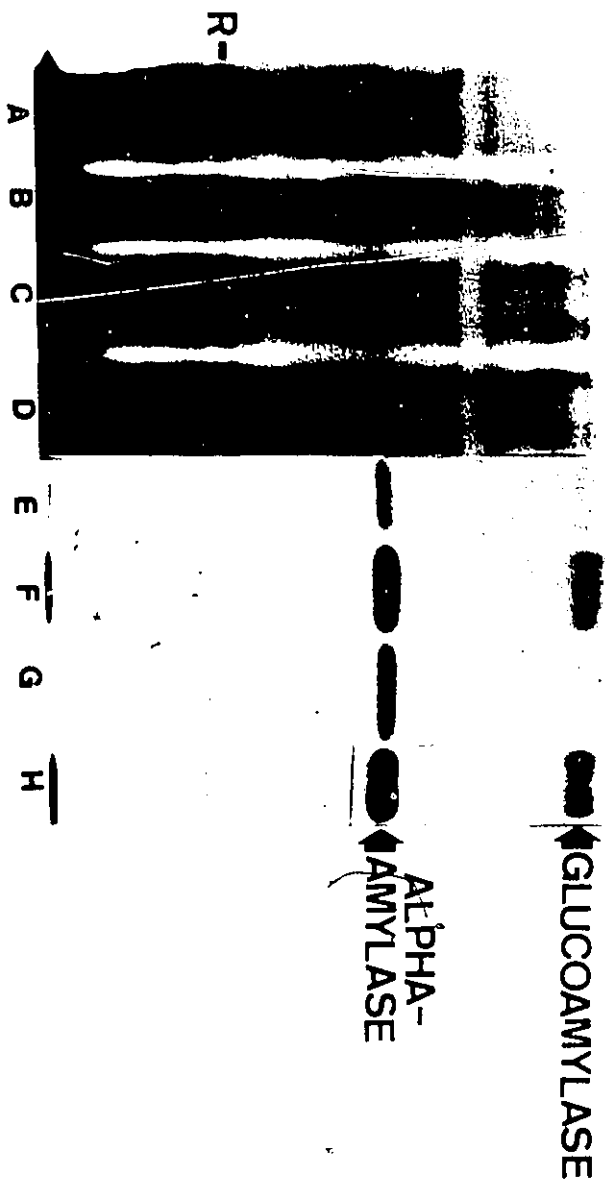


TABLE 7

Repression of Glucoamylase Biosynthesis by Glucose and cAMP

Medium	Glucoamylase protein levels <sup>a</sup> (CPM/ml) x 10 <sup>-3</sup>	
	Extracellular	Intracellular
Glucose	6.1 ± 0.25	0.9 ± 0.03
Starch	25.0 ± 1.25	4.6 ± 0.20
Starvation	27.1 ± 1.30	4.1 ± 0.20
Starch + cAMP	11.0 ± 0.70	2.5 ± 0.12

<sup>a</sup>Conditions for cell growth, protein labelling and analysis were as described for Fig. 10. After fluorography, bands corresponding to glucoamylase were excised and counted by liquid scintillations. The results are presented as average ± standard deviation based on 5 replicates.

vation media. On the other hand, in the presence of glucose, enzyme synthesis was repressed by a factor of about four. cAMP when added to the starch medium, inhibited the glucoamylase biosynthesis by about 50% (Fig. 10; Table 7). The proportional decrease in the intracellular levels of glucoamylase in the presence of cAMP ruled out the possibility that the decrease observed in the extracellular levels of enzyme may be due to the effect of cAMP at the level of secretion rather than biosynthesis. cAMP had no significant effect on the glucose-repressed biosynthesis of glucoamylase by A. awamori and its inhibitory effect on the biosynthesis of this enzyme in response to starch medium was specific as additions of other nucleotides (AMP, ADP, ATP, cGMP or GMP) at similar concentrations had no significant effect (data not shown).

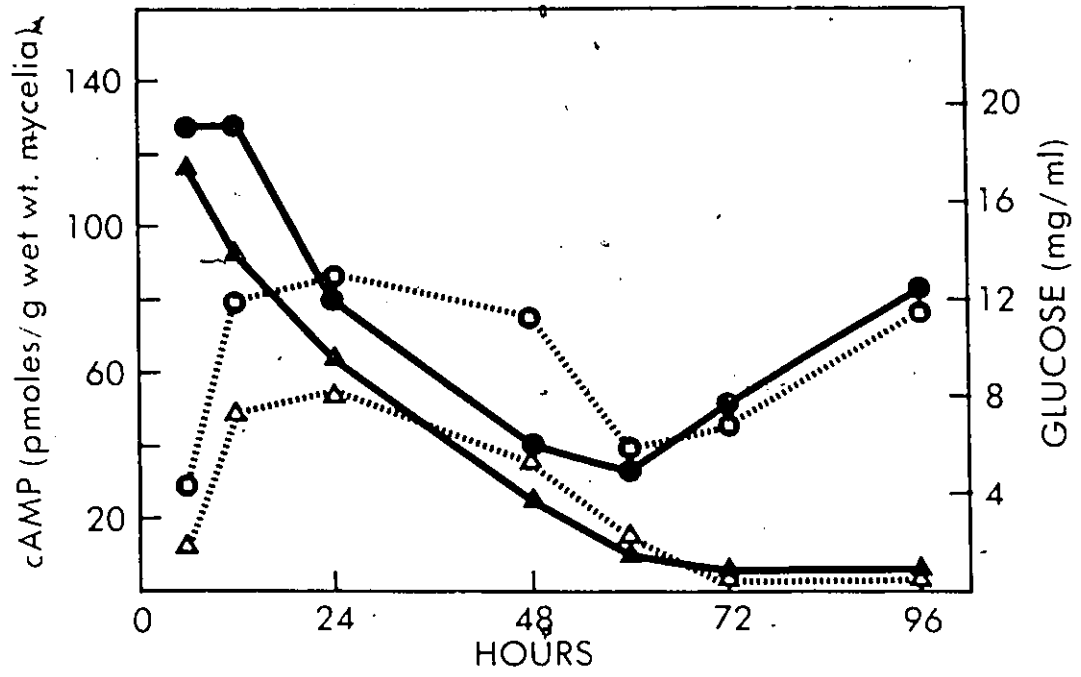
### 3.2.3 Correlation of intracellular cAMP levels and glucose concentration in the culture medium:

To determine whether there was any relationship between glucose catabolite repression and intracellular cAMP levels, the latter were measured at different intervals during the growth of A. awamori on starch or glucose. Fig. 11 shows a positive correlation between intracellular cAMP and glucose levels in the medium upto 72 hours of growth. At 6 hours of growth, low glucose concentration in the starch medium and high glucose concentration in the glucose medium corresponded to about five fold higher cAMP levels in the latter medium. At about 60 hours, when the glucose was

Figure 11. Fluctuations of intracellular cAMP levels during A. awamori growth in glucose and starch media.

The fungus growth and cAMP extractions and determinations were done essentially as described in Materials and Methods.

Symbols Used: Growth on starch - open symbols and broken lines  
Growth on glucose - closed symbols and full lines  
Intracellular cAMP levels - circles  
Glucose concentration in the culture medium - triangles.



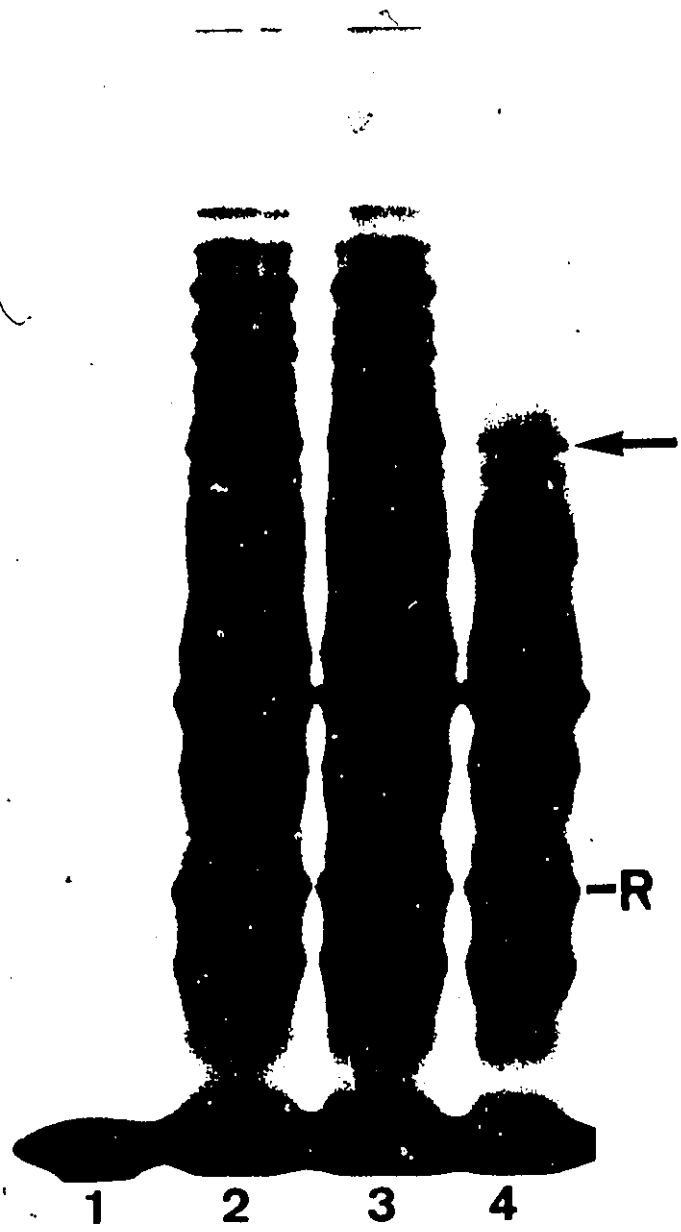
almost depleted from both media, cAMP levels were low and almost identical. Interestingly, the elevated synthesis of glucoamylase by A. awamori grown in either medium (Fig. 7 and Fig. 8) corresponded to the periods of lowest intracellular cAMP levels. These results indicate that the catabolite repression regulated synthesis of glucoamylase may be mediated by cAMP.

#### 3.2.4 Analysis of glucoamylase specific mRNA levels in fungal cells in response to various carbon sources:

The glucoamylase specific mRNA concentration was analysed by measuring its template activity in a cell free translation system. Total RNA was isolated from the fungal cells actively synthesizing glucoamylase in response to glucose, starch or starvation medium (basal medium without any carbon source). Equal amounts of total RNA were translated in a reticulocyte cell free translation system in the presence of [<sup>35</sup>S]-methionine. Total translation products were electrophoretically separated and protein bands were visualized by fluorography. The glucoamylase specific protein bands were identified by the positions of immunoprecipitated protein bands. A typical fluorograph of in vitro translation products is shown in Fig. 12. To quantitate glucoamylase specific translatable mRNA, the glucoamylase protein bands from in vitro translation product gels were excised and counted. To ensure equal input of RNA in translation products, the counts of all glucoamylase bands were standardized with reference to an arbitrary protein of about 30 000 molecular weight (R band in Fig. 12), levels of which do

Figure 12. Fluorograph of SDS-PAGE showing in vitro synthesis of glucoamylase.

Pregrown mycelia (4 g wet weight) were shifted to 100 ml of the indicated media. After 2 hours, total cellular RNA was isolated from the cells as described in Materials and Methods. Equal amounts of RNA (4 g) from each sample were translated in a reticulocyte cell free translation system and proteins were labeled with [<sup>35</sup>S]-met. About 150 000 CPM incorporated into protein (except the control where equivalent volume of the reaction mixture was used) from each sample were analysed by SDS-PAGE and fluorography as described in Materials and Methods. Lanes: 1, control (-RNA); 2, starch RNA; 3, starvation RNA; 4, glucose RNA. The position of a glucoamylase specific immunoprecipitated protein band (determined from Fig. 13) is indicated by an arrow. R in the fluorograph refers to an unknown protein that seems to be constitutively synthesized. This protein band was routinely used as a reference for quantitative analysis of the glucoamylase mRNA template activity. (Exposure was for 72 h).



not seem to be affected by growth conditions. As indicated by the data shown in Table 8, the glucoamylase translatable activities in RNA samples from starch and starvation media samples were essentially similar and about two fold higher than observed in RNA from glucose medium samples. These results indicate that glucose repression of the glucoamylase biosynthesis operates at least partially at the level of its translatable mRNA concentration.

### 3.2.5 Effect of exogenous cAMP on the glucoamylase specific mRNA levels in fungal cells grown in starch medium:

To understand the molecular basis of cAMP repression of the glucoamylase synthesis, the glucoamylase specific mRNA levels were analysed by in vitro translation assay system as described above. The levels of glucoamylase specific mRNA were found to be about 50% reduced in RNA from the cells in starch + cAMP medium than in the RNA from cells in starch medium (Fig. 13 and Table 8). Exogenously added cAMP lowered the glucoamylase specific mRNA concentration in the cells in starch medium to the levels observed in the cells in glucose medium.

### 3.2.6 Northern blot analysis of glucoamylase specific mRNA:

To find out whether processing of the glucoamylase gene transcript plays any role in the regulation of its gene expression, the glucoamylase specific mRNA was analysed by Northern blot analysis. Northern blots of RNA samples from the cells in glucose, starch or starvation medium when probed with radio-labelled glucoamylase gene probe, revealed a major band of about 2.3 kb (Fig.

TABLE 8

Analysis of In vitro Translatable Glucoamylase Specific mRNA  
Levels in A. awamori in Response to Various Carbohydrates in the  
Culture Media

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RNA Sample	Relative <u>in vitro</u> template activity (CPM/ $\mu$ g RNA) <sup>1</sup>
Glucose	1810 $\pm$ 107
Starch	3800 $\pm$ 210
Starvation	3900 $\pm$ 195
Starch + cAMP	2015 $\pm$ 150

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<sup>1</sup>Results are expressed as an average  $\pm$  S.D. of 7 experiments.

Figure 13. Fluorograph of SDS-PAGE of in vitro translated proteins showing the effect of cAMP on the glucoamylase mRNA levels.

Experiment was carried out essentially as described in legend to Fig. 12. Lanes: 1, control; 2, glucose RNA; 3, starch RNA; 4, starch + cAMP RNA. Lane 5, shows the position of immunoprecipitated in vitro synthesized glucoamylase. R-refers to the reference protein. (Exposure - 48 h).



1

2

3

4

5

-R

14). A band at a similar position was seen in the blots from starch and cAMP samples (Fig. 15). These data show that processing of the glucoamylase gene transcript does not play a significant role in the regulation of glucoamylase gene expression. In addition, the relative intensities of glucoamylase specific mRNA bands in various RNA samples indicate that glucose and cAMP repress the levels of glucoamylase mRNA in the cells.

### 3.2.7 Effect of culture conditions on the stability of glucoamylase specific mRNA

To find out whether the reduction in glucoamylase specific transcripts in the glucose or starch + cAMP medium cells was due to reduction in the rate of gene transcription or the stability of the transcripts, decay rates of glucoamylase mRNA were determined. RNA in the cells was labelled with [<sup>3</sup>H]-uracil for one hour and the label was chased for 2,4,6 and 8 hours. Radiolabelled RNA, isolated from the cells after various chase periods, was hybridized to glucoamylase specific genomic DNA insert in pGARI immobilized on nitrocellulose filters. The specific hybridizable radioactivity was determined and plotted as the percentage of glucoamylase mRNA remaining as a function of time. These data shown in Fig. 16 show that decay kinetics of glucoamylase mRNA are essentially similar under different culture conditions with mRNA half life of about 4.6 hours. These results suggest that stability of glucoamylase mRNA does not play a significant role in the regulation of gene expression of this enzyme and the reduction in

Figure 14. Northern blot analysis of glucoamylase specific mRNA. Total cellular RNA (10  $\mu$ g) denatured by glyoxalation was separated on 1.2% agarose gel and transferred to nitrocellulose filters as described in Materials and Methods. The blot was hybridized to radiolabelled (0.5  $\mu$ g;  $1 \times 10^8$  CPM/ $\mu$ g) glucoamylase gene specific DNA insert cloned in plasmid pGARI. After 12 hours of hybridization, the blot was washed and exposed to Kodak XAR-5 X-ray film with intensifying screen. Lanes: A, glucose RNA; B, starch RNA and C, starvation RNA. Position of 28S and 18S RNA is indicated by bars. (Exposure 12 hours).

-28S

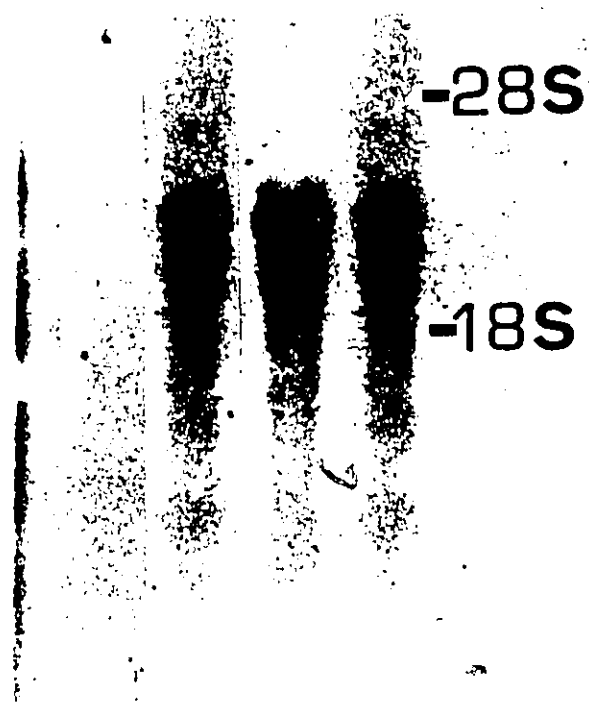
-18S

A B C

Figure 15. Effect of cAMP on the processing of glucoamylase specific gene transcript.

Experiment was carried out as mentioned in legend to Fig. 14. Lanes: A, glucose RNA; B, starch RNA and C, starch + cAMP RNA. (Exposure 18 hours).

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-28S

-18S

**a b c**

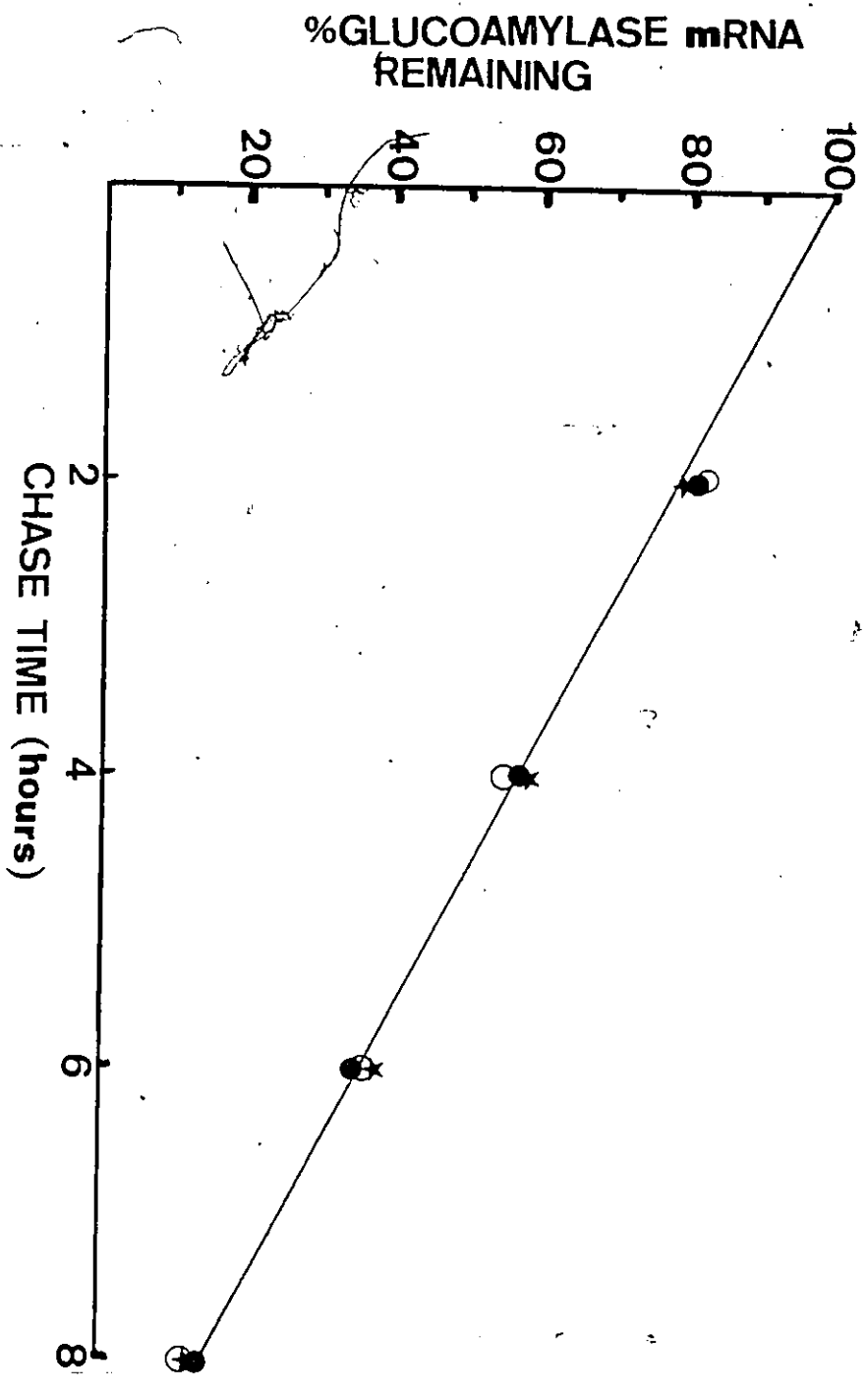
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Figure 16. Effect of glucose and cAMP on the stability of glucoamylase specific mRNA.

The fungal cells were pulse labelled for 1 hour with [<sup>3</sup>H]-uracil (50  $\mu$ Ci/ml). After 1 hour, further incorporation of label was stopped by the addition of 1000-fold excess of unlabelled uracil and the label was chased for 2,4,6 and 8 hours. Radiolabelled RNA from various chase periods was hybridized to the filters containing 5  $\mu$ g denatured immobilized glucoamylase specific gene insert from plasmid pGARI as described in Materials and Methods section. Specific hybridizable radioactivity was determined and plotted as percentage of glucoamylase mRNA remaining at each time period. Each point plotted in the Figure represents the average of duplicate filter hybridization using the RNA preparations from a single experiment.

Symbols:

- , RNA from starch medium cells;
- o o, RNA from glucose medium cells
- \*-\*, RNA from starch + cAMP medium cells.



glucoamylase specific gene transcripts in the glucose or starch + cAMP medium cells most likely result from the reduced rate of gene transcription in these culture media.

### 3.2.8 Dot blot analysis of glucoamylase specific mRNA:

To quantitate glucoamylase specific mRNA, RNA from various samples was dot-blotted in increasing concentrations. The blots were hybridized to radio-labelled glucoamylase gene probe in excess. Typical autoradiographs of dot blot hybridizations are shown in Fig. 17 and Fig. 18. Analysis of the blots indicated that glucoamylase specific mRNA levels were almost identical in the samples from cells in starch or starvation medium and were about 2 fold higher than the levels observed in the samples from the glucose medium (Fig. 19; Table 9). cAMP, like glucose, repressed the levels of glucoamylase specific mRNA by about two fold (Fig. 19; Table 9). These interpretations of dot blot analysis of glucoamylase specific mRNA concentration are obviously based on the assumption that the amount of RNA loaded onto filters was similar for all the samples. The contamination of RNA preparations with DNA, protein or carbohydrates can result in different amounts of RNA from each sample being loaded onto the filters. Under these conditions, the observed variation in glucoamylase mRNA concentration in different RNA samples may actually be due to the variation in input RNA. To eliminate this possibility, the input RNA should have been standardized with reference to some other mRNA sequences (internal control) which are known to be expressed

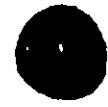
Figure 17. Dot blot analysis of glucoamylase specific mRNA levels. Total cellular RNA in 5XSSC was directly applied to nylon membranes in increasing concentration (1  $\mu$ g, 2  $\mu$ g, 3  $\mu$ g and 4  $\mu$ g) and crosslinked to the membrane by 3 min exposure of the blots to UV light. Blots were hybridized to radio-labelled glucoamylase specific gene probe (0.5  $\mu$ g,  $1 \times 10^8$  CPM/ $\mu$ g) for 48 hours as described in Materials and Methods. To ensure that the probe DNA was in large excess, 2  $\mu$ g of denatured cold probe DNA was added to the hybridization solution. After hybridization, blots were autoradiographed. Rows: A, RNA from glucose cells; B, RNA from starch cells; C, RNA from starvation medium cells.



Figure 18. Dot blot analysis of effect of cAMP on glucoamylase specific mRNA levels.

Experimental conditions were same as described for legend to Fig. 16. Rows: A, starch sample RNA; B, glucose sample RNA and C, starch + cAMP sample RNA. (Exposure 12 hours).

**A**



**B**



**C**



Figure 19. The glucoamylase specific mRNA concentrations in A. awamori cultured in glucose, starch, starch + cAMP or starvation medium.

The data plotted in this figure were obtained by dot blot hybridizations of total RNA with radiolabelled glucoamylase specific gene probe as described in Fig. 17. The dots on the filters were visualized by autoradiography. To quantitate the glucoamylase specific mRNA, dots were excised and counted by liquid scintillations. Each point represents the mean of three experiments carried out with different RNA preparations.

- ★-★ RNA from starvation medium cells
- RNA from starch medium cells
- RNA from glucose medium cells
- o-o- RNA from starch + cAMP medium cells

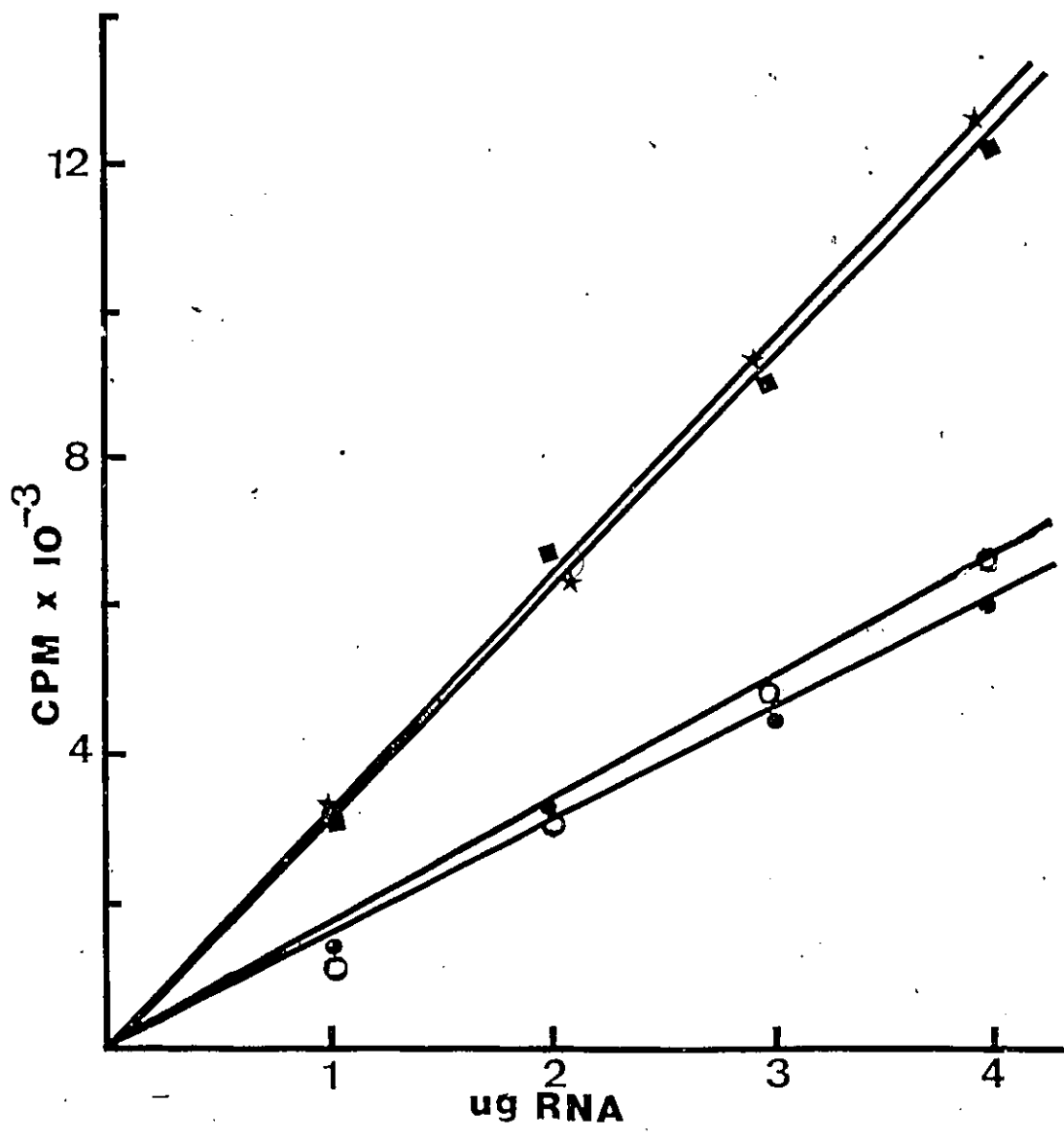


TABLE 9

Dot Blot Analysis of Glucoamylase Specific mRNA Levels During  
Repressed and De-repressed Synthesis of Glucoamylase in A. awamori

RNA Source (culture medium)	Glucoamylase specific mRNA levels (CPM/ $\mu$ g RNA) <sup>a</sup>
Glucose	1485
Starch	3355
Starch + cAMP	1540
Starvation	3360

<sup>a</sup>These results were obtained by linear regression analysis of the data shown in Fig. 19.

at the same level (constitutively expressed) under all culture conditions. Although internal control was not used in the quantitation of glucoamylase mRNA analysis, the fact that the three dot blot experiments using different RNA preparations gave similar results suggests that variation in input RNA was not the cause of observed results. Furthermore the levels of glucoamylase mRNA in various RNA samples determined by dot blot hybridizations were in agreement with the levels determined by in vitro translation assays. The similar results of glucoamylase mRNA quantitation by two different methods suggest that observed differences in glucoamylase mRNA levels were probably reflective of actual levels in A. awamori during various culture conditions.

### 3.3.0 Production of Multiple Forms of Extracellular Glucoamylases in A. awamori

#### 3.3.1 In vivo synthesis of glucoamylases:

To clarify the basis of glucoamylase multiplicity in A. awamori (see section 1.2.1 of Introduction), biosynthesis of glucoamylases by A. awamori was studied by pulse labelling of proteins with [<sup>35</sup>S]-met and chasing the label over the period of 48 hours. As shown in Fig. 20, after one hour of labelling, two forms of glucoamylases with apparent molecular weights of about 100 000 and 82 000 were detected in the culture medium. After 12 hours of chase, in addition to these two types, another form of glucoamylase with a molecular weight of about 94 000 was present. This form of glucoamylase most likely was derived from the high molecu-

lar weight type as indicated by the coincidental decrease in intensity of the 100 000 molecular weight glucoamylase protein band (Fig. 20). On the other hand, there was no significant change in the intensity of the 82 000 molecular weight glucoamylase protein band indicating that this type was not produced by degradation of the high molecular weight forms. After 48 hours of chase, only the 94 000 and 82 000 molecular weight species of glucoamylase were detected. Again the continued disappearance of label from the 100 000 molecular weight protein band resulted in the increase in intensity of the 94 000 molecular weight band while no significant change was observed in the 82 000 molecular weight glucoamylase protein band.

### 3.3.2 Existence of two types of glucoamylase specific mRNAs

As indicated by the above described results if two types of glucoamylase specific proteins are synthesized in vivo by A. awamori cells, then one can expect to detect the existence of two types of glycoamylase specific mRNAs in the fungal cells. Analysis of fungal RNA by in vitro translation as well as by hybridization analysis does show that there are two types of glucoamylase specific mRNA present in the fungal cells. Immunoprecipitations of glucoamylase specific polypeptides from total in vitro translation products showed the presence of two glucoamylase specific proteins with apparent molecular weights of about 71 000 and 68 000 respectively (Fig. 21A). The presence of two types of glucoamylase specific proteins in the in vitro translation products suggests that there are two types of glucoamylase related mRNAs in A. awamori cells.

Figure 20. In vivo synthesis of glucoamylases.

A. awamori was cultured in 50 ml flasks containing 10 ml of starch medium. Proteins were pulse labelled for 1 hour with [ $^{35}\text{S}$ ]-met (10  $\mu\text{Ci/ml}$ ) and further label incorporation was stopped by the addition of 1000 x fold excess of cold methionine. Extracellular labelled proteins in 0.5 ml samples taken at indicated times, were analysed by SDS-PAGE and fluorography as described in Materials and Methods. The molecular weight of protein bands shown in the figure was estimated by the relative mobilities of the standards run in parallel with the samples as described in Materials and Methods section 2.14. Lanes: 1, 1h (1 hour labelling; 0 hour chase) samples; 2, 12 h sample (1 hour labelling, 12 hour chase); 3, 48 h sample; 4, glucoamylase immunoprecipitation from lane 2 sample; 5, glucoamylases immunoprecipitated from lane 3 sample. (96 hours exposure).

**Mr (10<sup>-3</sup>)**

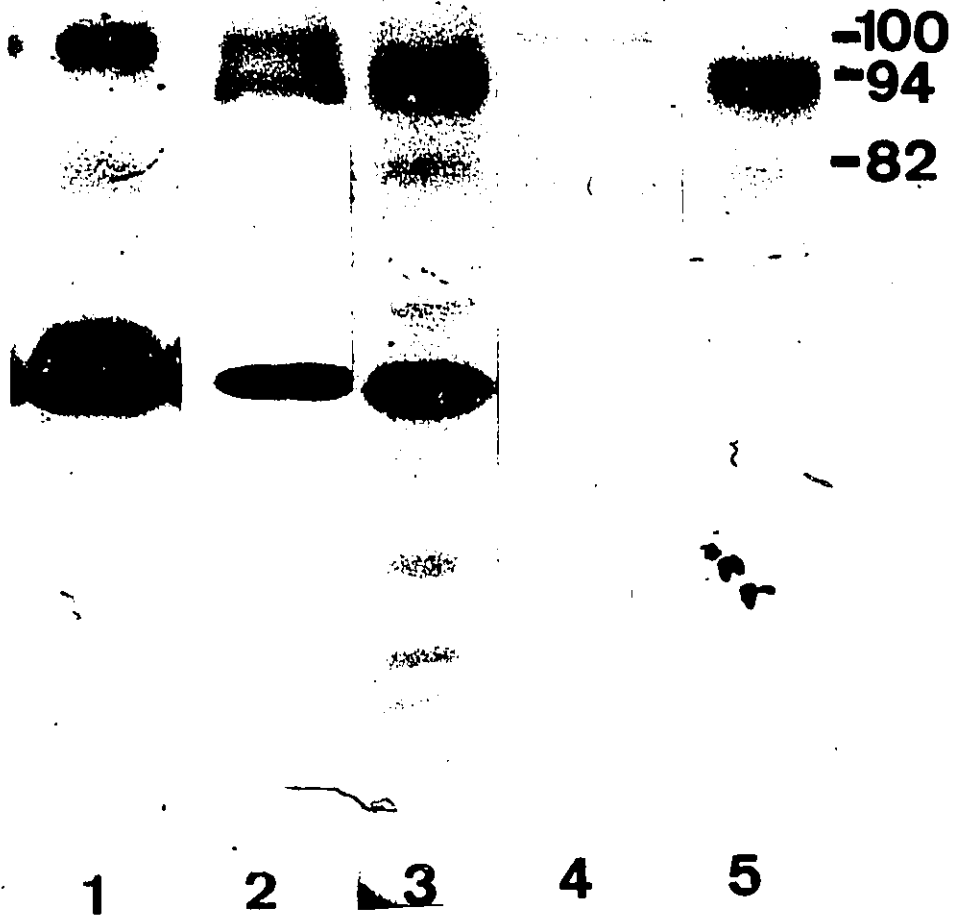
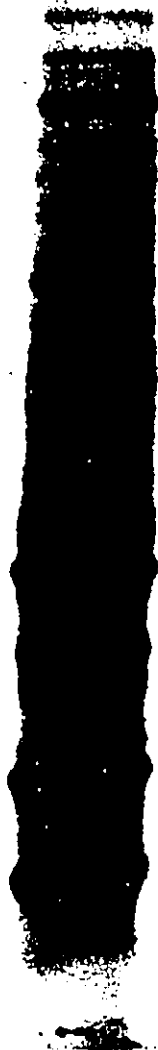


Figure 21. Analysis of glucoamylase specific mRNAs by in vitro translation and Northern blot analysis.

The figure presented here is a composite of lanes shown in Fig. 13 and Fig. 15. These lanes are presented again in this figure for the purpose of convenience and clarity of the text and are deemed to highlight the conclusion that there are two glucoamylase specific mRNAs present in A. awamori.

- A. In vitro translation: Lanes: 1, control (-RNA); 2, total translation products; 3, glucoamylase specific immunoprecipitated proteins from the total in vitro translation products.
- B. Northern blot showing the presence of two RNA species that hybridize to glucoamylase specific gene probe.

**A**



**1**

**2**

**3**

**B**



**-2.3Kb**  
**-2.1Kb**

This was further confirmed by the results of hybridization analysis of glucoamylase specific mRNA. The glucoamylase specific genomic DNA probe hybridized to two species of mRNA with sizes of about 2.3 kb and 2.1 kb (Fig. 21B) indicating that detection of two glucoamylase related proteins in the in vitro translation products was not simply due to non-specific immunoprecipitations with glucoamylase antibodies. Furthermore consistent with the in vivo and in vitro synthesis patterns of glucoamylases, larger (2.3 kb) mRNA species were found to be in much more abundance than the smaller (2.1 kb) species.

### 3.4.0 Regulation of $\alpha$ -Amylase Biosynthesis

#### 3.4.1 Synthesis of $\alpha$ -amylase during fungus growth in starch or glucose medium

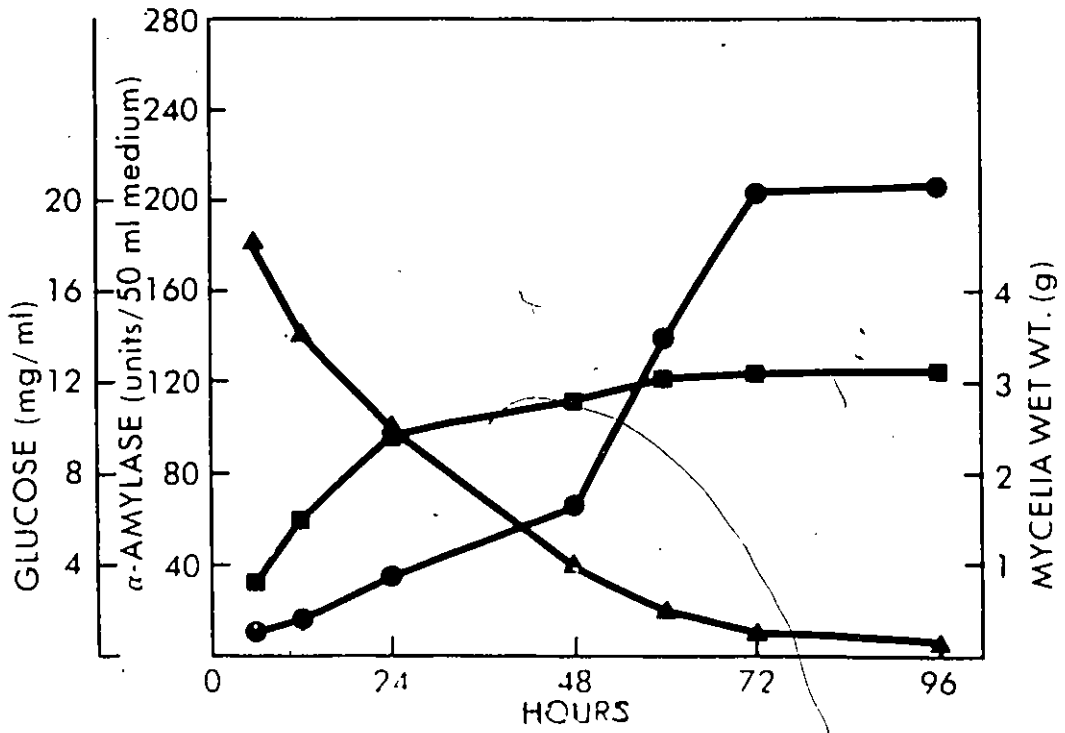
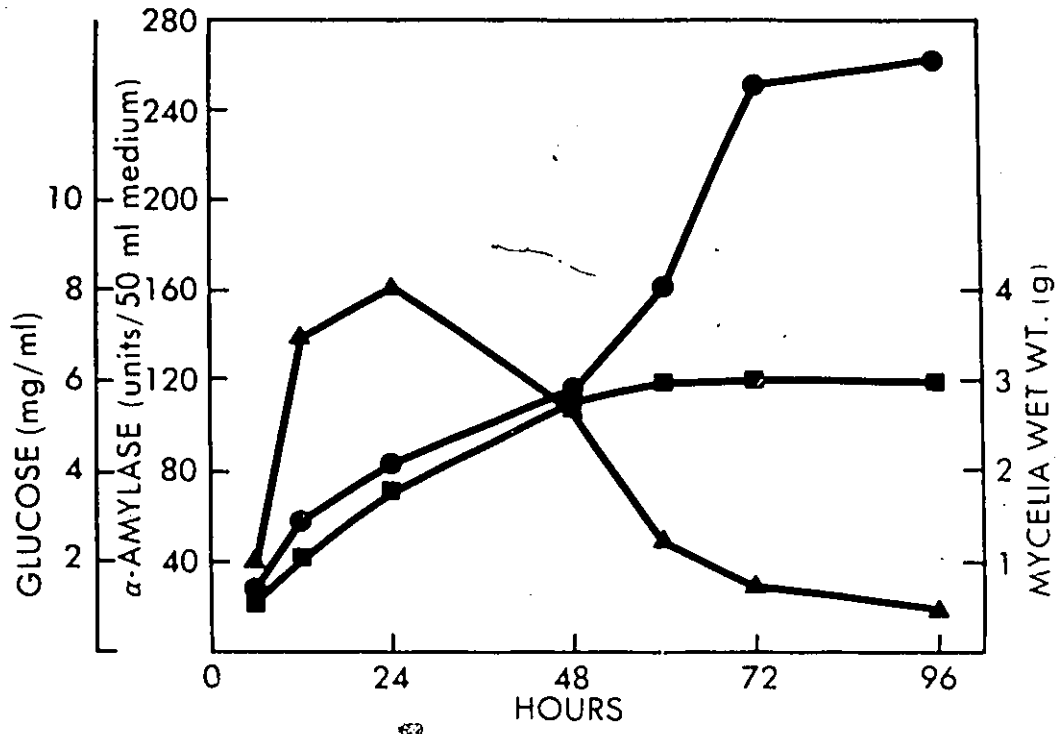
The patterns of  $\alpha$ -amylase production during the fungus growth in starch and glucose medium are shown in Fig. 22 and Fig. 23 respectively. The starch medium elaborated a sharp increase in  $\alpha$ -amylase enzyme activity levels during the first 12 hours of growth followed by an increase at low levels and another sharp increase occurring after about 48 hours of growth. In the glucose medium,  $\alpha$ -amylase enzyme levels increased with a low and steady rate for the first 48 hours followed by a sharp increase after 48 hours of growth (Fig. 23). Sharp increases in the  $\alpha$ -amylase enzyme levels in both starch and glucose media occurred when the glucose levels in the culture media were relatively low. The patterns of biosynthesis of  $\alpha$ -amylase enzyme protein as shown by

Figure 22. The patterns of  $\alpha$ -amylase production by A. awamori during its growth in starch medium.

The fungus was cultured in 250 ml flasks containing 50 ml of starch medium. At various intervals flasks were removed, mycelia separated by filtration and its mass was measured as an indicator of cell growth. The culture filtrates were dialyzed against 0.05M sodium acetate buffer, pH 5.3 and the  $\alpha$ -amylase activity in 0.5 ml aliquots was assayed as described in Materials and Methods.

- $\alpha$ -amylase activity
- cell mass
- ▲-▲- glucose levels in the culture medium

Figure 23. The pattern of  $\alpha$ -amylase production by A. awamori during its growth in glucose medium culture conditions and symbols same as in Fig.



in vivo labelling studies (Fig. 9) paralleled the increase in enzyme activity, thus eliminating the possibilities that the observed increase in enzyme activities may be due to activation of some  $\alpha$ -amylase precursor or delayed secretion of the enzyme upon glucose exhaustion. The identical rates of  $\alpha$ -amylase protein synthesis as indicated by the incorporation of [ $^{35}\text{S}$ ]-met into  $\alpha$ -amylase specific proteins in the absence or during the exhaustion of glucose from the culture media (Table 10) suggest that  $\alpha$ -amylase synthesis does not require any exogenous inducer.

To further investigate whether the increase in  $\alpha$ -amylase synthesis after 48 hours of fungus growth was due to glucose exhaustion itself or dictated by some developmentally regulated events, the fungal cells after 36 hours of growth were exposed to various conditions of growth. Equal amounts of pregrown A. awamori mycelia were shifted to starch, glucose or starvation media. The  $\alpha$ -amylase enzyme activities in the culture media were measured after 4 hours of incubation. The enzyme activity levels in starch and starvation media were almost identical and were about four fold higher than observed for glucose medium (Table 11). These results indicate that no exogenous inducer is required for the production of extra-cellular  $\alpha$ -amylase in A. awamori and that glucose acts as a catabolite repressor. The nature of glucose repression of enzyme activity was further investigated by in vivo labelling studies. Proteins were labelled by adding [ $^{35}\text{S}$ ]-met to the culture media. Both intracellular and extracellular proteins were separated by SDS-PAGE.  $\alpha$ -Amylase specific protein bands were

TABLE 10

Relative Rates of  $\alpha$ -Amylase Biosynthesis During A awamori  
Growth in Glucose or Starch Medium

<sup>a</sup>[<sup>35</sup>S]-met incorporation into extracellular  
 $\alpha$ -amylase protein/g cell mass (wet weight)/h

Growth (h)	Glucose Medium	Starch Medium
	CPMX10 <sup>5</sup>	
6	4.5	19.9
24	4.6	4.4
60	22.3	21.4

<sup>a</sup>Protein bands corresponding to  $\alpha$ -amylase in gel of Fig. 9 were excised, dissolved in NCS tissue solubilizer and counted by liquid scintillations.

identified by the position of immunoprecipitated  $\alpha$ -amylase protein bands. Both intracellular and extracellular enzyme proteins had identical molecular weights of about 54 000, indicating the synthesis of  $\alpha$ -amylase in its mature form. Incorporation of label into the extracellular  $\alpha$ -amylase specific protein bands was found to be about four fold higher in starch and starvation media samples than in glucose medium samples (Table 11). Similarly the intracellular  $\alpha$ -amylase protein levels were about four fold higher in starch and starvation media samples than in glucose medium. Taken together, these results demonstrate that the glucose repression of  $\alpha$ -amylase enzyme levels occurs at the level of protein biosynthesis. Higher levels of enzyme activities observed in starch and starvation media result from the derepression of the enzyme synthesis in response to lack or absence of readily utilizable carbon source in these media.

#### 3.4.2 Repression of $\alpha$ -amylase biosynthesis by exogenous cAMP:

Exogenous cAMP (4 mM) when added to the starch medium, repressed the levels of extracellular  $\alpha$ -amylase enzyme activity by about 50%. The results of in vivo protein synthesis studies (Table 11) indicated that cAMP represses the extracellular enzyme levels by repressing the  $\alpha$ -amylase protein biosynthesis. The proportional decrease in intracellular  $\alpha$ -amylase protein levels ruled out the possibility that cAMP may be bringing about its repressive effect, by somehow affecting the rate of secretion of the enzyme. The repressive effect of cAMP was similar to the effect of glucose on

TABLE 11

Repression of  $\alpha$ -Amylase Biosynthesis by Glucose and cAMP

Medium	<sup>a</sup> Extracellular $\alpha$ -amylase enzyme activity levels (units/g cell mass/4 h)	<sup>b</sup> <u>In vivo</u> $\alpha$ -amylase synthesis (CPM incorporated/ml) x 10 <sup>-3</sup>	
		Extracellular	Intracellular
Glucose	4.3 ± 0.3	21.6 ± 1.05	4.5 ± 0.23
Starch	18.1 ± 0.1	90.0 ± 3.50	17.2 ± 0.66
Starvation	21.0 ± 0.1	91.6 ± 4.58	17.1 ± 0.75
Starch + cAMP	9.5 ± 0.9	42.1 ± 1.90	8.6 ± 0.60

<sup>a</sup>Enzyme activity in the culture media was determined by measuring the amount of reducing sugars produced by  $\alpha$ -amylase action on starch.

<sup>b</sup>In vivo labelled proteins were separated by SDS-PAGE and detected by fluorography as described in the legend to Fig. 10.  $\alpha$ -Amylase specific protein bands were excised, solubilized in NCS tissue solubilizer and counted by liquid scintillations. The counts were corrected for recovery using immunoprecipitated  $\alpha$ -amylase band as reference standard.

All the results are presented as an average ± S.D. of 5 replicates.

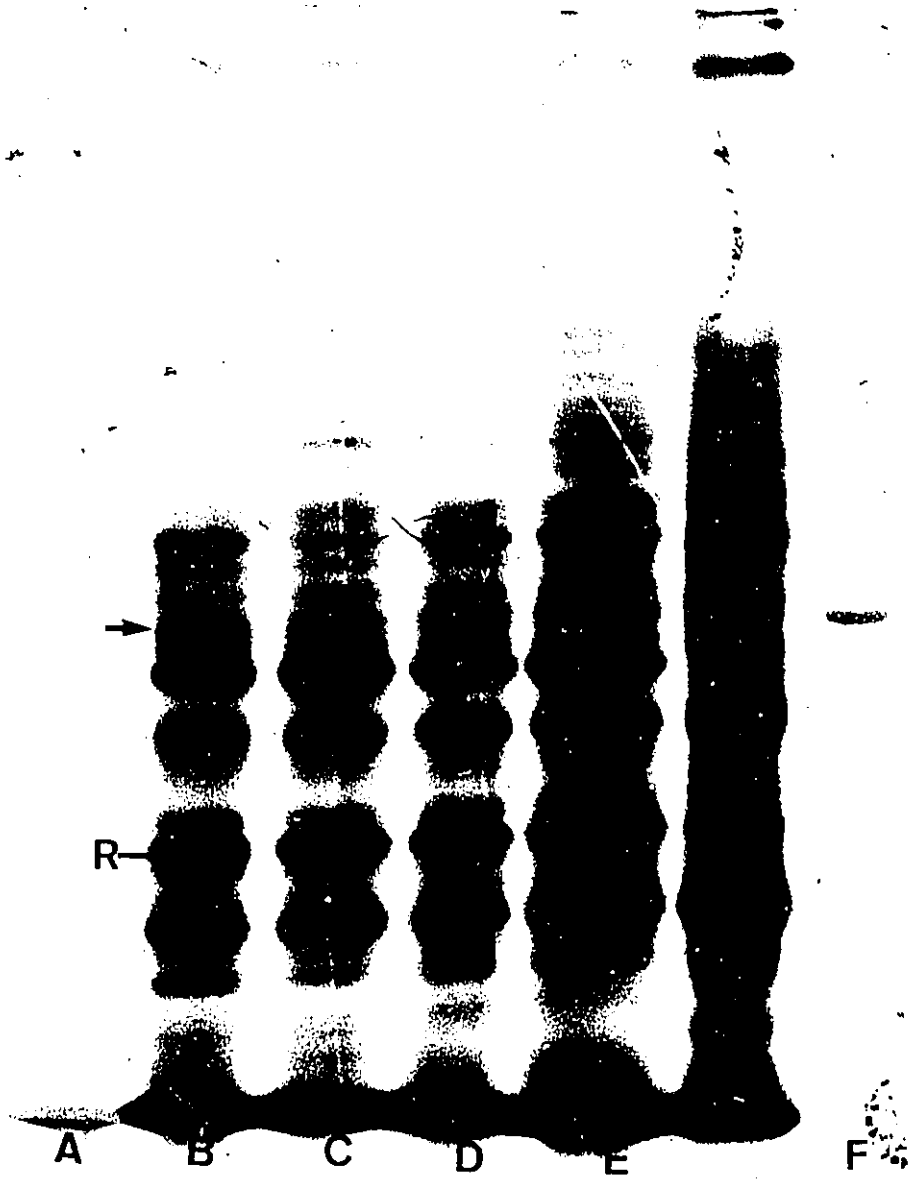
the biosynthesis of  $\alpha$ -amylase although repression by 4 mM cAMP was about half that effected by glucose.

### 3.4.3 Analysis of $\alpha$ -amylase specific mRNA concentration:

$\alpha$ -Amylase specific mRNA concentration was analysed by measuring its template activity in a cell free translation system. Total RNA was isolated from fungal cells actively synthesizing  $\alpha$ -amylase in response to glucose, starch or starvation media. Equal amounts of total RNA were translated in reticulocyte translation system and proteins were labelled with [ $^{35}$ S]-met. Translation products were analysed by SDS-PAGE followed by fluorography.  $\alpha$ -Amylase specific protein bands in the translation products were identified by the position of immunoprecipitated  $\alpha$ -amylase band. A typical fluorograph of in vitro translation products is shown in Fig. 24. In vitro synthesized  $\alpha$ -amylase was found to be about 52 000 in molecular weight, compared to the in vivo synthesized enzyme of about 54 000. The differences in the molecular weights may be due to the lack of glycosylation of in vitro synthesized proteins. To quantitate  $\alpha$ -amylase specific translatable mRNA,  $\alpha$ -amylase protein bands from in vitro translation products were excised and counted. To ensure equal input of RNA in translation products, the counts of all  $\alpha$ -amylase bands were standardized with reference to an arbitrary protein of about 30 000 molecular weight (R band in Fig. 24) which does not seem to be affected by the culture conditions. As indicated by the data shown in Table 12, there were no significant differences in  $\alpha$ -amylase translatable

Figure 24. Fluorograph of SDS-PAGE showing in vitro  $\alpha$ -amylase synthesis.

Total cellular RNA isolated from glucose, starch, starch + cAMP or starvation medium grown fungal cells was translated and analysed essentially as described in legend to Fig. 12. Lanes: A, control (-RNA); B, glucose sample RNA; C, starch sample RNA; D, starch + cAMP sample RNA; E, starvation sample RNA. Lane E shows the position of  $\alpha$ -amylase, immunoprecipitated from starch sample RNA directed in vitro translation products. R on the fluorograph denotes the reference protein band. (Exposure 36 hours).



A

B

C

D

E

F

R



TABLE 12

Analysis of In vitro Translatable  $\alpha$ -Amylase Specific mRNA  
Levels Under the Conditions of Repressed and Derepressed Synthesis  
of the Enzyme by A. awamori

RNA sample	Relative <u>in vitro</u> translatable activity (CPM/ $\mu$ g RNA) <sup>a</sup>
Glucose	4420 $\pm$ 265
Starch	4675 $\pm$ 318
Starvation	4800 $\pm$ 235
Starch + 4 mM cAMP	4300 $\pm$ 310

<sup>a</sup>Total cellular RNA was translated in vitro and translation products were analysed as described in the text and for Fig. 24.

Results are presented as an average  $\pm$  S.D. of 9 experiments with different RNA preparations.

activities in RNA samples from different cultures. These results suggest that catabolite repression of  $\alpha$ -amylase biosynthesis observed in vivo, operates at the level of translation of mRNA.

3.4.4 Effect of exogenous cAMP on the  $\alpha$ -amylase specific mRNA levels:

To investigate whether cAMP brings about the repression of  $\alpha$ -amylase biosynthesis by the same mechanisms as glucose, its effect on  $\alpha$ -amylase mRNA levels was studied.  $\alpha$ -Amylase specific mRNA levels were measured by using the in vitro translation assay method. As shown by the data in Table 12, cAMP did not affect the concentration of  $\alpha$ -amylase mRNA in the cells although it was shown to repress (section 3.4.2) the  $\alpha$ -amylase protein synthesis. This further strengthens the finding that  $\alpha$ -amylase biosynthesis is controlled at the translational level.

## CHAPTER IV

### DISCUSSION

#### 4.1.0 Purification and Properties of Amylolytic Enzymes

##### 4.1.1 Purification

Most of the Aspergillus species produce  $\alpha$ -amylase, glucoamylase and  $\alpha$ -glucosidase enzymes. Usually all of these enzymes are found in the extracellular medium which should be advantageous for the purification of these enzymes, as not too many protein species are expected to be present in the culture medium. However, in practice, purification of these enzymes proves to be a rather formidable task. In addition, most of the methods used for purification result in relatively very low recoveries (Mitsue et al., 1979; Pazur, 1972; Yamasaki et al., 1977). All of these methods involve DEAE-cellulose column chromatography at one or the other stage during the purification. Unfortunately,  $\alpha$ -amylase and glucoamylase activities are not completely separated by anion-exchange chromatography on DEAE-cellulose. This necessitates several cycles of DEAE-cellulose chromatography and often additional purification steps for the preparation of homogeneous enzyme proteins. The use of several steps or cycles of fractionations in enzyme purification inevitably results in low recoveries. For instance, a method described by Yamasaki et al., (1977) for the purification of A. awamori glucoamylase involves several steps including DEAE-cellulose chromatography, Sephadex gel filtration and preparative gel electrophoresis. Because of the

necessity of several steps to get electrophoretically homogeneous enzyme preparation, the final recovery using this scheme was around 40%. Analysis of the purification profile of glucoamylase reported by these authors clearly shows that most of the loss in recovery of enzyme activity occurs during DEAE-cellulose chromatography step. The protocol described for glucoamylase purification by Mitsue et al., (1979) involves at least nine steps including DEAE-cellulose chromatography and several gel filtration cycles using DEAE-sephadex as well as Sephadex gels. Using this procedure almost 90% of the enzyme is lost during the steps involving DEAE-cellulose or DEAE-sephadex fractionations resulting in final recovery of less than 2% of the enzyme protein. In comparison, the procedure described by Pazur (1972) for purification of glucoamylase enzyme from A. niger culture medium is relatively simple involving ethanol precipitation and two cycles of DEAE-cellulose chromatography. Analysis of the purification profile reported by Pazur (1972) using this three step procedure indicates that like the forementioned methods, almost 50% of the enzyme activity is lost during the DEAE-cellulose chromatography steps. The problem with DEAE-cellulose chromatography stems from the fact that the amylolytic enzymes tend to adsorb non-specifically to the cellulose matrix along with adsorption to DEAE ligand. The wide spread of elution patterns of  $\alpha$ -amylase and glucoamylase from DEAE-cellulose column as shown by data in Table 1 is indicative of the specific and non-specific interaction of these enzymes with DEAE ligand and cellulose

matrix. Adsorption of both  $\alpha$ -amylase and glucoamylase to cellulose has also been reported by Kennedy and Court (1983). The use of polystyrene resin as a matrix for anion-exchange chromatography eliminates the problems of adsorption to cellulose and affects the separation of these enzymes taking advantage of differences in their ionic properties. The method described in Materials and Methods (section 2.2) for purification of  $\alpha$ -amylase and glucoamylase enzymes essentially is a modification of the method described by Pazur for the purification of glucoamylase from A. niger. The method described by Pazur (1972) involves ethanol precipitation followed by two cycles of anion-exchange chromatography on DEAE-cellulose. The method described in this thesis involves ethanol precipitation to concentrate the proteins in the culture filtrates. In addition, ethanol precipitation may also affect separation of glycoproteins from non-glycoproteins if present in the culture medium. The ethanol is obviously more effective in the precipitation of carbohydrate carrying molecules such as glycoproteins and nucleic acids. The ethanol precipitation step is followed by gel filtration with Sephacryl-200. This step although does not separate  $\alpha$ -amylase and glucoamylase (Fig. 1) but is very effective in separation of  $\alpha$ -glucosidase enzyme from  $\alpha$ -amylase and glucoamylase enzymes. The removal of  $\alpha$ -glucosidase is crucial if one is to use step-wise salt gradient in the subsequent anion-exchange chromatography of these enzymes. After gel filtration step,  $\alpha$ -amylase and glucoamylase proteins are separated from each other by anion-exchange chromatography on

resin column. This method of purification is relatively simple and results in the recovery of enzyme proteins almost approaching 80-90% (Table 2). Furthermore, the effectiveness of stepwise salt gradient for the elution of enzyme proteins from anion-exchange column (Table 1) renders this method very suitable for large scale purification of  $\alpha$ -amylase and glucoamylase enzymes.

#### 4.1.2 Properties of glucoamylase

The properties of glucoamylase (Table 3 and Table 4) were essentially similar to those reported in the literature and mentioned in the Introduction (section 1.2.1).

#### 4.1.3 Properties of $\alpha$ -amylase

$\alpha$ -Amylase from A. awamori was found to be a single polypeptide of about 54,000 molecular weight (Fig. 2). Most of the  $\alpha$ -amylases from both bacterial (Kindle, 1983) as well as fungal sources (Arai et al., 1968; Yakubi et al., 1977) are in the 50,000-60,000 molecular weight range. The isoelectric point of  $\alpha$ -amylase at pH 4.2 (Fig. 3) was slightly higher than the values reported for acid-stable and acid unstable forms of  $\alpha$ -amylase from A. niger (3.44 and 3.75 respectively, Arai et al., 1968) and  $\alpha$ -amylase from A. oryzae (4.0, Yakubi et al., 1977).  $\alpha$ -Amylase from A. awamori was similar to acid unstable  $\alpha$ -amylase from A. niger and  $\alpha$ -amylase from A. oryzae in most of the biochemical properties i.e., optimal pH between 4.7 and 5.0; pH stability between 4.0 and 6.5 (Fig. 4); optimal temperature for activity around 50°C; heat stability up to 45°C (Fig. 5). The  $K_m$  value of 1.0 g.L<sup>-1</sup> for

starch (pH 5.3, 37°C; Fig. 6) was also similar to the values reported for A. niger and A. oryzae  $\alpha$ -amylases (Arai et al., 1968; Nitta et al., 1971). In variance with A. oryzae  $\alpha$ -amylase, glucose had no effect on the enzymatic activity of  $\alpha$ -amylase although maltose showed uncompetitive inhibition with a  $K_I$  value of 20.05 g.L<sup>-1</sup> (Fig. 6). In the case of  $\alpha$ -amylase from A. oryzae, glucose has been shown to be a competitive inhibitor of starch hydrolysis (Nitta et al., 1971). As is typical of fungal  $\alpha$ -amylases, the A. awamori enzyme displayed no requirement of Ca<sup>++</sup> for its activity or stability (Table 5). In contrast, most of the bacterial  $\alpha$ -amylases require Ca<sup>++</sup> for both activity and stability (Kindle, 1983). It appears that fungal  $\alpha$ -amylases are quite similar in physico-chemical properties which is a complete departure from the situation in bacteria.

#### 4.2.0 Regulation of Glucoamylase Biosynthesis

##### 4.2.1 Regulation at the cellular level

There are a number of reports in the literature regarding the cellular level of regulation of fungal glycosidases. In general, these enzymes are inducible by their substrates or substrate analogues and are repressed in the presence of preferred carbon sources for growth such as glucose. Thus  $\alpha$ -amylase of Aspergillus (Angelova et al., 1980; Feniksova et al., 1965),  $\alpha$ -glucosidase and invertase in Schizosaccharomyces (Schlanderer and Dellweg, 1974), maltase of Saccharomyces (Federoff et al., 1983), cellobiase (Eberhardt and Beck, 1973),  $\beta$ -galactosidase (Bates et

al., 1967) and invertase (Metzenberg, 1962) of Neurospora are some of the examples of the glycosidic enzymes regulated in this manner. On the other hand,  $\alpha$ -amylase (Adams and Deploey, 1976),  $\beta$ -glucosidase (Borgia and Sypherd, 1977) of Mucor,  $\alpha$ -glucosidase and  $\alpha$ -amylase of Lipomyces starkeyi (Kelly et al., 1985) are examples of the fungal glycosidases which are non-inducible and solely regulated by catabolite repression.

Based on the studies dealing with patterns of increase in activity (Fig. 7 and Fig. 8) as well as glucoamylase specific protein synthesis (Fig. 9 and Fig. 10), the glucoamylase synthesis in A. awamori seems to be regulated solely by catabolite repression. Similar rates of synthesis of the enzyme in the absence or during exhaustion of readily utilizable carbon source glucose regardless of the presence or absence of starch (Table 5 and Table 6), indicate that induction does not play any role in the regulation of this enzyme. Catabolite repression of the enzyme synthesis seems to be only partial as during repressive conditions the enzyme is still synthesized at about one fourth the rate observed during de-repression (Table 6 and Table 7). A previous report by Hayashida (1975) describing delayed synthesis of glucoamylase by A. awamori in glucose medium compared to starch medium is in agreement with the conclusion that enzyme synthesis is solely regulated by glucose repression. The glucoamylase enzyme synthesis in A. niger has also been reported to be sensitive to catabolite repression (Barton et al. 1969, 1972). In variance to my results regarding regulation of A. awamori glucoamylase, the A. niger

enzyme was reportedly inducible by  $\alpha$ -glucans (Barton et al., 1969, 1972). However, in my opinion, the data used to draw this conclusion were insufficient to distinguish between induction and de-repression. In fact a report by Paszczynski et al., (1985) does suggest that glucoamylase synthesis in A. niger may actually be regulated as discussed here for the A. awamori enzyme. The synthesis of glucoamylases in other fungal organisms studied so far also seem to be regulated solely by catabolite repression. For instance glucoamylase synthesis in Neurospora has been shown to be non-inducible and sensitive to glucose repression (Sigmund et al., 1985). However, unlike the glucoamylase of A. awamori the synthesis of which seems to increase 4-5 fold during derepression, Neurospora glucoamylase synthesis is stimulated more than 100 fold during release from glucose repression. The glucoamylase synthesis in Saccharomyces species seems to be growth related and does not exhibit a requirement for any specific exogenous inducer (Pretorius et al., 1986). The glucose is a weak repressor of glucoamylase synthesis in Saccharomyces as the enzyme synthesis is repressed only 2-3 fold in the presence of glucose (Pretorius et al., 1986).

Although cAMP has been shown to be involved in the regulation of synthesis of several fungal glycosidases, its role in the regulation of synthesis of amylolytic enzymes of Aspergilli has never been reported. The inhibitory effect of cAMP on the synthesis of glucoamylase as shown by the data of Fig. 10 and Table 7 suggests that glucose catabolite repression of glucoamylase syn-

thesis in A. awamori may also be mediated by cAMP. The negative modulation of glucoamylase synthesis by cAMP is in contrast to its reported positive effect on the synthesis of  $\alpha$ -glucosidase and invertase in Schizosaccharomyces (Schlanderer and Dellweg, 1974), trehalases of A. nidulans (Zonneveld, 1980) and Saccharomyces (van der Platt, 1974) or its well established effect on glycosidases of prokaryotes (Botsford, 1981). However, catabolite repression of  $\beta$ -glucosidase in Mucor (Borgia and Sypherd, 1977) and an extracellular protease in Neurospora crassa (Hansen and Marzluf, 1975) has been shown or suggested to be mediated by cAMP in a similar fashion as described here for glucoamylase of A. awamori. In addition, as in Mucor (Paznokas and Sypherd, 1975), intracellular cAMP levels in A. awamori were found to be in positive correlation with repressive conditions of enzyme synthesis (Fig. 11). Elevated cAMP levels in response to high glucose concentration in the culture medium have also been reported to occur in A. nidulans (Zonneveld, 1976, 1980), A. niger (Alobaidi and Berry, 1980) and A. oryzae (Yurkevich, 1985). On the basis of the observed repressive effect of both glucose and exogenous cAMP as well as the existence of a positive correlation between intracellular cAMP and glucose levels in the culture medium, it is not unreasonable to conclude that the catabolite repression regulated synthesis of glucoamylase in A. awamori is probably mediated by cAMP.

#### 4.2.2 Regulation at molecular level

Modulation of protein synthesis in eukaryotes can be brought about at the levels of gene transcription, mRNA processing, transport, stability, translation or post translation processes. Regulation at the level of all these processes is known to occur in eukaryotic organisms (Darnell Jr., 1982). My results regarding the glucoamylase synthesis in A. awamori suggest that catabolite repression regulated synthesis of this enzyme is brought about at the levels of both transcription and translation. During the de-repression of enzyme synthesis, the glucoamylase specific mRNA levels were about two fold higher than under repressed enzyme synthesis conditions (Table 8 and 9). These differences in mRNA levels most likely are caused by the higher rate of glucoamylase gene transcription during the de-repression as there were no apparent differences in mRNA processing (Fig. 14) or stability (mRNA half life 4.6 hours; Fig. 16) under different conditions of enzyme synthesis. However, two fold differences in mRNA levels do not explain the observed four fold stimulation of glucoamylase protein synthesis during the de-repression (Table 6 and Table 7). Differential mRNA translation efficiencies during repression and de-repression of glucoamylase synthesis are most likely to cause an additional two fold difference in enzyme protein levels during these conditions. Although post-translational processes (e.g. stability of the enzyme) can not be entirely ruled out, experimental data do not lend to this view. In fact comparison of the relative in vitro and in vivo glucoamylase mRNA template efficiencies (Table 13) clearly show that glucoamylase mRNA is

translated more efficiently during de-repression than the conditions of its repressed synthesis. Interestingly, the efficiency of translation during de-repression is, as expected, about two fold higher than under the repressive conditions. Regulation of the glucoamylase gene expression in A. awamori both at the transcriptional and translational level as indicated by my results contrasts with the reported transcriptional controls of several fungal glycosidases studied so far. Thus catabolite repressible genes of galactose (Guarente et al., 1982) and maltose (Charron et al., 1986) utilization in Saccharomyces have been suggested to be regulated at the transcriptional level. Similarly catabolite repression of invertase (Carlson and Botstein, 1982) and alcohol dehydrogenase (Denis and Galo, 1986) in Saccharomyces also seem to be mediated at the level of transcription. Among the filamentous fungi, the most extensively studied amidase gene (Hynes et al., 1985) and the qa gene cluster (genes encoding enzymes for the utilization of quinic acid as sole source of carbon or nitrogen during conditions of starvation; Giles et al., 1985) also seem to be regulated predominantly at the level of transcription. The regulation of glucoamylase of A. awamori both at transcriptional and translational level contrasts with the reported transcriptional control of this enzyme in Saccharomyces (Pretorius et al., 1986).

As suggested by the cellular level studies of glucoamylase gene expression, glucose catabolite repression of this enzyme in A. awamori does seem to be mediated by cAMP. As would be expected, exogenous cAMP mimicked the effect of glucose on the glucoamylase gene transcription by repressing it by about two fold during the

TABLE 13

Relative Translation Efficiencies of mRNA Templates of  
 $\alpha$ -Amylase and Glucoamylase Enzymes

	% of total proteins synthesized			
	Repressed synthesis		De-repressed synthesis	
	In vivo	In vitro	In vivo	In vitro
$\alpha$ -Amylase	1.84	1.47	7.55	1.56
Glucoamylase	0.49	0.56	2.10	1.20

conditions of de-repression (Fig. 13, Fig. 17 and Table 8 and Table 9). Like glucose, cAMP had no effect on the processing (Fig. 15) or stability (Fig. 16) of the glucoamylase specific mRNA. Exogenous cAMP at 4mM concentration consistently repressed in vivo glucoamylase synthesis by about two fold (Table 7) which is similar to its effect on the rate of gene transcription. Whether it mediates the glucose repression of glucoamylase mRNA translation efficiency remains to be clarified. Although our knowledge about the molecular basis of cAMP mediated gene expression of fungal glycosidases is almost entirely lacking, cAMP has been shown to be both negative and positive modulator of several cAMP responsive genes in the fungus Dictyostelium, an organism most studied in terms of cAMP mediated gene expression among fungi. For instance cAMP has been shown to repress the transcription of M4-1 gene (unknown function; Kimmel and Carlisle, 1986) and the discoidin-1 gene (Williams et al., 1980) in Dictyostelium. Similarly three heat shock protein genes referred to as hsp72A, hsp72B and hsp41 in Saccharomyces have also been reported to be negatively regulated by cAMP (Shin et al., 1987). On the other hand, cAMP has been shown to be a positive modulator of two developmentally regulated cystein proteinases in the fungus Dictyostelium (Presse et al., 1986). Reportedly, cAMP modulates the expression of these proteinase genes by enhancing the gene transcription by about 2-3 fold (Press et al., 1986). In addition to transcription, cAMP perhaps also enhances the stability of at least proteinase II mRNA (Datta et al., 1986).

#### 4.2.3 Production of Multiple Form of Extracellular Glucoamylases in A.

##### awamori

The results of in vivo labelling studies (Fig. 20) indicate that two forms of glucoamylases with apparent molecular weights of 100,000 and 82,000 are synthesized de novo. Post-translational processing of the higher molecular weight form leads to the formation of the commonly isolated form of glucoamylase with a molecular weight of about 94,000. As the NH<sub>2</sub>-terminal amino acid (alanine) of this form of glucoamylase is the same as the first codon (following the signal sequence codons) of glucoamylase specific mRNA (Boel et al., 1984a; Nunberg et al., 1984), this form must be generated by deglycosylation of the higher molecular weight (100,000) form which appears first in the culture medium. In fact, Yoshino and Hayashida (1978) have already provided the direct evidence for the presence and involvement of glycosidases in the formation of various types of glucoamylases in A. awamori. The variation in molecular weights reported for glucoamylases from the same organism by different authors (Pazur et al., 1971; Svensson et al., 1982) may actually be caused by the extent of deglycosylation at the time of purification. However, unlike the hypothesis forwarded by Yoshino and Hayashida (1978), all forms of glucoamylase do not seem to be produced by post-translational degradation of a single prototype. The existence of two glucoamylase specific mRNAs as demonstrated by cell-free translation (Fig. 21A) and Northern blotting (Fig. 21B) is compatible with my conclusion that two forms of glucoamylases are synthesized de novo.

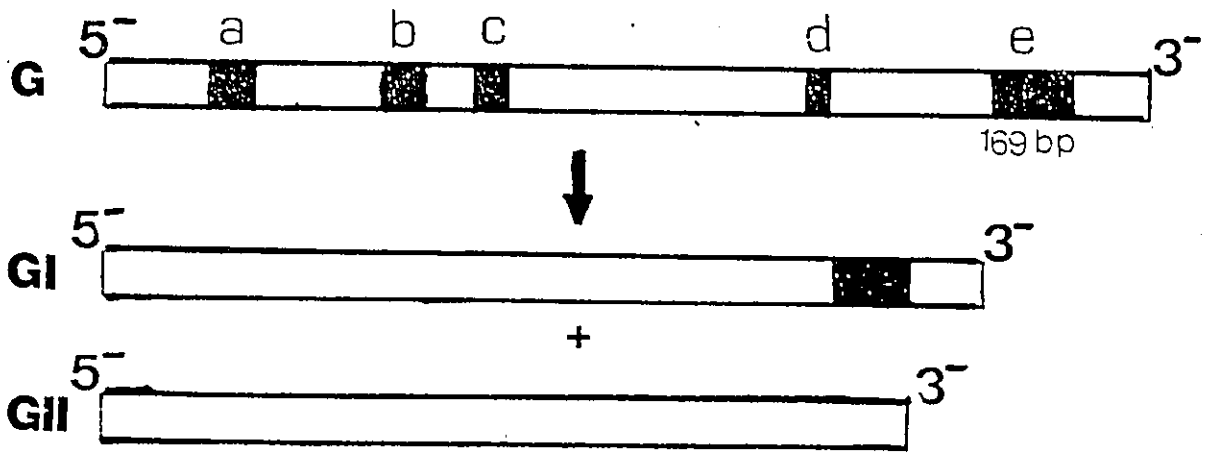
A. awamori has been shown to possess only a single copy of the glucoamylase gene (Nunberg et al., 1984). Hence, two types of glucoamylase specific mRNAs must be generated by either differential transcription of the same gene or differential processing of a single gene transcript. In A. niger, differential processing of a single gene transcript as illustrated in Fig. 25, leads to the formation of two glucoamylase specific mRNAs (Boel et al., 1984a). In GI mRNA one intron of 169 bases towards the 3'-end is retained while in the GII mRNA, this intron is processed out. As the glucoamylase gene structure is identical in both A. awamori and A. niger (Boel et al., 1984b; Nunberg et al., 1984), two glucoamylase specific mRNAs present in A. awamori differing by about 200 bases (Fig. 21B) are most likely formed by a mechanism similar to the one illustrated in Fig. 25. However S1 mapping and primer extension studies should be carried out to confirm this possibility. The production of different proteins or isoforms by the differential processing of a single gene transcript is a very common phenomenon in eukaryotes (Carlson and Botstein, 1982; Rogers et al., 1980). However, the functional significance of differential processing of the glucoamylase gene transcript and the formation of two glucoamylases (one major and one minor) is not clear.

#### 4.3.0 Regulation of $\alpha$ -Amylase Biosynthesis

##### 4.3.1 Regulation at cellular level

The patterns of  $\alpha$ -amylase synthesis during fungus growth in starch (Fig. 22). or glucose (Fig. 23) indicate that  $\alpha$ -amylase

Figure 25. Probable mechanism of formation of two glucoamylase specific mRNAs and hence two types of glucoamylases. G denotes the initial glucoamylase gene transcript with five introns (black boxes, a-e). GI and GII are mature mRNAs presumably formed by differential processing of the initial gene transcript.



synthesis in A. awamori is regulated by catabolite repression. The similar rates of  $\alpha$ -amylase synthesis upon glucose exhaustion or in the absence of glucose in either glucose or starch medium (Table 10) indicate that an exogenous inducer such as starch is not required for  $\alpha$ -amylase synthesis in A. awamori. These conclusions are further supported by the studies of  $\alpha$ -amylase synthesis by pre-grown mycelia. The synthesis of  $\alpha$ -amylase in the pre-grown mycelia is repressed by about fourfold in the presence of glucose (Table 11). About four fold stimulation of  $\alpha$ -amylase synthesis observed in the starch medium is essentially due to de-repression rather than induction as the enzyme is synthesized at similar rates even in the starvation medium which lacks any carbon source. The catabolite repression of  $\alpha$ -amylase synthesis is only partial as the enzyme is synthesized at low levels even in the presence of readily utilizable carbon source such as glucose.  $\alpha$ -Amylase synthesis in A. oryzae has also been reported to be sensitive to catabolite repression (Angelova et al., 1980). However, unlike  $\alpha$ -amylase of A. awamori as reported here, A. oryzae enzyme seems to be inducible by  $\alpha$ -glucans (Angelova et al., 1980; Erratt et al., 1984; Yakubi et al., 1977). It is interesting to note the existence of these different modes of regulation of  $\alpha$ -amylase synthesis in closely related species of Aspergillus. Different modes of regulation of amylase synthesis in various species of Bacillus are also known to exist. Thus, the synthesis of  $\alpha$ -amylases in Bacillus subtilis (Coleman, 1967; Heineken and O'Connor, 1972), B. licheniformis (Saito and Yamamoto, 1975;

Thirunavukkarasu and Priest, 1980) has been reported to be regulated solely by catabolite repression. On the other hand  $\alpha$ -amylase of B. stearothermophilus (Welker and Campbell, 1963) is regulated both by induction and catabolite repression.

As discussed for glucoamylase,  $\alpha$ -amylase synthesis in A. awamori, is also repressed by exogenous cAMP (Table 11). The repressive effect of exogenous cAMP during the conditions of de-repressed  $\alpha$ -amylase synthesis (e.g. in starch medium) and the positive correlation of intracellular cAMP levels and glucose concentration in the culture medium (Fig. 11, discussed in section 4.2.1) suggests that catabolite repression of  $\alpha$ -amylase synthesis is probably mediated by cAMP. cAMP has also been reported to be involved in the regulation of  $\alpha$ -amylase synthesis in Bacillus licheniformis (Saito and Yamamoto, 1975). But its role in the regulation of  $\alpha$ -amylase synthesis in bacilli in general, is presently controversial as attempts to detect intracellular cAMP or enzymes responsible for its synthesis and degradation in several bacilli have been unsuccessful (Priest, 1977).

#### 4.3.2 Regulation at molecular level

Catabolite repression and de-repression of  $\alpha$ -amylase synthesis in A. awamori seem to be mediated at the translational level.  $\alpha$ -Amylase mRNA concentration, as analyzed by its in vitro template activity, is not affected by the type of carbon source in the culture medium (Fig. 24, Table 12), although presence of glucose represses the protein synthesis at least by four fold (Table 11).

The results of differential hybridization of cDNA library (Appendix 2) also lead to the same conclusion that there is no difference in  $\alpha$ -amylase mRNA levels during A. awamori growth in starch or glucose medium. The observed differential rates of protein synthesis can be caused by either less efficient translation of  $\alpha$ -amylase mRNA during repression or high efficiency of translation during the de-repression. As indicated by Table 13, the relative in vitro and in vivo translation efficiencies of  $\alpha$ -amylase mRNA templates during repression are almost similar. But during de-repression in vivo translation efficiency is about five fold higher than in vitro translation efficiency. This suggests that  $\alpha$ -amylase mRNA is translated in vivo much more efficiently during the de-repression of  $\alpha$ -amylase synthesis than under the conditions of repression. Translational control seems to be a common phenomenon for  $\alpha$ -amylases that are regulated by catabolite repression. Thus catabolite repression regulated synthesis of  $\alpha$ -amylases in Bacillus amyloliquefaciens (Gould et al., 1975), B. subtilis (Semets et al., 1973) and Aspergillus oryzae (Angelova et al., 1983; Yurkevich et al., 1984) involves at least some elements of translational control. Erratt et al., (1984) have suggested that  $\alpha$ -amylase biosynthesis in A. oryzae is regulated at the transcriptional level. However, only four fold stimulation of  $\alpha$ -amylase mRNA reported by these authors does not explain about 20 fold stimulation of protein synthesis during  $\alpha$ -amylase induction in this organism. Most likely a high translational efficiency during induction phase results in an additional five fold stimu-

lation of  $\alpha$ -amylase synthesis under these conditions. Several glucose repressible fungal genes or their regulators have been recently reported to be regulated at the translational level. Thus glucose repressible synthesis of cytochrome oxidase subunit III in the yeast Saccharomyces has been shown to be regulated at the translational level (Costanzo and Fox, 1986). Similarly ADRI, a positive regulator gene for glucose repressible alcohol dehydrogenase of Saccharomyces, also has been reported to be regulated at the translational level (Denis and Galo, 1986). It seems that translational control is involved directly or indirectly in the mediation of catabolite repression in fungal organisms.

CHAPTER V

GENERAL DISCUSSION

5.1. Modulation of biosynthesis of  $\alpha$ -amylase and glucoamylase enzymes

The  $\alpha$ -amylase and glucoamylase enzymes of A. awamori both seem to be non-inducible and regulated at the cellular level solely by catabolite repression. Upon de-repression, the synthesis of these enzymes is stimulated by a factor of about 4-5 fold. The catabolite repression regulated synthesis of both of these enzymes seems to be mediated by cAMP. Interestingly, cAMP was found to be a negative regulator of these enzymes in A. awamori compared to its well documented positive effect on gene expression in prokaryotes. Surprisingly, the similarities in gene expression of  $\alpha$ -amylase and glucoamylase enzymes in A. awamori are restricted only to the cellular level, as different molecular mechanisms of gene regulation were found to be operative in each case. Thus the gene expression of  $\alpha$ -amylase seems to be controlled at the level of mRNA translation. On the other hand, the analysis of enzyme activity, protein and mRNA levels strongly suggest that the glucoamylase gene expression is regulated at the levels of both transcription and translation. The repressive effect of exogenous cAMP on the transcription of the glucoamylase gene suggests that catabolite repression of these enzymes is probably mediated by cAMP dependent or related processes. Although about 50% repression of glucoamylase gene transcription by exogenous cAMP is sufficient to

explain its similar effect on the glucoamylase protein synthesis, I believe the translational effect of glucose repression on glucoamylase gene expression may also be mediated by cAMP dependent mechanisms. Exogenous cAMP appears to partially repress the synthesis of  $\alpha$ -amylase and the gene expression of this enzyme seems to be regulated at the translational level. If cAMP, indeed, is involved in the repression of synthesis of this enzyme, it has to be doing so at the level of translation of mRNA. The inability of exogenous cAMP to completely overcome or counteract the processes mediating translation during de-repression may explain its only partial repression of  $\alpha$ -amylase and glucoamylase enzymes.

The data presented in this thesis is limited to the elucidation of modes of regulation of synthesis of  $\alpha$ -amylase and glucoamylase enzymes. How these modes of regulation such as transcription or translation etc. are mediated, is another question. Whatever the mechanisms of regulation of these modes, cAMP appears to be playing some role. The mechanisms by which cAMP mediates gene expression in eukaryotes seem to be different than the ones operative in prokaryotes. It is now well established that in prokaryotes, cAMP brings about its effect via a cAMP receptor protein (CRP or CAP). The cAMP-CRP complex modulates the gene expression of cAMP responsive operons by interacting directly with their promoter sequences (Ullman and Danchin, 1983). On the other hand, there is substantial evidence that the effect of cAMP on the responsive eukaryotic genes is mediated by cAMP dependent protein

kinases (Montminy et al., 1986). The cAMP-dependent protein kinases are activated by cAMP and presumably bring about the changes in gene expression by affecting the phosphorylation states of some regulatory proteins. Whether cAMP, when involved, mediates the fungal gene expression in a manner similar to prokaryotes or higher eukaryotes remains to be clarified. The analysis of structure and expression of several fungal genes e.g. maltase (Charron et al., 1986), invertase (Sarokin and Carlson, 1985), galactose utilization (Guarente et al., 1982) genes of yeast Saccharomyces, acetamidase gene of Aspergillus nidulans (Hynes et al., 1985) and qa gene cluster (genes required for utilization of quinic acid as sole source of carbon and nitrogen) in Neurospora crassa (Giles et al., 1985) has revealed that fungal genes, like higher eukaryotic genes, are controlled by multiple regulatory circuits. These regulatory elements include both trans and cis acting elements. The trans acting elements (usually proteins) act on the cis elements (usually certain nucleotide sequence stretches) to modulate the gene expression. These cis elements which can be several hundred bases away from the transcription initiation sites can be considered to some extent as equivalent to enhancer elements in higher eukaryotes. Keeping this information in view, it is very unlikely that cAMP by itself or in conjunction with some regulatory protein would regulate the transcription of glucoamylase gene in A. awamori by acting directly on the promotor sequences of this gene. It is more likely that it brings about its effect by activating some protein kinase which in return is involved in phosphory-

lation of some regulatory protein. In fact, the recent demonstration that the SNFI gene which is required for de-repression of several glucose repressible genes in Saccharomyces, encodes a protein kinase (Celenza and Carlson, 1986) which may be bringing about its effect by phosphorylation of regulatory proteins, lends a strong support to the view that cAMP may be mediating gene transcription in A. awamori by activating a similar protein kinase.

Although expression of several eukaryotic genes have been shown to be regulated at the translational level (Darnell Jr., 1982), the mechanisms affecting the control at this level are just beginning to be understood. The translation initiation factors, particularly eIF2 (Ochoa et al., 1981) eIF4 (Ray et al., 1986) as well as 5'-end non-coding sequences of mRNAs (McGarry and Landquist, 1985; Rosen et al., 1986; Tzamarias et al., 1986) are postulated to be involved in translational controls. The translational control is thought to be brought about by other regulatory proteins that interact with either components of eIF or the 5'-end of mRNAs. For instance eIF-2 stimulatory protein (ESP) interacts with eIF-2 to enhance the translational efficiency of globin mRNA in erythrocytes (Ochoa et al., 1981). While a transactivator protein (TAT III) enhances the translation of T-lymphotropic virus III mRNA by acting at the 5'-end (Rosen et al., 1986). Similarly PET 494 gene product has been shown to enhance the translation of cytochrome c oxidase III mRNA in Saccharomyces by acting at the 5'-end of the mRNA (Costanzo and Fox, 1986). So it is possible

that translational control of  $\alpha$ -amylase and glucoamylase in A. awamori is also mediated by one or all of these mechanisms. As cAMP seems to be involved in the modulation of translation efficiencies of these mRNAs, it is possible that cAMP dependent phosphorylation - dephosphorylation of some of the components of translational machinery plays a major role. In fact it has been shown that phosphorylation of the  $\alpha$ -subunit of eIF-2 inhibits globin mRNA translation in erythrocytes (Haro et al., 1982). I find it tempting to speculate that during repression of  $\alpha$ -amylase and glucoamylase synthesis in A. awamori, a cAMP dependent protein kinase is activated which phosphorylates some regulatory protein. This regulatory protein when phosphorylated somehow (interacting with eIF or 5'-end) lowers the translation efficiencies of  $\alpha$ -amylase and glucoamylase mRNA templates. While during de-repression an active phosphatase dephosphorylates this regulatory protein and when dephosphorylated this protein enhances the translational efficiencies of these templates. This would explain why exogenous cAMP is unable to completely overcome the de-repression of these enzymes. Because under these conditions cAMP dependent phosphorylation is being counteracted by phosphatase dependent dephosphorylation. Alternatively it is possible that during different conditions these genes are transcribed from different initiation sites, a phenomenon that could also be mediated by cAMP. Because of the differences in their 5'-end non-coding sequences, the translation efficiencies of these mRNAs may vary. Multiple initiation sites of transcription in eukaryotes is a

common phenomenon (Kozak, 1983, see pages 29-30). In fact, Nunberg et al., (1984) has shown the occurrence of multiple initiation sites of glucoamylase gene transcription in A. awamori. Whether the translational efficiencies of mRNAs are regulated by the generation of different 5'-ends during the conditions of repressed and de-repressed enzyme synthesis remains to be seen. Regardless of the mechanisms underlying the gene expression of  $\alpha$ -amylase and glucoamylase enzymes in A. awamori, my studies (highlighted by the data of Table 13) strongly suggest that the translational control plays a major role in the modulation of  $\alpha$ -amylase and glucoamylase gene expression in A. awamori. Probably translational control mechanism of gene expression allows the organism to respond quickly to the environmental changes. In fact several eukaryotic genes which are involved or are required for quick adaption to the environmental changes have been shown to be regulated predominantly at the translational level. For instance the heat shock response in fungal as well as higher eukaryotic organisms is predominantly mediated at the translational level. Survival of organisms at temperature higher than normal temperature requires a quick response and it has been shown at least in Xenopus oocytes (Bienz and Gurdon, 1982), Drosophila (McGarry and Lindquist, 1985) and in Saccharomyces (Shin et al., 1987) that this is achieved by enhancing the translational efficiencies of pre-existing heat shock protein mRNAs. Interestingly heat shock protein mRNAs are also known to be translated selectively and with enhanced efficiency during nutritional

stress as seems to be the case for two amylolytic enzymes in A. awamori. The employment of translational control by eukaryotic organisms for quick adaptation to the environmental changes is further demonstrated by among others the insulin, apolipoprotein E and ferritin genes in animals. The insulin controls glucose levels in blood and hence its synthesis and secretion is required to change quickly according to the changes in blood glucose. The glucose dependent synthesis of pro-insulin is mainly achieved by enhancing the translational efficiency of its pre-existing mRNA (Itoh and Okamoto, 1980). Apolipoprotein E is required for uptake of lipoproteins by hepatic and other tissues. High lipid diet is known to stimulate the synthesis of apolipoprotein E at least in mice without affecting its mRNA levels (Reue et al., 1984). Similarly upon administration of iron, the rat liver has been shown to increase the synthesis of ferritin several fold by enhancing the translational efficiency of pre-existing mRNAs (Aziz and Munro, 1986).

#### 5.2. cAMP and catabolite repression of $\alpha$ -amylase and glucoamylase enzymes

In the preceding discussion, cAMP was discussed as a probable mediator of catabolite repression regulated synthesis of  $\alpha$ -amylase and glucoamylase enzymes in A. awamori. This conclusion was arrived at basically because of the following observations:

- a) Intracellular cAMP levels in A. awamori were found to fluctuate in a positive correlation with the glucose levels in the culture medium

- b) Like glucose, exogenous cAMP had a repressive effect on the synthesis of  $\alpha$ -amylase and glucoamylase enzyme proteins
- c) Exogenous cAMP had an effect similar to glucose on the glucoamylase specific mRNA levels in A. awamori cells.

These observations merely suggest but are not sufficient to demonstrate unequivocally that the catabolite repression of these two amylolytic enzymes in A. awamori is actually mediated by cAMP dependent processes. The possibility that high intracellular cAMP levels during catabolite repression conditions are effect rather than cause of catabolite repression can not be ruled out by the data presented in this thesis. This seems to be the case at least in prokaryotes where it has been suggested that a catabolite modulator factor is a direct modulator of catabolite repression and often observed negative relationship between cAMP and catabolite repression results from the inhibitory effect of this factor on the adenylate cyclase (Ullman, 1985). The activation of adenylate kinase and increase in intracellular cAMP levels in A. awamori may also result from the secondary effect of glucose repression. At least in Saccharomyces glucose has been shown to increase the synthesis of hexokinase II which in return brings about the repression of catabolite sensitive genes (Entian, 1986). Although never demonstrated, this hexokinase enzyme which is phosphorylated in its active form may activate the membrane bound adenylate cyclase by phosphorylation of this enzyme through phosphate transfer reaction similar to the one suggested for phosphoenolpyruvate-phosphotransferase phenomena in prokaryotes (Kornberg, 1981).

Although externally added cAMP repressed the protein synthesis of both  $\alpha$ -amylase and glucoamylase enzymes in A. awamori, there is no evidence that cAMP was bringing about this effect by getting into the cells and affecting some intracellular processes. At least in Dictyostelium it has been shown that exogeneous cAMP binds to a membrane receptor which results in the activation of adenylate cyclase hence increase in intracellular cAMP levels (Oyama and Blumberg, 1986). Whether Aspergilli have a membrane bound receptor for cAMP is not known. However, the ability of exogenous cAMP to somehow transduce the signal suggests that Aspergilli may also have receptor protein for cAMP or cAMP is somehow able to enter the fungal cells. Regardless of the mechanism by which exogenous cAMP enters or affects the intracellular cAMP pool, its repressive effect on these two amylolytic enzymes does not necessarily suggest that it is a mediator of catabolite repression of these enzymes. If catabolite repression in Aspergilli also involves hexokinase II enzyme as shown for Saccharomyces, then cAMP may bring about the apparent repression by affecting the hexokinase II enzyme. Hexokinase II enzyme is known to be activated by cAMP dependent protein kinase. Under the conditions of repression i.e. presence of glucose a protein kinase is active and phosphorylates the hexokinase II enzymes. In the absence of glucose, exogenous cAMP may activate the kinase by binding to the regulatory unit thus resulting in activation of hexokinase II and hence catabolite repression. However, it is very likely that glucose may actually be bringing

about its repressive effect on the sensitive genes through both cAMP and hexokinase II. As mentioned above the glucose increases the synthesis of hexokinase II enzyme in Saccharomyces but for its activity this enzyme needs to be phosphorylated which requires a cAMP-dependent protein kinase. Thus entry of glucose into the cells can be envisaged as a trigger for increase in hexokinase II levels as well as cAMP levels.

If cAMP is indeed involved in modulation of gene expression in fungi or for that matter any eukaryotic organism, it is generally believed to be doing so by activating protein kinases rather than directly affecting the gene activity. Indirect role of cAMP in modulation eukaryotic gene expression, is obviously dictated by the existence of multiple regulatory elements for eukaryotic genes. The transcription of fungal genes is also modulated by the interaction of several regulatory proteins with the 5'-end sequences of the genes (see introduction section). Thus phosphorylation-dephosphorylation states of these regulatory proteins obviously can affect the interaction of these proteins among each other or with 5'-end sequences (called upstream activator sequences in yeast) thereby bringing about the gene modulation. cAMP dependent kinase can bring about this phosphorylation and dephosphorylation of proteins through either a cascade of events leading to activation of certain phosphorylases or phosphatases or direct phosphorylation of regulatory proteins. With few exceptions nature of the regulatory proteins is not known. Isolation and characterization of the regulatory proteins is crucial to deter

mine whether their activity is in fact modulated by cAMP dependent phosphorylation and dephosphorylation. There is some evidence that phosphorylation-dephosphorylation of regulatory proteins plays some role in the mediation of catabolite repression at least in yeast. The genetic studies have shown that a product SNFI gene is required for the expression of a number of glucose sensitive genes in yeast (Celenza and Carlson, 1986). The sequence analysis of this gene has shown that it encodes a protein which is homologous to cAMP-dependent protein kinase (Celenza and Carlson, 1986). Although the mechanism by which this gene affects the expression of glucose sensitive genes is not known, the nature of the gene product suggests that it probably brings about its effect by phosphorylation of some other regulatory proteins.

In addition, to identification and characterization of regulatory proteins, the demonstration of role of cAMP in the mediation of catabolite repression requires the identification of sequences that are targets of these regulatory proteins. This approach has been used to establish a role of cAMP in the modulation of expression of several eukaryotic genes (Comb et al., 1986; Datta et al., 1986; Montminy et al., 1986; Pears and Williams, 1987). The most but not all cAMP responsive genes of higher eukaryotes contain TGACGTCA sequences located at 70700 bases upstream of transcription initiation site. The analysis of upstream sequences required for cAMP induction of proteinase genes in Dictyostelium has shown that these cAMP responsive sequences are different in the fungal organisms than in the higher eukaryotes (Pears and Williams, 1987). The analysis of extensive

upstream sequences of glucoamylase gene is required to determine whether it contains any cAMP responsive sequences.

The results described in this thesis indicate that translational control plays a major role in the modulation of gene expression of  $\alpha$ -amylase and glucoamylase enzymes. If catabolite repression of these enzymes in A. awamori is mediated by cAMP dependent processes then obviously some of these processes are translation apparatus related. As has been discussed in preceding section, there is enough evidence that suggests that phosphorylation-dephosphorylation of some proteins involved in translation plays a key role in the modulation of translational control. However to my knowledge there is no evidence that demonstrates that this phosphorylation-dephosphorylation is related to cAMP-dependent protein kinases. To establish the role of cAMP in the modulation of translational control of amylolytic enzymes in A. awamori, one needs to identify translation related regulatory proteins, determine their state of phosphorylation-dephosphorylation during various conditions of enzyme synthesis and study their effects using in vitro translation assay systems.

It is apparent from the preceding discussion that a lot remains to be learned about the precise mechanisms of gene regulation in filamentous fungi. Our knowledge about the regulatory elements controlling such processes as transcription and translation in these organisms is almost non-existent. cAMP mediated gene expression of  $\alpha$ -amylase and glucoamylase enzymes in A. awamori provides an excellent model system for elucidation of the mechanisms of gene regulation in filamentous fungi. Recent developments in fungal transformation systems (Ballance et al., 1983; Yelton et al., 1984) and growing interest in these organisms for heterologous gene expression certainly would go a long way in adding to our knowledge about regulation of gene expression in these organisms.

APPENDIX 1

Optimization of Rabbit Reticulocyte in vitro

Translation System

In vitro translation assay system is oftenly used to characterize mRNAs by virtue of their translation products. The relative abundance of the translation product of a given mRNA can be used as an indirect measure of the levels of that mRNA species in a mixture of mRNAs. Rabbit reticulocyte and wheat germ in vitro translation systems are commonly employed for in vitro translation of mRNAs from a wide variety of sources. A major drawback of wheat germ in vitro translation system lies in its incapability to synthesize high molecular weight proteins. Rabbit reticulocyte in vitro translation system not only is very efficient in the translation of a wide variety of mRNAs, it is also capable of translating unusually long mRNAs coding for proteins upto 200,000 in molecular weights. Hence rabbit reticulocyte in vitro translation system is considered as the system of choice for in vitro translation of mRNAs. The efficiency of in vitro translation systems depends upon, among other factors, the optimal concentration of  $K^+$  and  $Mg^{++}$  ions. The commercially available in vitro translation system preparations are usually optimized for the efficient translation of most mRNAs from various sources. However the optimal concentration of  $K^+$  and  $Mg^{++}$  varies from mRNA to mRNA, hence it is imperative to determine the optimal

concentrations of these ions for mRNAs from a given source. Failure to do so may result in inefficient translation as well as production of truncated peptides which is totally undesirable if quantitation of mRNAs is to be done by this assay system.

#### Optimal $K^+$ concentration

The effect of  $K^+$  concentration, in the range of 100-233 mM on in vitro translation of fungal mRNAs was studied. As shown by the data of Figure 26, the increase in  $K^+$  concentration upto 180 mM resulted in almost linear increase in efficiency of translation (indicated by the incorporation of [ $^{35}$ S]-met into proteins) of fungal mRNAs. The optimal concentration of  $K^+$  ions for in vitro translation of A. awamori mRNAs with rabbit reticulocyte translation system was in the 180-200 mM range. The translation efficiency was considerably reduced below or above this range of  $K^+$  concentration.

#### Optimal $Mg^{++}$ concentration

Figure 27 shows the effect of  $Mg^{++}$  concentration on the translation efficiency of fungal mRNAs by rabbit reticulocyte in vitro translation system. The optimal  $Mg^{++}$  concentration was found to be around 1.20 mM. Although there was reduction in the efficiency of in vitro protein synthesis with the  $Mg^{++}$  concentration below or above 1.20 mM, the effect was not as severe as observed for  $K^+$  concentration.

Figure 26. The effect of  $K^+$  concentration on in vitro translation efficiency of A. awamori RNA by rabbit reticulocyte cell free translation system.

In vitro translation reaction mixtures consisted of 70% (v/v) commercial reticulocyte lysate (Amersham), 2  $\mu$ g of total fungal RNA and 10  $\mu$ Ci [ $^{35}$ S]-met in a total volume of 10  $\mu$ l.  $Mg^{++}$  ion concentration of the reaction mixtures was 1.2 mM and  $K^+$  concentration as indicated. After allowing the translation to occur for 1 hour at 30°C, 1  $\mu$ l aliquots of translation mixtures were spotted on Whatman-3 filter squares (25 mm<sup>2</sup>) in duplicate. After air drying, the filters were submerged in 10% TCA chilled with ice for 5 minutes. The filters were then transferred to a boiling bath of 5% TCA solution. After 10 minutes in boiling bath the filters were washed consecutively in 5% TCA solution, 95% ethyl alcohol and acetone. The filter were dried and assayed for radioactivity by liquid scintillations. Each point plotted in the figure represents the average of two independent translation assays with the same RNA preparation.

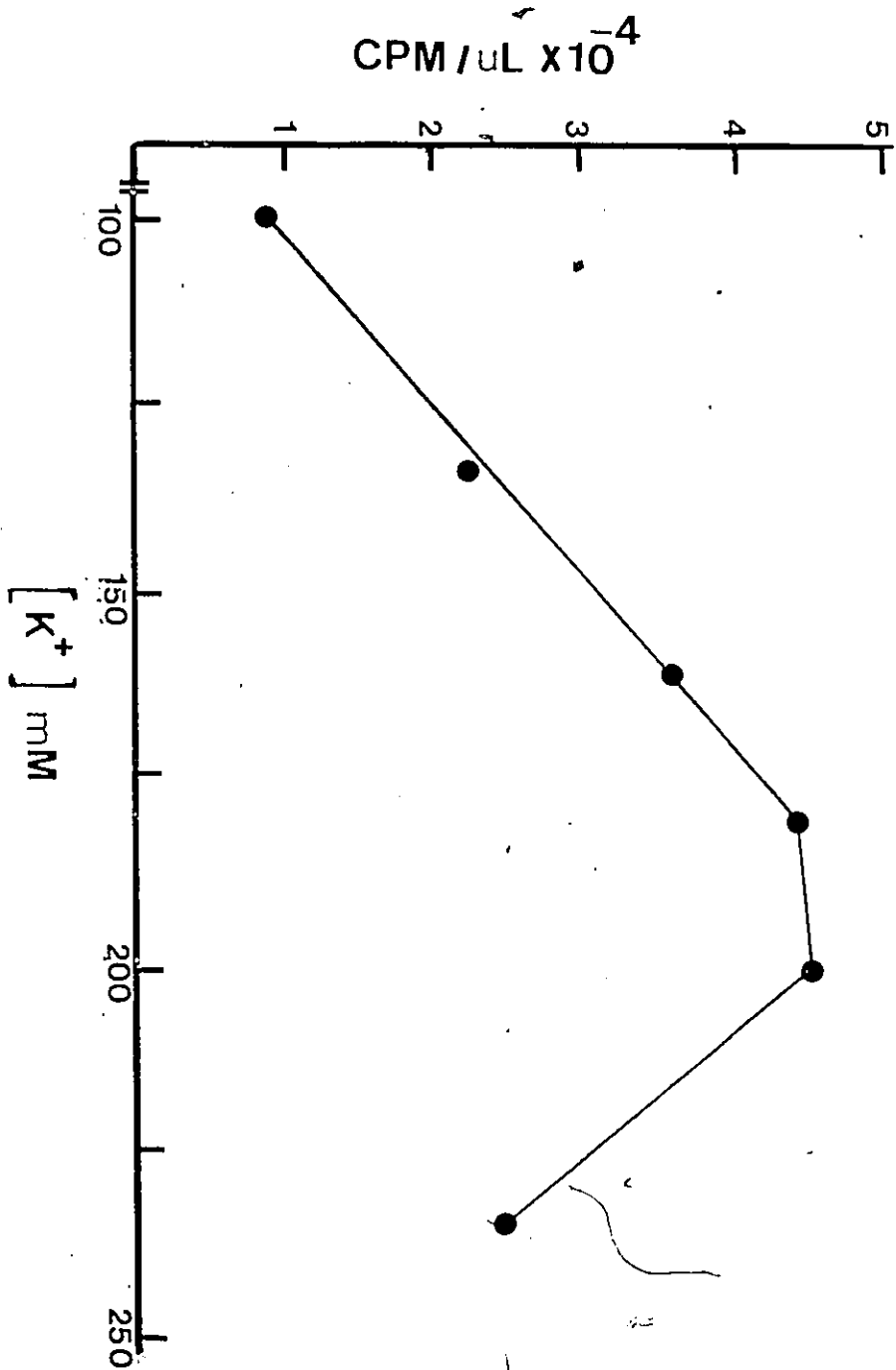
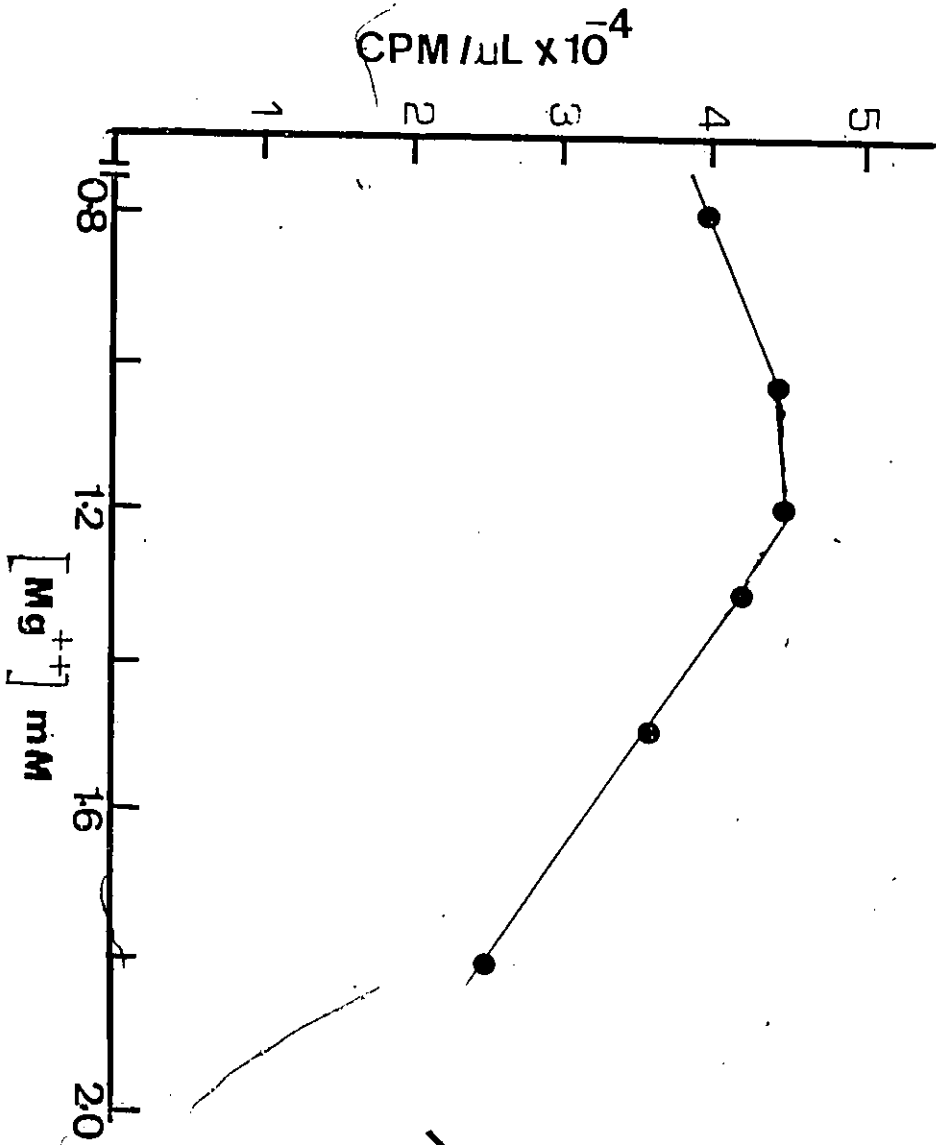


Figure 27. The effect of  $Mg^{++}$  concentration on in vitro translation efficiency of A. awamori RNA by rabbit reticulocyte cell free translation system.

In vitro translation assays were done as described in legend to Fig. 26 except that  $K^+$  concentration in the reaction mixture was adjusted to 180 mM and  $Mg^{++}$  concentration was as indicated.

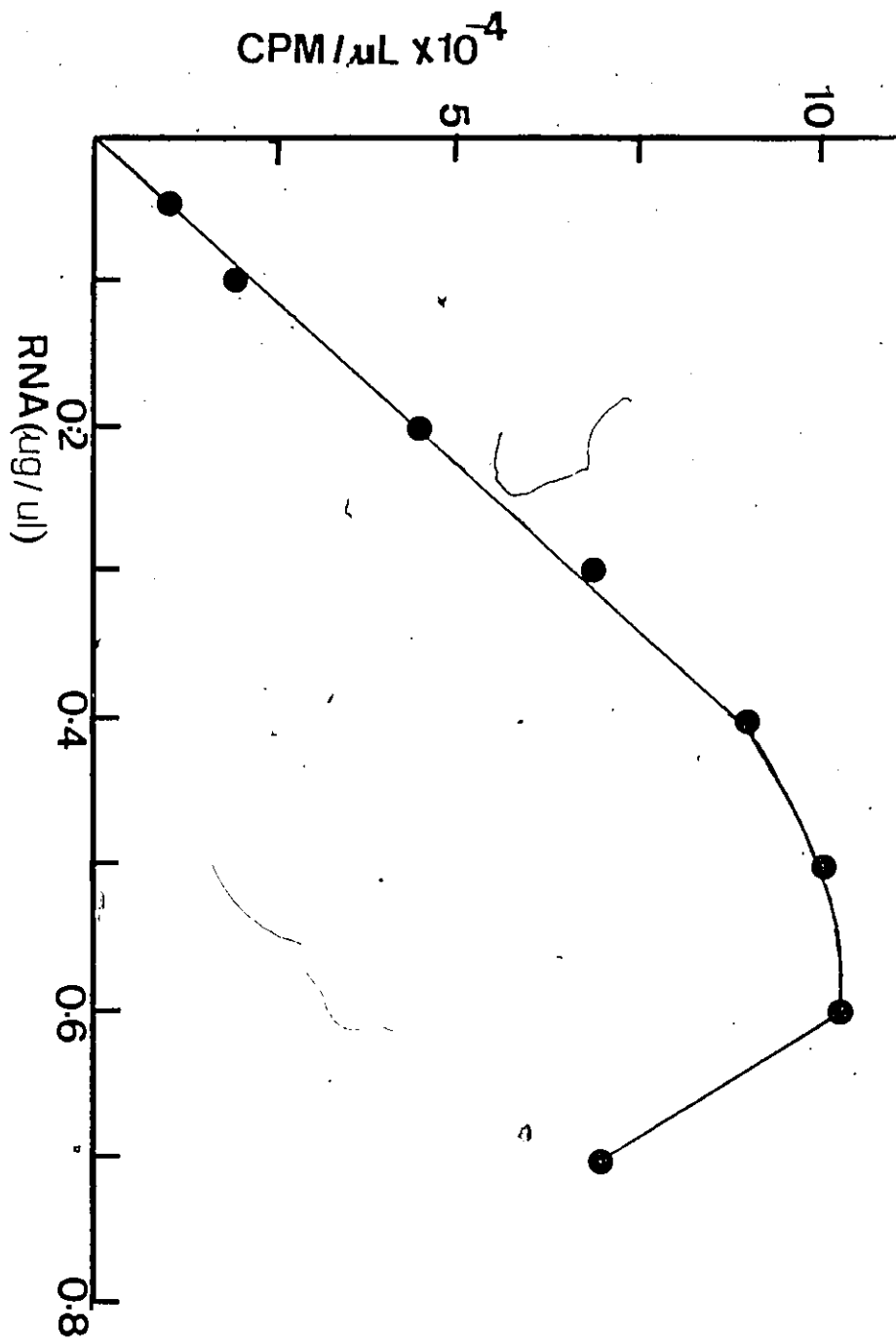


Optimal RNA concentration

It is imperative that the amount of RNA used for in vitro translation assay be below the saturation point, if in vitro translation assay is to be used for the quantitation of a given mRNA species. As in vitro translation assay was used for mRNA quantitations throughout this study, its capacity for translation of total RNA was determined. There was linear response to input RNA upto 0.4  $\mu\text{g}/\mu\text{l}$  under the assay conditions (Fig. 28). The system was saturated with RNA concentration over 0.4  $\mu\text{g}/\mu\text{l}$  and too high concentration of RNA was inhibitory to the translation.

Figure 28. The relationship between RNA input and response of rabbit reticulocyte cell free translation system.

In vitro translation of RNA was carried out as described in the legend to Fig. 26 except that  $K^+$  and  $Mg^{++}$  concentration were adjusted to 180 mM and 1.2 mM respectively.



## APPENDIX 2

### Construction and Screening of cDNA Library

The purpose of construction of cDNA library (described in Materials and Methods) was to isolate clones containing  $\alpha$ -amylase and glucoamylase specific sequences for the use in hybridization analysis of their respective mRNAs. To this end, clones were first subjected to differential hybridization analysis followed by hybrid selection analysis of the candidate clones.

#### Screening by differential hybridizations

The rationale behind the differential hybridizations screening is that if a cDNA library prepared from mRNAs representing the desired sequences is hybridized to mRNA probes containing all the common sequences but differing in the level of desired sequences then the clones showing differential hybridization signals have high probability of containing the desired sequences. This method of screening cDNA libraries has been successfully used to obtain among others, the clones of galactose inducible sequences in Saccharomyces (St. John and Davies, 1979). SV40 inducible sequences in mouse cell (Scott et al., 1983) and interferon inducible sequences in human cells (Friedman et al., 1984).

In vitro protein synthesis studies indicated that rate of  $\alpha$ -amylase and glucoamylase synthesis in A. awamori was 4-5 fold higher with the fungus growth in starch medium compared to its growth in glucose medium. If these differential rates of enzyme synthesis were due to differences in their mRNAs concentrations

under these conditions of growth, then mRNA preparations from the cells cultured in these media can be used as differential probes. A cDNA library was constructed using the Poly A<sup>+</sup> RNA from starch medium cells. PolyA<sup>+</sup> RNA from starch medium contains among others the sequences specific for  $\alpha$ -amylase and glucoamylase proteins hence these sequences should also be represented in the cDNA library. About 1000 cDNA clones were transferred to nitrocellulose filters in duplicate. One set of filters was hybridized with <sup>32</sup>P-labelled 1st-strand cDNA synthesized from glucose medium cells poly-A<sup>+</sup> RNA and the second set was probed with the 1st-strand cDNA prepared from the starch medium cells poly-A<sup>+</sup> RNA. The intensities of hybridizations were visualized by autoradiography. Twenty eight clones showing at least three fold more hybridization intensities with starch cDNA probe compared to glucose cDNA probe (Fig. 29) were selected for further analysis.

#### Analysis of clones by hybrid selection

Twenty eight clones selected by differential screening were analysed for the sequences specific for  $\alpha$ -amylase and glucoamylase proteins. Recombinant plasmids of each clone were isolated, linearized by EcoRI restriction and immobilized on separated nitrocellulose filters. The filters carrying the immobilized plasmid DNA were hybridized to PolyA<sup>+</sup> RNA isolated from the starch medium cells according to a procedure described by Maniatis et al (1982). Hybridized RNA was eluted from each filter and translated in vitro using rabbit reticulocyte cell free translation system. In vitro translation products were analyzed by

SDS-polyacrylamide gel electrophoresis and immunoprecipitations. The results of hybrid selection experiments (data not shown) indicated that none of the clones selected by differential hybridization screening contained sequences related to either  $\alpha$ -amylase or glucoamylase enzymes. Assuming all of mRNA sequences present in poly-A<sup>+</sup> RNA (used for cDNA synthesis) were proportionally represented in the cDNA library as well as in the hybridization probes, it seems that there was not a significant difference in the levels of  $\alpha$ -amylase and glucoamylase specific mRNA sequences in the cells grown in glucose or starch medium. Then the observed 4-5 fold differences in the levels of  $\alpha$ -amylase and glucoamylase enzymes during A. awamori growth on glucose and starch is most likely caused by some other processes (e.g. differential translational efficiencies) rather than the differences in their mRNA levels.

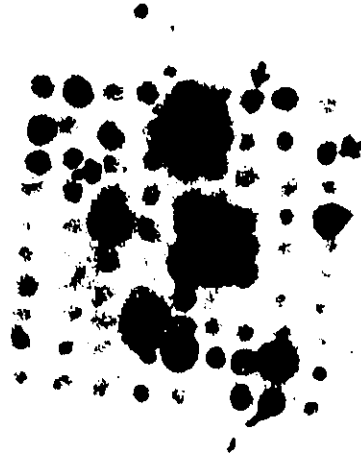
Figure 29. A typical autoradiograph showing differential hybridization intensities of cDNA clones.

The preparation of cDNA library, hybridization probes and hybridization conditions have been described in Materials and Methods and in the text. Briefly, cDNA clones were generated from the poly-A<sup>+</sup> RNA isolated from the starch medium cells. The clones were grown in duplicate on nitrocellulose filters. After lysis and immobilization of colony DNA, each set of filters was hybridized to <sup>32</sup>P-labelled 1st-strand cDNA probe prepared from poly-A<sup>+</sup> of glucose (G) or starch (S) grown cells. To avoid ambiguity in evaluation of differential hybridization intensities, glucose related cDNA probe used was in slight excess compared to starch related cDNA probe (9.0 x 10<sup>6</sup> CPM to 8.0 x 10<sup>6</sup> CPM in a final volume of 30 ml). The clones showing stronger hybridization signals with starch related probe (positive clones) than glucose related probe are marked by arrows.

**G1**



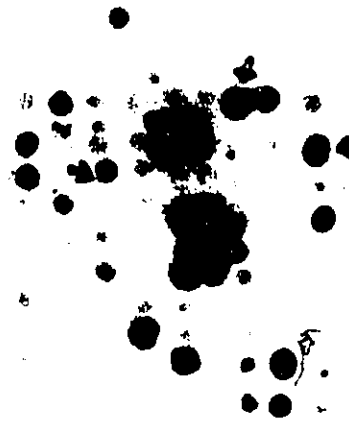
**G2**



**S1**



**S2**



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