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**Deletion analysis of the starch binding domain from *Aspergillus*
glucoamylase**

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Iowa State University, 1992

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Ann Arbor, MI 48106**

Deletion analysis of the starch binding domain
from Aspergillus glucoamylase

by

Luojing Chen

A Dissertation Submitted to the
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GENERAL INTRODUCTION

Explanation of Dissertation Format

This dissertation contains two papers. Proceeding them is a general introduction and literature review to the work undertaken. The references for the introduction can be found at the end of the thesis. The first paper is written in the form of a research paper and is composed of material adapted from two published papers. This work defines the starch binding region of Aspergillus glucoamylase (GA) by fusion of the C-terminal region of glucoamylase to β -galactosidase from E. coli. The second paper is written in a form suitable for publication discussing deletion analysis of the starch binding region of Aspergillus glucoamylase expressed in Saccharomyces cerevisiae. The two papers are followed by a General Summary and then by an Appendix.

Literature Review

In this introduction, the literature relevant to the work undertaken is reviewed. It is composed of two sections: (1) Aspergillus glucoamylase native starch binding domain, and (2) fusion proteins and their recovery. In the first section, an introduction to Aspergillus glucoamylase is given and information covering the starch binding domain of glucoamylase is presented. In the second section, different protein fusion systems are described. Based on this information, genetic

deletion analysis of Aspergillus glucoamylase was undertaken to define the starch binding region (tail). Application of the starch binding tail to promote purification of fusion proteins was also investigated.

Aspergillus glucoamylase starch binding domain

Glucoamylase (1, 4- α -D-glucan glucohydrolase, EC 3.2.1.3.) is an exo-enzyme that cleaves glucose units from the non-reducing end of starch and related polysaccharides (Reilly, 1979). It was first mentioned in about 1938 as an enzyme and characterized in 1951 (Panjunath et al., 1983). Glucoamylase (GA) hydrolyzes α -1,4-glucosidic linkages; it also hydrolyzes α -1,6- and α -1,3- glucosidic linkages, but at much slower rates (Pazur et al., 1962; Nikolov et al., 1989). Glucoamylase is an industrially important enzyme.

Glucoamylase has been found in numerous microorganisms, animals, and plants (French et al., 1950; Tsujisaka et al., 1958; Kawamura et al., 1968; Seetharam et al., 1970; Eggermont et al., 1969). In recent years, there has been considerable attention paid to fungal glucoamylases, because of their industrial usefulness (Saha et al., 1989). The enzyme is used in conjunction with α -amylase and isomerase (glucose isomerase) in the conversion of cornstarch into high fructose sweeteners. Most fungal glucoamylases exist in multiple forms (Pazur et al., 1971; Hayashida et al., 1978; Yamasaki et al., 1977 a; Hayashida et al., 1975; Takahashi et al., 1983). Glucoamylase from Aspergillus niger exists in two major forms: GAI and GAII (Lineback et al., 1969; Pazur et al., 1971; Svensson et al., 1982; Pazur et al., 1971). The molecular

weights of GAI and GAII are in the range of 82 KDa and 68 KDa respectively (Boel et al., 1984). GAI is synthesized as a precursor of 640 amino acid residues, which then is cleaved to produce the 616 residues-long mature enzyme (Nunberg et al., 1984), GAII is identical to GAI from amino acid residue 1 to 512 but lacks the C-terminal 104 amino acids of GAI. It was reported that the GAII form is generated either by limited proteolysis of the large GAI (Svensson et al., 1986) or by a mechanism of mRNA splicing (Boel et al., 1984). Glucoamylase GAI and GAII from Aspergillus niger have been characterized and the amino acid sequences have been determined (Svensson et al. 1983) (Fig.1).

Glucoamylase from A. niger is thought to be composed of three functional regions. 1) The first 470 residues from the amino terminus are the active catalytic region; 2) from residues 441 to 512 is a highly O-glycosylated region linked through mannose to the hydroxyl groups of serines and threonines in the polypeptide chain (Svensson et al., 1983; Gunnarsson et al., 1984). The function of the carbohydrates has been shown to be involved in stability of the enzymes (Pazur et al., 1971). Evans et al. (1990) reported that deletion of more than 30 amino acids from the C-terminal end of GAII resulted in diminution or loss of enzyme activity on starch plates. The O-glycosylated region may also be involved in transport, since fusion of the O-glycosylated region from a yeast glucoamylase promoted secretion of the fusion protein from yeast into the extracellular media (Yamashita, 1989); 3) from residue 513 to 616 is the carboxy terminal domain involved in binding to native starch (Hayashida et al., 1975; Svensson et al., 1982; Pazur et al., 1980;

```

1   ATLDSWLSNE ATVARTAILN NIGADGAWVS GADSGIVVAS PSTDNPDYFY

51  TWTRDGLVL KTLVDLFRNG DTSLLSTIEN YISAQAIVQG ISNPSGDLSS

101 GAGLGEPKFN VDETAYTGSW GRPQRDGPAL RATAMIGFGQ WLLDNGYTST

151 ATDIVWPLVR NDLSYVAQYW NQTGYDLWEE VNGSSFFTIA VQHRALVEGS

201 AFATAVGSSC SWCDSQAPEI LCYLQSFWTG SFILANFDSS RSGKDANTLL

251 GSIHTFDPEA ACDDSTFOPC SPRALANHKE VVDSFRSIYT LNDGLSDSEA

301 VAVGRYPEDT YYNGNPWFLC TLAAAEQLYD ALYQWDKQGG LEVTDVSLDF

351 FKALYSDAAT GTYSSSSSTY SSIVDAVKTF ADGPVSIVET HAASNGSMSE

401 QYDKSDGEQL SARDLTWSYA ALLTANNRRN SVVPASWGET SASSVPGTCA
      * **

451 ATSAIGTYSS VTVTSWPSIV ATGGTTTTAT PTGSGSVTST SKTTATASKT
      **      ** * * * * * * * * * * * * * * * *

501 STSTSSTSCT TPTAVAVTFD LTATTTYGEN IYLVGSISQL GDWETSDGIA
      ***** * * -----

551 LSADKYTSSD PLWYVTVTLP AGESFEYKFI RIESDDSVIEW ESDPNREYTV
      -----

601 PQACGTSTAT VTDWTR
      -----

```

Fig. 1 The one-letter amino acid sequence of *A. niger* glucoamylase (Svensson et al., 1983(b)). Symbols: * - O-glycosylated residues, ---- raw starch binding tail region.

Hayashida et al., 1982). The only functional difference between GAI and GAII is that GAI has the ability to bind to and digest native starch while GAII does not (Svensson et al., 1982). Knowles et al. (1987) reported that a variety of cellulases have features similar to Aspergillus glucoamylase in their protein architecture. In these enzymes, either the N- or the C-terminal substrate-binding domain is linked to a separate catalytic domain by a region rich in serine and threonine. Recently, the crystal structure of the glucoamylase-I' from Aspergillus awamori var. kawachi has been determined (Honzatko et al., personal communication, 1991). Glucoamylase-I' consists of the entire catalytic domain plus the N-terminal half of the O-glycosylated domain. The crystal structure of glucoamylase-I' has shown 13 α -helices with limited regions of antiparallel β -strands in the vicinity of the activity site. The N-terminal half of the glycosylated domain is in an extended conformation, wrapping around the catalytic domain.

The enzymatic digestion of native starch is of considerable industrial importance (Saha et al., 1989). Multiple forms of glucoamylases separated and purified from a number of sources were investigated for native starch digestion. Hayashida (1975) reported Aspergillus awamori var kawachi produced three types of glucoamylase, but only the largest form of glucoamylase hydrolyzed native starch. Yamasaki et al. (1977b) obtained two forms of glucoamylase from Mucor rouxianus and one of them was 3-fold more active in native starch digestion than the other one. Iizuka et al. (1978) also reported one of the two forms of glucoamylase from M. kaoliang can hydrolyze native

starch more rapidly than the other one. This is also true for glucoamylases isolated from Rhizopus (Svensson et al., 1982) and Aspergillus oryzae (Shortle et al., 1981). It was shown for glucoamylase from Rhizopus (Takahashi et al., 1982; Takahashi et al., 1985) that two minor forms of this enzyme differ from its major form by the absence of part of the N-terminal sequence, and only the major form has the ability to bind to starch granules. As is generally the case with fungal glucoamylases, only the larger form, GAI, possesses the capacity to adsorb onto and digest native starch, while both GAI and the smaller GAII are essentially equally active towards soluble polysaccharide and oligo-saccharide substrates (Ueda et al., 1983).

Several glucoamylases have been studied in order to elucidate the mechanisms of native starch adsorption, elution and digestion. Medda's group found different behaviors of native starch adsorption, elution and digestion between Aspergillus and Rhizopus niveus glucoamylases (Medda et al., 1982a; Medda et al., 1982b; Saha et al., 1983). Dalmia et al. (1990) studied native starch binding of Aspergillus niger glucoamylase in detail. Starch adsorption properties of GAI and GAII were investigated as a function of pH, temperature, ionic strength and enzyme concentration of the reaction mixture. It was found that GAI has much higher affinity ($K_{ad}=49$ ml/g starch) to starch granules than GAII ($K_{ad}=1.5$ ml/g starch) which lacks the C-terminal domain of GAI. Adsorption of GAI from A. niger and A. awamori to starch was affected by pH, with maximum adsorption occurring around pH 3.5. Ueda et al. (1983) studied Endomycopsis fibuligera glucoamylase and found its behavior of

starch binding, elution and digestion similar to that of Rhizopus niveus glucoamylase, but quite different from Aspergillus. Saha et al. (1983) studied starch adsorption and elution behavior of Aspergillus glucoamylase I. They concluded that hydroxy groups of the starch molecules are involved in the adsorption of GAI onto native starch.

Native starch degradation by glucoamylase may require its ability to adsorb to the native starch granules (Yoshino et al., 1978). GAI from several sources adsorbs well to native starch and has much stronger native starch-digesting activity than the corresponding GAII from the same organisms that adsorbs only weakly (Yoshino et al., 1978; Miah et al., 1977a; Saha et al., 1979). Dalmia et al. (1990) found that the surface concentration of bound GAI was five times higher than that of GAII and after a period of incubation GAI released almost five times more glucose from starch granules than did GAII. The investigation for some other enzymes also indicated that native starch degradation may be related to enzyme's ability to adsorb onto the starch granules. Koba et al. (1986) studied the adsorption of malt and bacterial α -amylases to native starch and its relation to native starch digestion. The malt α -amylase showed a higher hydrolytic activity than the bacterial α -amylase and was adsorbed more readily onto native starch. Walker et al. (1978) found that the ability of a particular α -amylase to digest maize starch granules was associated with its ability to adsorb onto the granules.

In addition, the degradation of native starch by a glucoamylase may be related to its debranching activity (Ueda et al., 1981; Miah et al., 1977b; Ueda et al., 1975). GAI of Aspergillus niger has a strong

debranching activity and is highly active in native starch digestion. GAII, however, has a very weak debranching activity and is weak in native starch digestion (Ueda et al., 1974). The same phenomenon of high debranching activity and high activity in native starch digestion was observed in Rhizopus sp. and in the multiple forms of glucoamylase of A. oryzae (Ueda et al., 1975; Mitsue et al., 1979; Saha et al., 1979; Miah et al., 1977a). Although it is clear that adsorption to starch is required for native starch hydrolysis, the significance of debranching activity is not well understood.

The amino acid sequences of putative starch binding domains from eight different starch-degrading enzymes were compared (Svensson et al., 1989) to elucidate possible structure-function relationships (Fig.2). The C-terminal 100 amino acid of GAI shows significant homology with the other putative starch binding domains and several specific amino acid residues occurred in all eight sequences. Svensson et al. (1989) hypothesized that this conserved region constitutes a self-contained domain involved in native starch binding. It was suggested that the invariable specific amino acid residues might be required for native starch binding function. The sequence of the starch binding domain of GAI is fairly homologous to the starch binding domains of cyclodextrin glucanotransferases. The 3-dimensional structure of the starch binding domain may therefore be similar to that of the known structure of these glucanotransferase domains (Hofmann et al., 1989; Klein et al., 1991).

		I	II
		-----	-----
gaRh	9	- vgl dsyn ydgstfsg-kiyv-27	45-dnwnnngntiaaysapisgsnyeywtf -72
α Sli	442	- QTSASFHV NATTAWGENIYVTGDQAALGNWDPARALKL-----	DPAAYPVWK1-489
β Cth	420	- IPVTFTINNATYYGQNVYIVGSTSDLGNWNTTYARGP-----	ASCPNYPTWTI-469
gaAN	514	- AVAVTFDLTATTTYGENIYLVGSISQLGDWETSDGIALSAD---	KYTSSDPLWYV-565
M α Bs	581	- SVVFTVKSAPPTNLGDKIYLTGNIPELGNWSTDTSGAVNNAQGPLLAPNYPDFY-635	
MTPs	430	- VSVSFRCDNGATQMGDVYAVGNVSQLGNWSPAAALRL-----	TDTSGYPTWKG-478
CAIB	587	- VTVRFVINNATTALGQNVFLTGNVSELGNWDPNNAIGPMYNQ---	VVYQYPTWYY-638
CKpn	530	- QSINFTCNNGYTI SGQSVYIIGNIPQLGGWDLTKAVKI-----	SPTQYPQWSA-577
Consensus		T G G LG W	P W
		II	III IV
		-----	-----
gaRh	73	- sasingikefyikyev-----	sgktyydnnsanvgvst-104
α Sli	490	- DVPLAAGTPFQYKYLKDA--	GKAVWESGANRTATVGTT---GALT LNDTWRG*538
β Cth	470	- TLNLLPGEQIQFKAVKIDSS--	GNVTWEGGSNHTYTVPTS---GTGSVTITWQN*519
gaAN	566	- TVTL PAGESFEYKFIRIESD--	DSVEWESDPNREYTVPQACGTSTATVTDWR*616
M α Bs	636	- VFSVPAGKTIQFKFFIKRAD--	GTIQWENG SNHVATTPTG---ATGNITVTWQN*684
MTPs	479	- SIALPAGQNEEWKCLIRNEANATQVRQWQGGANNSLTPSE----	GATTVGRL*526
CALB	639	- DVSVPAGQTIEFKFLKKQG---	STVTWEGGANRTFTTPTS---GTATVNVNWQP*686
CKpn	578	- SLELPSDLNVEWKCVKRNETNPANVEWQSGANNQFNSND-----	TQTTNGSF*625
Consensus		K	W N

Fig. 2 Comparison of C-terminal sequences of amylases and cyclodextrin glucoanotransferases. Gaps are indicated by dashes. Possible errors in predicated positions are +/- 2. Asterisks signify the C-terminal residues of the protein. α Sli, α -amylase from *Streptomyces limosus*; β Cth, β -amylase from *Clostridium thermosulfurogenes*; gaAn, glucoamylase from *Aspergillus niger*; M α Bs, maltogenic α -amylase from *Bacillus stearothermophilus*; MTPs, maltotetraose-forming amylase from *Pseudomonas stutzeri*; CALB, CGTase from alkalophilic *Bacillus* sp. strain 1011; CKpn, CGTase from *Klebsiella pneumoniae*. The N-terminal starch-binding region from *Rhizopus* glucoamylase (gaRH) is shown in lower case. Four areas of sequence similarity (I-IV) are shown. (The figure is from Svensson et al. (1989)).

The mechanism of native starch adsorption by glucoamylase is not understood yet (Svensson et al., 1986), although some work on this protein has been done. In 1982 Hayashida et al. reported the presence of a native starch affinity-site in GAI of A. awamori. They suggested that the site may play a role in the initial step of the process of native starch digestion. Recently, Hayashida et al. (1989, a, b) reported that this starch affinity site of GAI consists of 45 amino acids, from residue 471 to residue 515. However, this finding is contradictory to the fact that GAII, which contains almost all of these residues, is unable to bind to native starch and digest it. Thus the starch-binding domain must include some or all of the 104 C-terminal amino acids of GAI. Recently, Belshaw et al. (1991) reported isolating the starch binding domain of A. niger GAI by digestion with protease VIII. The isolated starch binding domain including amino acids 471-616 was shown to bind to native starch and contained a portion of the glycosylated region isolated by Hayashida et al. (1989a,b). One possible explanation for the above observations is that the O-glycosylated region contributes to native starch binding, but in a very minor way compared to the starch binding domain. Thus, by itself, Hayashida's 45 amino acid domain is functional and binds starch, but probably not at high levels.

The nucleotide sequences of six different glucoamylases have been determined: 1) from Saccharomyces diastaticus (Yamashita et al., 1985a); 2) Saccharomyces cerevisiae (Yamashita et al., 1985b); 3) Rhizopus oryzae (Ashikari et al., 1986); 4) Saccharomycopsis fibuligera (Itoh et

al., 1987); 5) A. niger (Svensson et al., 1983b); and 6) A. awamori (Nunberg et al., 1984). Glucoamylases from A. niger and A. awamori are identical. The glucoamylase gene of A. awamori has been cloned (Nunberg et al., 1984) and the original glucoamylase gene isolated from A. awamori contained five introns which had to be removed from the gene for expression in yeast (Nunberg et al., 1984). Glucoamylase was successfully processed, secreted and glycosylated when introduced into the yeast host Saccharomyces cerevisiae (Innis et al., 1985). Recently, Cole et al. (1988) constructed improved vectors resulting in higher expression of glucoamylase in yeast. This has provided a convenient system for genetic manipulation of Aspergillus glucoamylase.

Fusion proteins and their recovery

DNA cloning techniques have allowed in vitro fusions of genes or gene fragments in a predictable manner. Expression of such gene fusions gives rise to hybrid polypeptides which may retain the functional activities of one or both proteins. The first described examples of gene fusion were small peptides, such as somatostatin (Itakura, 1977) and insulin (Goeddel, 1979). Since the fusion proteins (the resulting hybrid proteins) containing a well-characterized component (such as β -galactosidase) can be identified easily, it facilitates the analysis of basic biological processes such as gene regulation and detection of cellular localization of gene products (Malcolm et al., 1983; Marston et al., 1986). It has also been demonstrated that by gene fusion some small foreign peptides are stabilized in E. coli (Marston et al., 1986)

that otherwise might be rapidly degraded by host proteases. Gene fusions can also be used to facilitate protein purification.

Purification of polypeptides produced from cloned genes is a very important aspect of biotechnology. Using genetic engineering techniques, it is possible to add a C or N-terminal polypeptide "tail" to a target protein, so that this recombinant protein can be recovered and purified based on specificity of the tail. Such genetically engineered proteins can be designed to take advantage of affinity, ion-exchange, hydrophobic, metal-chelate and covalent separations, etc. The target protein can potentially be purified in a single step from a complex mixture of proteins by chromatography using an adsorbent that binds to the tail. After elution, some target proteins may need further chemical or enzymatic treatment to remove the tail in order to retain activity. The topic of recovery and purification of recombinant proteins by fusion tails has been excellently reviewed recently (Ford et al. 1991; Marston et al., 1986; Sassenfeld, 1990; Hammond et al., 1991). Fusion tails with different characteristics have been classified into four categories: (1) Affinity tails; (2) Ion-exchange tails; (3) Hydrophobic and covalent tails and (4) Metal-chelate tails.

Affinity tails The large protein β -galactosidase has been extensively used as a fusion protein to aid the purification of target proteins such as β -endorphin (Shine, 1980), lysozyme, gal-repressor (Ullman, 1984), the replication initiator protein of plasmid R6K (Germino, 1984), and erythropoietin (Nielson, 1988 ; Itakura et al., 1977). The purification of β -galactosidase fusions can be achieved by

affinity chromatography on immobilized P-amino-phenyl- β -D-thiogalactoside (TPEG) (Steers, 1971). Because β -galactosidase is easily detected by a colorimetric assay using o-nitrophenyl-thiogalactoside, large numbers of clones can be screened and different stages of recovery and purification can be monitored.

Fusion to protein A from Staphylococcus aureus is perhaps the best known example of a system for affinity purification of fusion proteins. Protein A binds to the constant region of immunoglobulins (Nilsson, 1985). The target protein can be genetically fused to the protein A and purified by affinity chromatography on immobilized immunoglobulin G (IgG)-Sepharose. Vectors are available for intracellular or periplasmic expression in E. coli or for extracellular secretion from Staphylococcus aureus (Moks et al., 1987a; Nilsson et al., 1990; Abrahmsen, 1985). The protein A fusion system has been used on a large scale for the purification of recombinant human IGF-I from a 1000 L culture of E. coli (Moks et al., 1987b), demonstrating the potential for scale-up of fusion protein recovery systems in downstream processing.

Recently, a dual-affinity gene fusion system has been developed for the isolation of extremely sensitive peptides in E. coli (Hammarberg, 1989). This system was designed to have synthetic IgG-binding domains at the N-terminus and an albumin-binding domain (Streptococcal protein G) at the C-terminus of the target protein, and has been very useful in the purification of small recombinant and sensitive proteins (Jansson et al., 1989).

Chloramphenicol acetyltransferase (CAT) and Glutathione-S-transferase (GST) are the other two enzymes that have been used as fusion tails. Human atrial natriuretic factor was fused to the C-terminus of CAT and purified using CAT substrate affinity chromatography (Benmett, 1983; Dykes, 1988; Knott, 1988). A number of antigens from Plasmodium falciparum were fused at the C-terminus to GST from Schistosoma japonicum, expressed in E. coli, and purified on immobilized glutathione (Smith et al., 1988). High expression vectors for GST fusion proteins are available (Smith et al., 1988) (Guan, 1991).

Recently, Cronan (1990) has constructed biotin-binding fusion proteins. The fusion tail providing a functional site contains 75 amino acids from the 1.3 S subunit of Propionibacterium shermanii transcarboxylase. Biotin-binding fusion proteins can be purified by their high affinity to avidin or streptavidin columns.

Another group of fusion tails have been constructed that facilitate protein purification by immunoaffinity. Krivi et al. (1985) reported that a recA-somatostatin fusion produced in E. coli could be purified using an immobilized anti-recA monoclonal antibody. Field et al. (1988) added a short antigenic peptide to the N-terminus of adenylyl cyclase (which is difficult to purify) and the resulting fusion protein was recovered using a Sepharose-bound monoclonal antibody raised against the tail. Hopp et al. (1988) have designed a tail which is an extremely hydrophilic peptide of 8 amino acids. Monoclonal antibodies specific for the tail have been raised to facilitate immunopurification. The C-terminal end of the tail provides an enterokinase cleavage site for

removal of the tail from the target protein after purification. The fusion of this tail with a number of interleukins and granulocyte macrophage colony stimulating factor (GM-CSF) has shown a high-efficiency one step immunopurification by immobilized monoclonal antibody.

Recently, two systems (cellulose binding domain and maltose binding protein) using complex carbohydrates as adsorbents have been reported (Ong, 1989; Bedouelle et al., 1988). The cellulose-binding domain from Cellulomonas fimi endoglucanase Cen A has been used to purify alkaline phosphatase from E. coli (Greenwood et al., 1989). Similarly the cellulose-binding domain from C. fimi exoglucanase Cex has been fused to β -glucosidase. Cellulose-binding domains bind to cellulose at very low salt concentrations and they can be eluted with water.

The periplasmic maltose-binding protein of E. coli has been used as a fusion tail (Guan, 1988; Bedouelle et al., 1988; Blondel et al., 1990). The purification is based on its high affinity to cross-linked amylose. The bound fusion protein can be eluted with maltose at a high yield. Both the cellulose-binding domain and maltose-binding protein use inexpensive complex carbohydrates as adsorbents, thus greatly lowering the cost of protein recovery and purification.

Ion-exchange tail Positively and negatively charged tails have been used for recovery and purification of target proteins by ion-exchange chromatography and other charge-based separation methods. The first example of the use of an ion-exchange tail was the purification of recombinant urogastron with a C-terminal tail containing five arginine

residues (Sassenfeld, 1984; Smith, 1984; Brewer, 1985). The fusion protein bound strongly to a cation-exchange matrix. Poly aspartic acid tails have also been used for recovery, increasing precipitation of the target protein β -galactosidase by the polyelectrolyte polyethyleneimine (Zhao et al. 1990; Parker et al. 1990). Similarly, Dalbøge et al. (1987) fused an N-terminal Ala-Glu dipeptide tail to human growth hormone (hGH) and the resultant protein was purified by anion-exchange chromatography.

Metal-chelate tails Metal-chelate chromatography is based on the interaction of specific amino acids (mainly histidine) with immobilized metal ions. Hochuli et al. (1988) fused two to six histidine residues to mouse dihydrofolate reductase and purified the fusion protein on a Ni^{2+} - chelate column. Other examples which involved Ni^{2+} metal-chelate chromatography to purify fusion proteins have also been reported (Smith et al., 1988; Liungquist et al., 1989; Jansson et al., 1989).

Other tails Hydrophobic interactions have been used for the purification of fusion proteins. Persson et al. (1988) fused 11 phenylalanine residues to β -galactosidase and the fusion protein was purified by phenyl-Superose chromatography. Similarly, they also reported (1988) that galactokinase fused with four cysteine residues bound specifically to thiopropyl Sepharose.

Tail cleavage

One of the main concerns in designing fusion proteins is whether to remove the fusion tail or not. Usually, the fusion tail must be removed

before the protein can be used. Especially for pharmaceutical applications, the precise removal of the fusion tail is required. However, in some cases, it may be possible to leave the tail on, depending on the purpose of the target protein and whether or not the tail interferes with the biological function of the target protein. There are both enzymatic methods and chemical methods for tail cleavage. The cleavage method chosen will depend on the composition, peptide sequence and stability of the target protein etc. A variety of cleavage methods have been summarized by several authors (Sassenfeld et al. 1990; Marston et al. 1986; Uhlen et al. 1990; Nagai et al. 1987). Table 1 lists some examples of specific cleavage methods.

Conclusions

So far, at least nine fusion-tail recovery systems designed to facilitate the purification of fusion proteins have been described. Although fusion-tail recovery systems are powerful tools for recovery and purification of recombinant proteins, there are some difficulties. The production of some recombinant proteins by microorganisms can be restricted by degradation, missprocessing, incorrect folding or conformation, or the production of insoluble aggregates (inclusion body formation). These problems may result in yield loss or loss of biological activity of the fusion protein. The challenges involved in fusion tail technology and its application from basic research to industrial production are both stimulating and important to the future of biotechnology.

Table 1: Examples of specific cleavage methods

Sequence recognized	Cleavage Method
-Asn -↓- Gly -	Hydroxylamine
-Asp -↓- Pro -	Acid pH
-Arg -↓- X _{aa} -	Carboxypeptidase B
-Lys -↓- X _{aa} -	Carboxypeptidase B
-Cys -↓- X _{aa} -	2 nitro-5-thio-cyanatobenzoate
-Gly - Pro - Arg -↓-	Thrombin
-X _{aa} -↓- His _(n)	Carboxypeptidase A
-Ile - Glu - Gly - Arg -↓-	Factor X _a
-↓-Ile - Val - Gly - Gly - Thr - Val	Factor X II _a
-Met -↓- X _{aa} -	CNBr
-Pro -X _{aa} -↓- Gly - Pro	Collagenase
-Trp -↓-X _{aa}	BNPS-skatole

Note: Arrow indicates site of cleavage and X_{aa} represents any amino acid residue.

PAPER 1: ADSORPTION TO STARCH OF β -GALACTOSIDASE FUSION PROTEINS
CONTAINING THE STARCH BINDING REGION OF ASPERGILLUS GLUCOAMYLSE

ABSTRACT

Five β -galactosidase fusion proteins (BSB133, BSB119, BSB103, BSB80 and BSBCD8) containing different sized fragments from the C-terminus of Aspergillus glucoamylase and one size-control fusion protein (BGA134) with 134 amino acids from another region of Aspergillus glucoamylase (amino acids 380-513) have been constructed. Affinity purified fusion proteins were tested for native starch binding. Fusion protein BSB119 containing 119 amino acids from the C-terminus of GAI had the strongest affinity ($K_{ad}=38$ ml/g starch) to starch granules among all fusion proteins when compared to a β -galactosidase control ($K_{ad}=1$ ml/g starch) and the size-control fusion protein ($K_{ad}=3.9$ ml/g starch). These results confirmed that the location of the starch binding domain is in the C-terminus of GAI and indicated that the 119 amino acid starch-binding tail is near the optimum functional size for fusion proteins expressed in E. coli. The fusion proteins BSB133 and BSB119 were also tested for starch binding in the presence of crude soluble cell extracts. It was found that both BSB133 and BSB119 can specifically and strongly bind to native starch granules and the bound BSB133 can be eluted from starch granules with similar purity to that achieved by affinity chromatography. These results indicate the potential application of native starch as an adsorbent to facilitate the purification or immobilization of fusion proteins containing the starch binding region.

INTRODUCTION

Glucoamylase (GA) from Aspergillus (EC 3.2.1.3) exists in two forms: GAI (amino acid 1-616) and GAII (amino acid 1-512) (Fig.1). Both GAI and GAII have the ability to hydrolyse soluble starch. However, only GAI has the unique capacity to bind to native (raw) starch granules (Svensson et al., 1982, 1986a). GAII, which lacks the C-terminal 104 amino acid residues of GAI, binds to native starch granules with very low affinity ($K_{ad} = 1.5$ ml/g of starch) in comparison to GAI ($K_{ad} = 49$ ml/g of starch) (Dalmia, 1990). It has been suggested that the carboxy tail (involving residues 513-616) of glucoamylase is involved in native starch binding (Svensson et al., 1982; Pazur et al., 1971; Pazur et al., 1980; Hayashida et al., 1982). This is also consistent with the observation that there is a significant amino acid sequence homology between the C-terminal domain of GAI and starch binding domains of several other putative starch binding enzymes (Svensson et al., 1989). Svensson et al. hypothesized that this conserved region constitutes a self-contained domain involved in native starch binding. However, the native starch binding domain of GAI has not yet been defined.

Native starch has been used as an adsorbent to purify a variety of starch-binding enzymes. Holmberg (1933) separated α -amylase from β -amylase by chromatography on starch gels. Thayer (1953) used native starch to purify microbial amylases. Saha (1988) also used native starch as an adsorbent to purify a thermostable β -amylase from Clostridium thermosulfurogenes. Starch has also been used to purify GAI

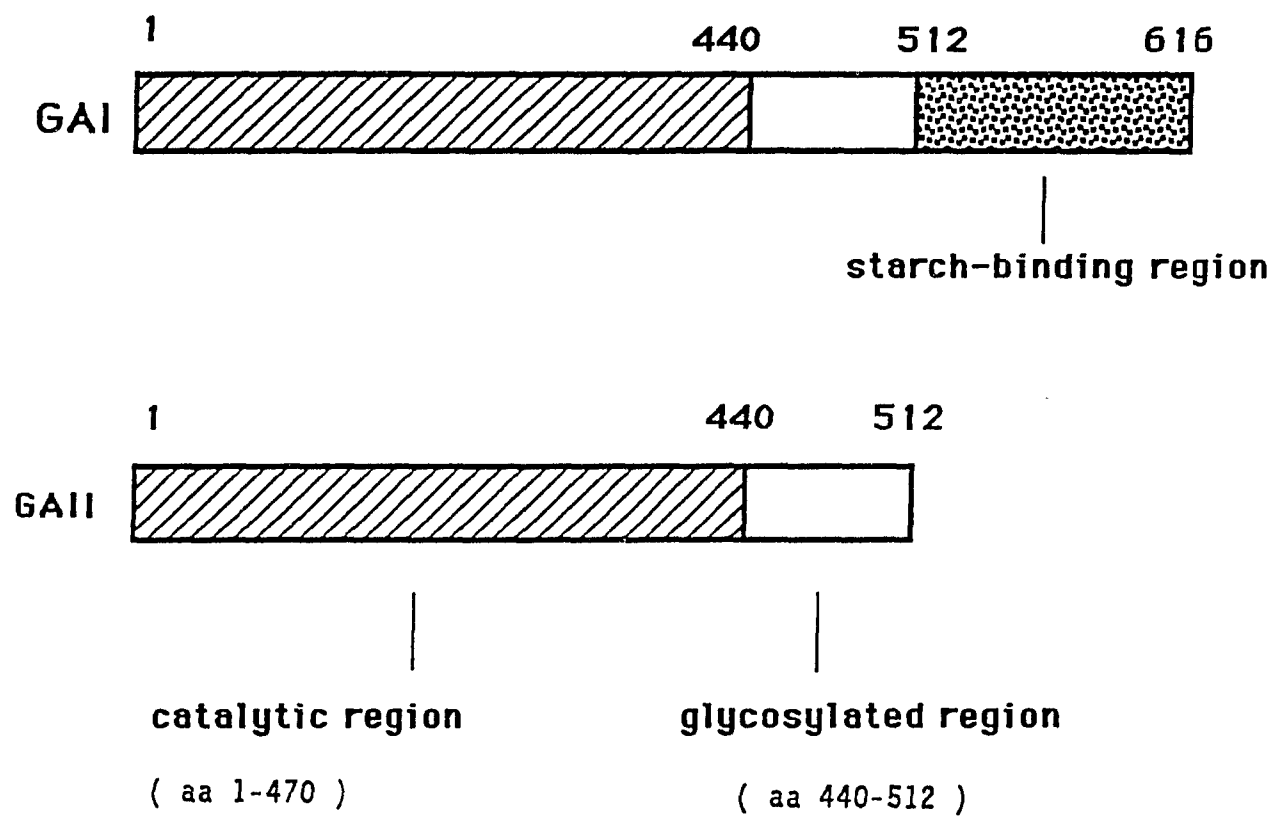


Fig. 1 Schematic amino acid sequence of GAI and GAII

from GAI_I based on the starch binding domain (Ueda et al., 1974). GAI bound to native starch can be eluted with a yield of > 90 % (Medda et al., 1982; Dalmia et al., 1990). There are some advantages to the use of native starch as an adsorbent over other fusion tail systems (reviewed by Ford et al. 1991). Native starch granules are very stable, inexpensive and non-toxic. The utilization of native starch might open a new market for corn starch and construction of a starch-binding chimeric protein offers a new genetic technique for the immobilization of proteins on an inexpensive inert and stable support (starch). Many enzymes of industrial importance have been immobilized on various types of supports (Stanley et al., 1978; Kucera et al., 1983). The adsorption of fusion proteins to starch through the starch binding domain may provide a simple technique for enzyme immobilization.

Based on the above information, we proposed to determine if the glucoamylase starch binding domain is functional when fused to an enzyme (β -galactosidase) expressed intracellularly in E. coli. If so, we proposed to define by deletion analysis how much of the domain is required for function. Finally we proposed to develop a simple one-step protein purification system based on adsorption of the starch binding domain to native starch. Fusion of the starch binding domain to a protein would permit the removal of recombinant protein from a crude extract by application of inexpensive starch as an adsorbent. It may also be possible to use a starch binding tail for immobilization of recombinant fusion proteins.

Gene fusions using the β -galactosidase structural gene lacZ have been used to study a variety of biological processes in both bacteria and higher organisms (Silhavy et al., 1985; Weinstock et al., 1983). β -galactosidase is a tetrameric enzyme containing four identical 116 kD subunits of 1021 amino acids (Welply et al., 1980; Kalnins et al., 1983). We chose β -galactosidase as the targeting protein for these experiments, because (1) convenient expression vectors exist. A series of vectors which allow the cloning of DNA into both ends of the lacZ gene such that active chimeric β -galactosidase is produced have been constructed (Rüther et al., 1983; Koenen et al., 1985). For example, the cloning vector pUR290 has unique BamHI, SalI, PstI, XbaI and HindIII cloning sites at the 3' end of the lacZ gene (Rüther, 1983). In-frame insertions in these site leads to the production of a fusion protein containing active β -galactosidase at the N-terminus and the peptide encoded by the inserted DNA at the C-terminus (Fig.2); (2) It has been demonstrated that the β -galactosidase gene can be fused with various proteins from different sources at both termini (Malcolm et al., 1983; Silhavy et al., 1985), since the N-terminal 25 amino acids and the C-terminal 2 amino acids are non-essential (Silhay et al., 1985); (3) The β -galactosidase fusion protein can be easily identified by its β -galactosidase enzymatic activity; (4) The purification of β -galactosidase fusion proteins can be easily achieved by affinity chromatography (Steers, 1971) and the purification steps can be monitored by a simple colorimetric test (Miller, 1972) and (5) β -galactosidase itself basically does not bind to or digest native starch.

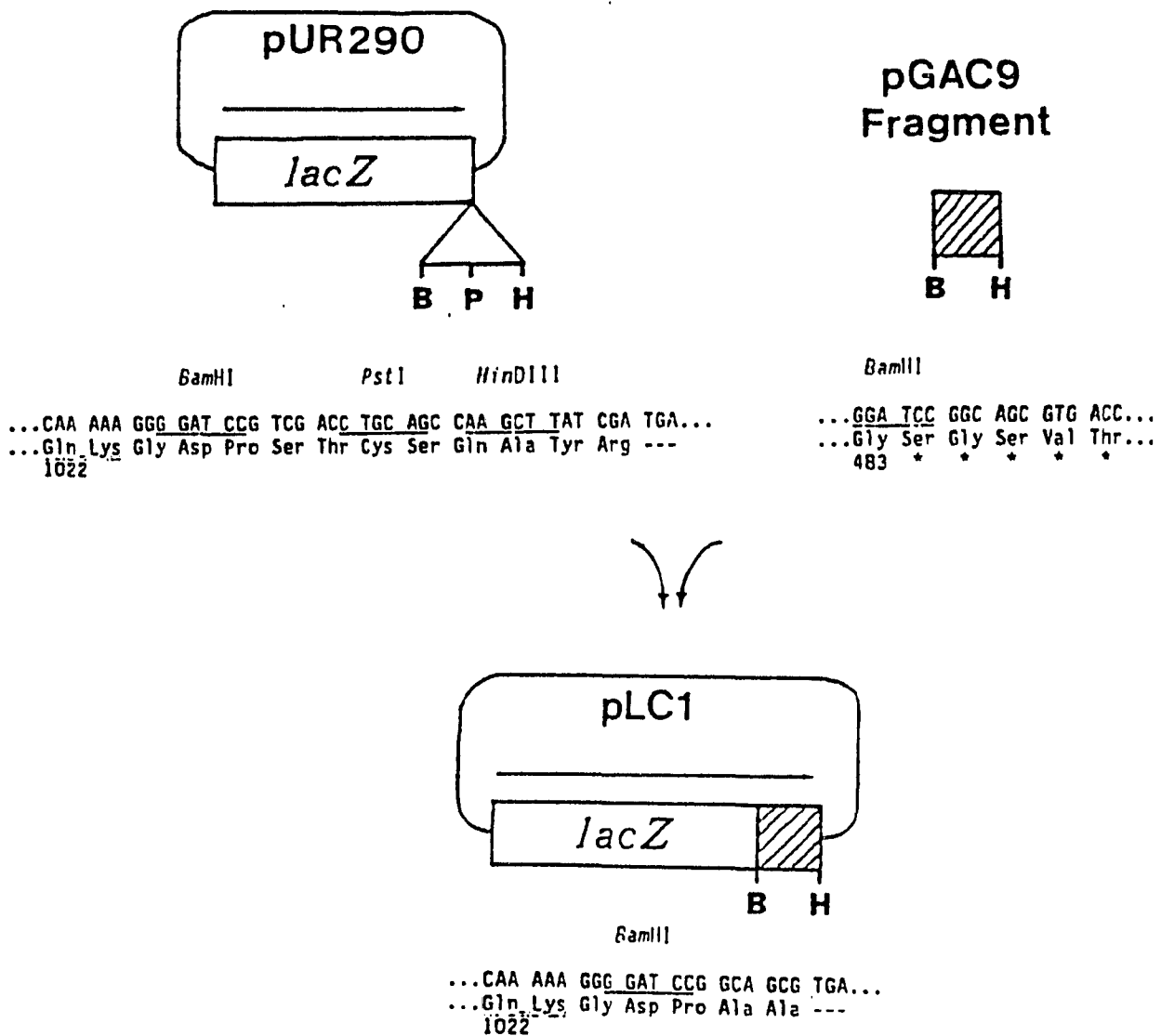


Fig. 2 Construction of plasmids encoding β -galactosidase fusion proteins containing glucoamylase starch binding region. Construction of plasmid pLC1 is shown as an example. Vector pUR290 encodes the wild-type β -galactosidase gene (*LacZ*) and plasmid pGAC9 provides fragment encodes the glucoamylase starch binding region.

MATERIALS AND METHODS

Strains, plasmids and growth conditions

The Escherichia coli host strain Y1089-1 was derived from strain Y1089 (Δ lacU 169, proA⁺, Δ lon, araD 139, strA, hflA 150, [Chr::Tn10] (pMC9) (Young et al., 1983) by eliminating pMC9 during growth in non-selective medium.

The plasmids pUR290 and pGAC9 were used. pUR290 (Fig. 2) encoding full-length β -galactosidase (Rüther et al., 1983) was a generous gift from Dr. Müller-Hill . The plasmid pGAC9 (Fig.3) which encodes the glucoamylase gene from Aspergillus awamori (Innis et al., 1985) was a generous gift from Cetus Corporation. The total size of plasmid pGAC9 is 12.78 kilobases. The plasmid contains the replication origin of pBR322 which allows proliferation of the plasmid in E. coli and the yeast 2 μ origin which allows the vector to replicate in S. cerevisiae. The glucoamylase gene was inserted between a yeast enolase promotor and terminator. The plasmid also contains selectable markers for both E. coli and yeast.

Construction of fusion proteins

The vector pUR290 (Rüther et al., 1983) was used for the construction of gene fusions encoding β Gal::GA fusion proteins. Standard methods for DNA manipulation were used (Sambrook et al., 1989). This plasmid has unique BamHI and HindIII sites at the 3' end of the lacZ gene. The fusion proteins were constructed by cloning segments

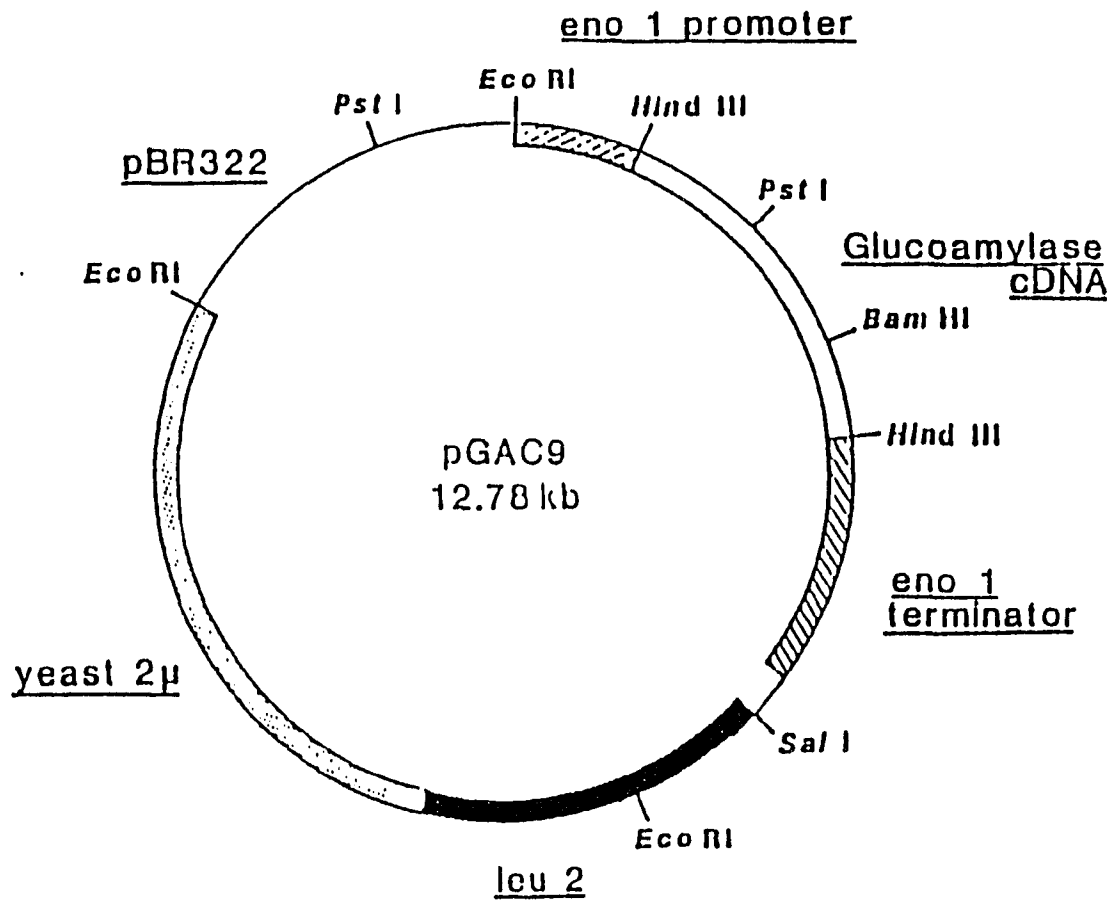


Fig. 3 The plasmid pGAC9 received from Cetus Corporation (Innis et al., 1985)

of the Aspergillus glucoamylase gene into these sites of the vector. Plasmid pLC0 was constructed by ligating BamHI/HindIII cut pUR290 with a 0.55-kb BamHI-HindIII fragment from pGAC9. The reading frame at the fusion junction of pLC0 was corrected by filling-in the cohesive ends at the BamHI site and religating. The resultant plasmid pLC1 encodes the fusion protein BSB133. The plasmid pLC2, which encodes the fusion protein BSBCD8, was constructed by inserting a SpeI linker containing stop codons in three frames (5'-CTAGACTAGTCTAG, New England Biolabs) into the SalI site near the 3' end of the glucoamylase coding region of pLC1. The SalI site was blunted first and then ligated with the linker. After this, plasmid pLC3 was constructed which encodes a size-control fusion protein BGA134. Plasmid pLC3 was made by ligating pUR290 with a 0.85 kb XmnI-HindIII fragment (encoding glucoamylase residues 380-513) from pRE513 (Evans et al., 1990). The XmnI site of the fragment and the BamHI site of pUR290 were blunted before ligation. Plasmid pLC4 was made by removing a 45-bp BamHI-NheI GA fragment from pLC1 followed by conversion of the cohesive ends to blunt ends and ligation. Plasmid pLC4 encodes fusion protein BSB119. Plasmid pLC5, which encodes fusion protein BSB103 was constructed by cloning a 0.45-kb BstXI-HindIII fragment from the glucoamylase gene of pGAC9 into pUR290, using a BstXI-BamHI adaptor (5'-GATCCTGAGATCTCGCC-3'/5'-AGATCTCAG-3') (synthesized at the Iowa State University Nucleic Acid Facility). Plasmid pLC6 encoding fusion protein BSB80 was constructed in two steps. First, a new BamHI site was generated in the glucoamylase gene by removing the 3'overhang of a pre-existing ClaI site with S1 nuclease, followed by insertion of a

BamHI linker (New England Biolabs). Creation of the BamHI site allowed isolation of a 0.39-kb BamHI-HindIII fragment that was then subcloned into the multiple cloning sites (MCS) of pUR290. The junction nucleotide sequences in the constructed plasmids pLC0, pLC1, pLC3 were verified by the dideoxy method (Chen et al., 1985) using a primer (5'-GCGGAATTCCAGCTGAG) homologous to the DNA sequence near the 3' end of the lacZ gene. The primer was synthesized at the Iowa State University Nucleic Acid Facility. The universal M13 primer was used to sequence the fusion junctions of pLC2, pLC5, pLC6 which had been subcloned into M13 vectors for DNA sequencing.

Plasmids pLC1, pLC2, pLC3, pLC4, pLC5, pLC6 and pUR290 were transformed into the protease-deficient (Lon-) E. coli strain Y1089-1 for expression of BSB133, BSBCD8, BGA134 (as a size-control), BSB119, BSB103, BSB89 and a β -galactosidase control, respectively.

Purification of fusion proteins

Transformed cells containing pLC1, pLC2, pLC3, pLC4, pLC5, pLC6 or pUR290 were grown to mid-log phase in LB liquid medium (Sambrook, et al., 1989) containing 100 ug ampicillin/ml, and harvested by centrifugation at 3,000 x g for 5 min. Pellets were resuspended in 0.1M NaOAc (pH 6.0) buffer and then disrupted by sonication. A crude soluble enzyme preparation was obtained following centrifugation at 23,700 x g for 20 min.

Purification of the enzymes from the crude soluble preparation was achieved using the affinity matrix P-aminophenyl β -D-

thiogalactopyranoside-agarose (Sigma) following the two-step method of Steers et al. (1971). Both purified enzymes and crude enzyme extracts were assayed for total protein concentration by the method of Bradford (1976) and for β -galactosidase activity by the method of Miller (1971).

Starch binding and elution

Adsorption of fusion proteins to native starch was assayed by a procedure modified from Medda et al. (1982) (Fig.4). Linear adsorption isotherms indicate the apparent equilibrium distribution of fusion proteins between the solid phase (bound) and the liquid phase (unbound) at various protein concentrations. A crude soluble enzyme preparation from untransformed Y1089-1 host cells containing 2.1 mg/ml of protein was prepared following the procedure outlined in Purification of Fusion Proteins. Small aliquots of each purified fusion protein (BSB133, BSB119, BSBCD8, BGA134 and β -galactosidase, respectively) were then added separately to the soluble extract to yield fusion protein concentrations of 0.1 mg/ml, 0.3 mg/ml and 0.5 mg/ml. The concentration of the soluble host-cell proteins remained essentially the same in each sample i.e. at 2.1 mg/ml. Aliquots of 1 ml containing the protein mixtures were added to 0.1g of washed starch and then were shaken at 4°C for 20 min. Samples were centrifuged (17400 xg) and the protein concentration of the supernatant was assayed by the method of Bradford (1976). The amount of bound and free enzyme was determined by subtracting the β -galactosidase activity (Miller, 1972) of the supernatant from the initial β -galactosidase activity. Enzyme

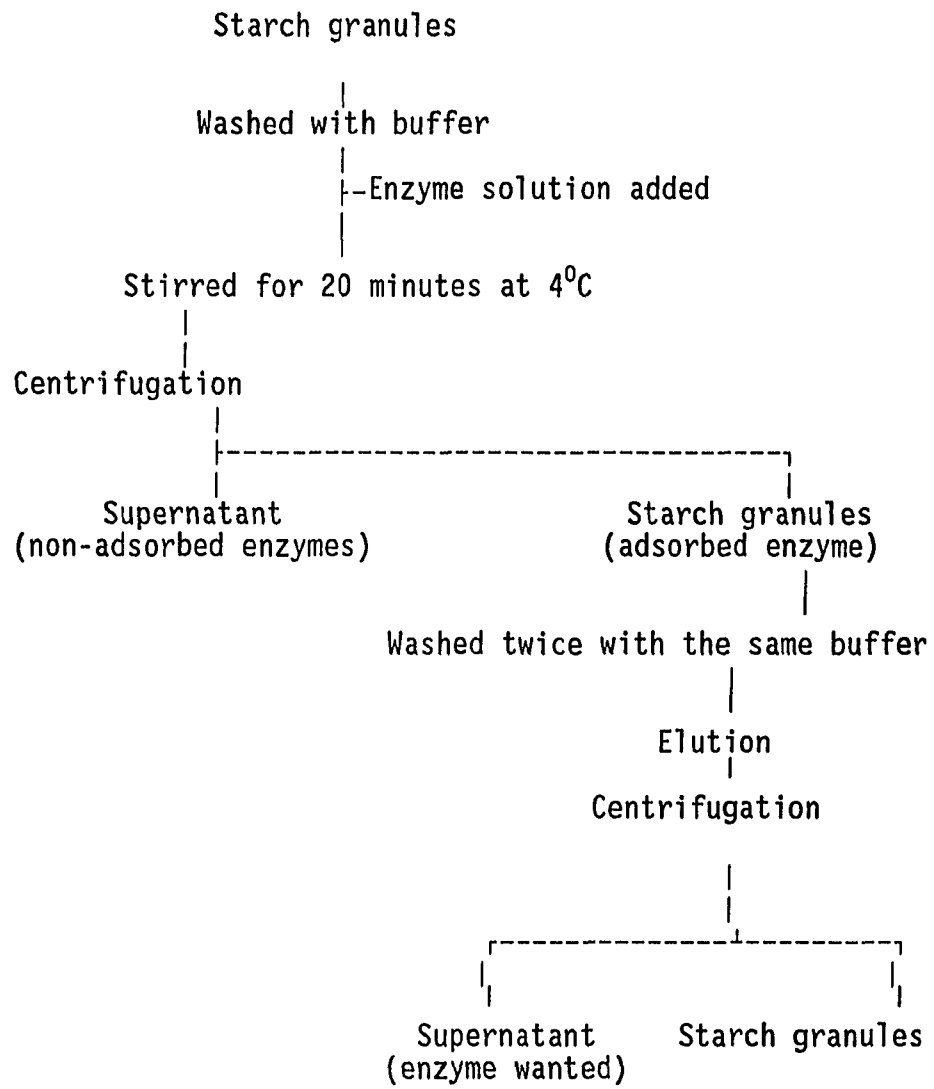


Fig.4 Procedure for native starch adsorption and elution

concentrations were calculated from the β -galactosidase activity, based on the specific activity of each fusion protein. Adsorption of purified fusion proteins to native starch also was assayed by a procedure modified from Medda et al. (1982). Native starch was prepared by washing with 0.1 M sodium acetate buffer (pH 6.0). Purified fusion proteins (in the washing buffer) were added to washed starch (0.1 g) in a 1-ml total volume with initial protein concentrations ranging from 0.1 to 0.5 mg/ml, and each mixture was shaken for 20 min. at 4°C. After centrifugation (17,400 xg) the protein concentration of the supernatant was assayed, and the amount of adsorbed protein was calculated from the difference between the initial and final protein concentrations in the supernatant. The amount of bound protein at equilibrium expressed as milligrams of protein per gram of starch was a linear function of the free (unbound) protein in the range of protein concentrations assayed. Adsorption constants (K_{ad}) were calculated from the slopes of the linear adsorption isotherms. A total of four initial concentrations per purified protein were used to obtain the average adsorption constant.

The bound enzyme was eluted from starch following the method of Medda et al. (1982) by resuspending the washed starch-enzyme complex in 1.0 ml of 0.05 M boric acid/ 0.1 M borax buffer, pH 8.2. The suspension was stirred for 5 min. at 4°C and centrifuged at 17400 x g. The supernatant was recovered and the protein concentration was assayed.

RESULTS AND DISCUSSION

Construction of fusion proteins

To define the starch binding domain and test the possibility of using starch to purify fusion proteins containing the starch binding tail, six β -galactosidase fusion proteins were made. The *E. coli* plasmid vector pUR290 was used for the construction and expression of six plasmids: pLC1, pLC2, pLC3, pLC4, pLC5, and pLC6 (Fig.5) (Fig.6). pLC1, pLC2, pLC4 and pLC6 encode, respectively, β -galactosidase fusion proteins BSB133 containing 133 amino acids from the C-terminus of glucoamylase (from amino acid 484 to 616), BSBCD8 (from amino acid 484-608 of glucoamylase), BSB119 (from amino acid 498 to 616 of glucoamylase), BSB103 (from amino acid 514 to 616 of glucoamylase), and BSB80 (from amino acid 537 to 616 of glucoamylase). pLC3 encodes BGA134 containing 134 amino acids from a different region of glucoamylase (from amino acid 380 to 513) and this protein served as a control fusion protein. Fusion protein BSB133 (including the 133 amino acid residues of the C-terminal domain of GAI) was first constructed, because it covered the entire GAI tail region (amino acid 513 - 516) and utilized a convenient cloning site (BamHI) on the glucoamylase gene. Further N-terminal deletions were made by utilization of convenient unique NheI, BstXI, and ClaI restriction enzyme sites in the glucoamylase gene (Fig.7). BSB119, BSB103 and BSB80 include, respectively, 119 amino acid residues, 103 amino acid residues and 80 amino acid residues of the C-terminal domain of GAI. Simultaneously, the C-terminal deletion fusion

pLC1 ...CAA AAA GGG GAT CGA TCC GGC...
 ...Gln Lys Gly Asp Arg Ser Gly...
 1023

pLC2 ...CAA AAA GGG GAT CGA TCC GGC...TCG ACT AGA CTA GTC TAG
 ...Gln Lys Gly Asp Arg Ser Gly...Ser Thr Arg Leu Val Stop
 1023 484 608

pLC3 ...CAA AAA GGG GAT CCT TTC GCC...CCC ACC TAG
 ...Gln Lys Gly Asp Pro Phe Ala...Pro Thr Stop
 1023 380 513

pLC4 ...CAA AAA GGG GAT CCT AGC AAG...
 ...Gln Lys Gly Asp Pro Ser Lys...
 1023 498

pLC5 ...CAA AAA GGG GAT CTC GCT GTG...
 ...Gln Lys Gly Asp Leu Ala Val...
 1023 514

pLC6 ...CAA AAA GGG GAT CCG ATC TCT...
 ...Gln Lys Gly Asp Pro Ile Ser...
 1023 537

pUR290 ...CAA AAA GGG GAT CCG TCG ACC TGC AGC CAA GCT TAT CGA TGA
 ...Gln Lys Gly Asp Pro Ser Thr Cys Ser Gln Ala Tyr Arg stop
 1023

Fig. 5 Plasmid constructions. The sequences at the junctions of the gene fusions are shown. Underlined amino acids are from β -galactosidase. Bold amino acids are from glucoamylase and are identified by their glucoamylase amino acid numbers.

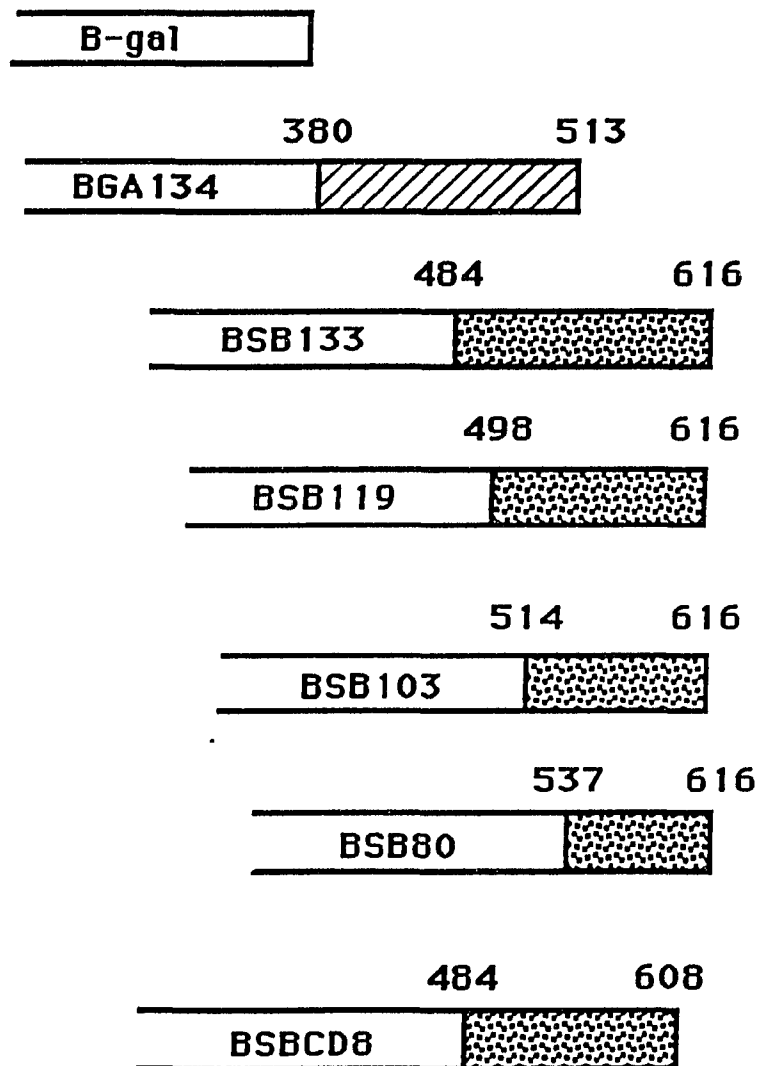


Fig. 6 β -galactosidase fusion proteins containing glucoamylase tails at the C-terminus. Numbers indicate glucoamylase amino acid residues.

Fig. 7 The C-terminal 238 amino acid residues of Aspergillus awamori glucoamylase (amino acid 371-616) in the one-letter code. Sequence information is from Nunberg et al. (1984). The approximate location of DNA restriction enzyme sites are indicated above the amino acid sequence.

protein BSBCD8 was constructed. BSBCD8 differs from BSB133 by the truncation of 8 amino acid residues at the C-terminus of glucoamylase. The six fusion proteins and a β -galactosidase control encoded by pUR290 were expressed in the *E. coli* host strain Y1089-1 (Materials and Methods). This strain is protease-deficient (*Lon*⁻) and contains a deleted *lacZ* gene, so it does not produce β -galactosidase itself.

Characterization of fusion proteins

Fusion proteins BGA134, BSB133, BSB119, BSB103, BSB80, BSBCD8 and the control β -galactosidase were isolated from *E. coli* and purified by affinity chromatography (Materials and Methods). The purified proteins were analyzed by SDS-PAGE (Fig.8) (Fig.9). All of the fusion proteins showed two major bands on SDS-PAGE. A larger band of 120-130 KDa represented the fusion protein monomers and a lower band of about 116 KDa was approximately the same size as a native β -galactosidase monomer. The occurrence of two bands was likely the result of proteolytic degradation. Both bands in western blots reacted positively with anti- β -galactosidase antibody, indicating that both were derived from the β -galactosidase fusion proteins (Fig.10)(Fig.11). Similar proteolysis has been found for other β -galactosidase fusion proteins (Rüther et al., 1983; Germino et al., 1984; Hellebust et al., 1988). Since β -galactosidase is a tetramer, it is possible that only a portion of the β -galactosidase fusion proteins contained four starch binding tails. Due to proteolysis, other intermediates containing three, two or one starch-binding tails may also have been present. The degree of

Fig. 8 SDS-PAGE of Purified Fusion Proteins. Samples (9.2 ug in lanes 1-3, 6.3 ug in Lane 4) were electrophoresed in a 7.5% polyacrylamide gel containing 0.1% SDS and stained by Coomassie brilliant blue dye according to the method of Laemmli (1970). Lane 1: BSB133; Lane 2: BSBCD8; Lane 3: BGA134; Lane 4: β -galactosidase from pUR290. The position of molecular weight standards are indicated.

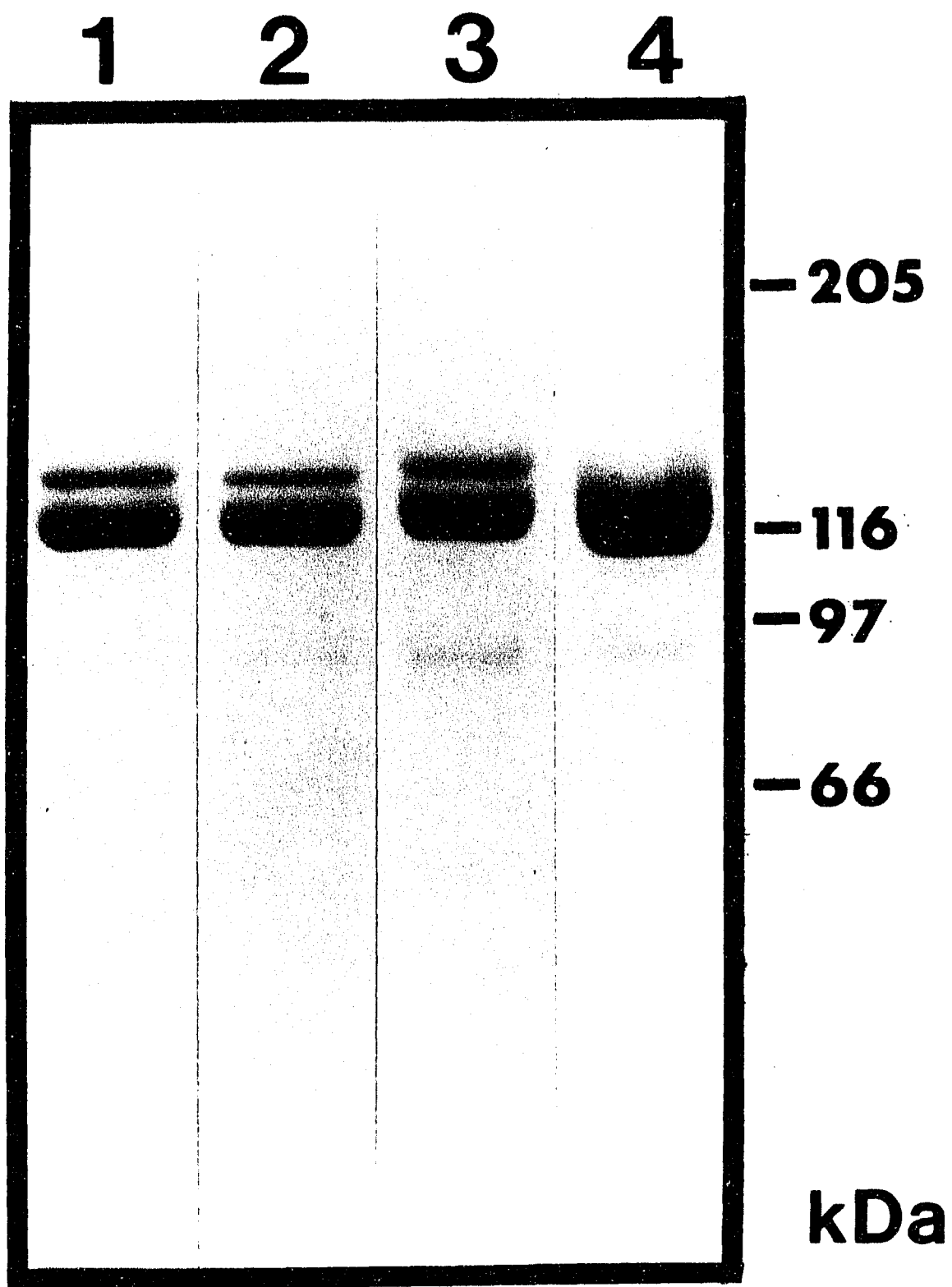


Fig. 9 SDS-PAGE of purified fusion proteins. Samples were electrophoresed in a 7.5% polyacrylamide gel containing 0.1% SDS and stained by Coomassie Brilliant Blue dye according to the method of Laemmli (1970). Lane 1: BSB119; Lane 2: BSB103; Lane 3: BSB80; Lane 4: β -galactosidase from pUR290. The position of molecular weight standards are indicated.

1

2

3

4



—205

—116

—97

—66

KDa

Fig. 10 Immunoblot analysis of purified fusion proteins. (a) SDS-PAGE of purified fusion proteins. Lane 1: β -galactosidase from pUR290; Lane 2: BSB133; Lane 3: BSBCD8; Lane 4: BGA134. Samples (7 ug) were electrophoresed in a 7.5% polyacrylamide gel containing 1% SDS and were stained with Coomassie brilliant blue dye (Laemmli et al., 1970). The Fusion proteins exhibited proteolytic degradation resulting in two major bands: a band of about 130 KDa representing full-length fusion protein monomers, and a band of about 116 KDa, approximately the size of wild-type β -galactosidase monomer. (b) Immunoblot (Blake et al., 1984) of purified fusion proteins. Lane 1: β -galactosidase from pUR290; Lane 2: BSB133; Lane 3: BSBCD8; lane 4: BGA134. Duplicate samples from the same gel shown in Fig. (a) were transferred to nitrocellulose using a Semi-Dry Electrophoretic Transfer Cell (BIO-RAD). The blot was probed with anti- β -galactosidase (Boehringer Mannheim Biochemicals). The probe was detected using anti-antibody conjugated to alkaline phosphatase (Boehringer Mannheim Biochemicals). Both the 130 KDa and 116 KDa bands reacted to the anti- β -galactosidase probe.

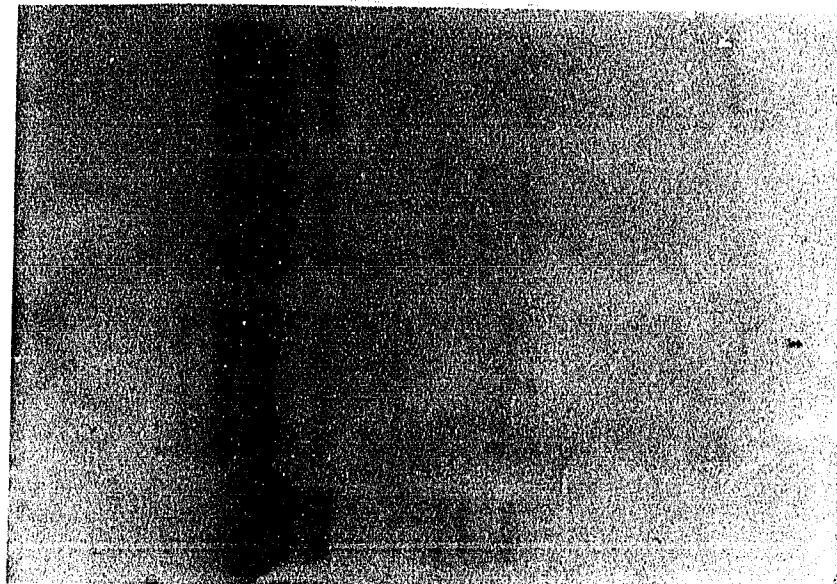
a

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3

4



b

1

2

3

4

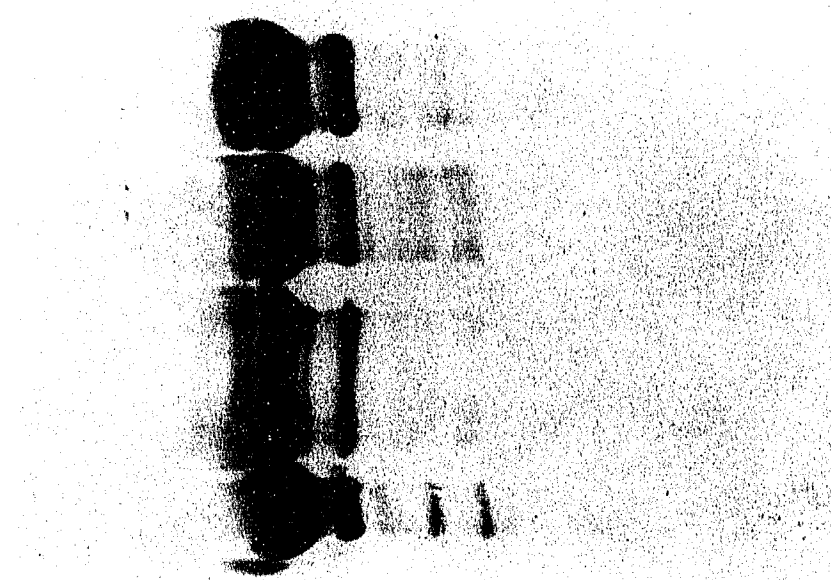


Fig. 11 Immunoblot analysis of purified fusion proteins. (a) SDS-PAGE of purified fusion proteins. Lane 1: BSB119; Lane 2: BSB103; Lane 3: BSB80; Lane 4: β -galactosidase from pUR290. Samples (7 ug) were electrophoresed in a 7.5% polyacrylamide gel containing 1% SDS and were stained with Coomassie brilliant blue dye (Laemmli et al., 1970). The fusion proteins exhibited proteolytic degradation resulting in two major bands: a band of about 130 KDa representing full-length fusion protein monomers, and a band of about 116 KDa, approximately the size of a wild-type β -galactosidase monomer. (b) Immunoblot (Blake et al. 1984) of purified fusion proteins. Lane 1: BSB119; Lane 2: BSB103; Lane 3: BSB80; Lane 4: β -galactosidase from pUR290. Duplicate samples from the same gel show in Fig. (a) were transferred to nitrocellulose using a Semi-Dry Electrophoretic Transfer Cell (BIO-RAD). The blot was probed with anti- β -galactosidase (Boehringer Mannheim Biochemicals). The probe was detected using anti-antibody conjugated to alkaline phosphatase (Boehringer Mannheim Biochemicals). Both the 130 KDa and 116 KDa bands reacted to the anti- β -galactosidase probe.

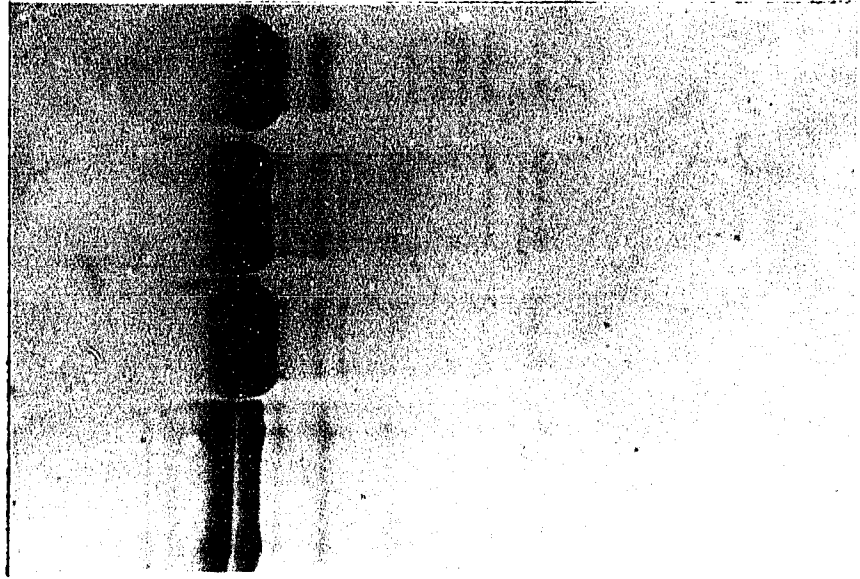
a

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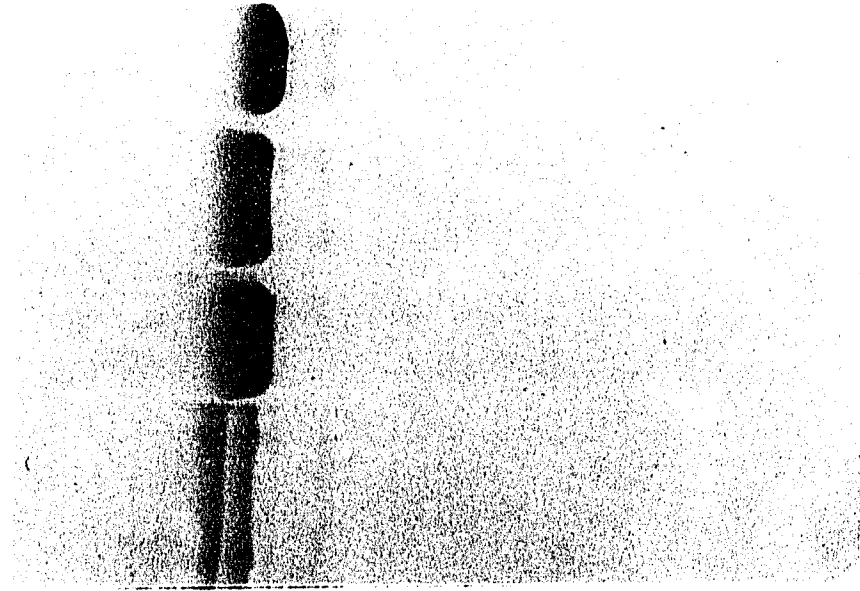
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4



proteolytic degradation was similar for BSB133, BSBCD8 and BGA134. Likewise, BSB119, BSB103, BSB80 also showed similar but less extensive proteolysis. Proteolysis was measured by densitometer reading of the SDS-PAGE bands. The differences between the 2 groups of fusion proteins in proteolytic degradation may have resulted from differences in the origin of the glucoamylase tail. BSB133, BSBCD8 and BGA134 all retain residues from 484 to 497 which are absent in the other fusion proteins (BSB119, BSB103, BSB80). It is possible that there is an amino acid sequence within this 14 amino acid long fragment which is sensitive to E. coli proteases.

The specific activity of the purified fusion proteins was assayed (Table 1) and proved to be similar to that of the β -galactosidase control. This indicated that the starch binding region when fused to β -galactosidase did not greatly interfere with the enzyme function. These results suggest that it may be possible to fuse the starch binding region to other enzymes without disrupting enzyme function.

Adsorption of fusion proteins to native starch

To test the starch-binding ability of the purified fusion proteins, a starch binding assay was performed (Fig.4). Fusion protein BSB133 adsorbed to starch with a higher affinity ($K_{ad} = 18 \text{ ml/g starch}$) than did β -galactosidase ($K_{ad}=1 \text{ ml/g starch}$). The different affinity between fusion protein BSB133 and control β -galactosidase can not be explained simply by the presence of additional amino acid residues at the C-terminus of β -galactosidase as shown by the low affinity to starch

Table 1. Specific β -galactosidase Activity of Fusion Proteins

Enzyme	Units / pmol
β -galactosidase	193
BSB133	154
BSB119	182
BSB103	152
BSB80	170
BSBCD8	142
BGA134	190

Note: β -galactosidase activity was assayed by the method of Miller (1972). The data from duplicate assays were averaged. In all cases, duplicate data points deviated less than 5 % from each other. The molarity of the enzyme (pmol) was calculated based on protein concentrations (determined by the method of Bradford, 1976) and the deduced M_r of each fusion protein.

($K_{ad}=3.9$ ml/g starch) of the similar-sized fusion protein (BGA134) containing residues from another part of Aspergillus glucoamylase (Fig.12). The low starch binding of BGA134 compared to BSB133 indicates that BSB133 contained a functional starch binding region. The fusion protein BSB119 with a further 14 amino acid residues deleted from the N-terminus of the glucoamylase segment showed the highest affinity ($K_{ad}=38$ ml/g starch) among all β -galactosidase fusion proteins constructed (Fig.13). BSB119 adsorbed to native starch with an affinity more than 2-fold higher than that of BSB133 ($K_{ad}=18$ ml/g starch). Increased stability shown by the higher percentage (52%) of full-sized monomers in BSB119 compared to BSB133 (28%) (as determined by densitometer reading) may account for the increased starch adsorption of BSB119. Adsorption isotherms for BSB119 generated over a wide range of initial concentrations of the enzyme were performed by Kusnadi (Chen et al., 1991b). The loading capacity of starch for BSB119 (36.5 mg of protein /g starch) was 10-fold greater than that of the β -galactosidase control. This result indicates that BSB119 adsorbs strongly to starch granules when compared to the β -galactosidase control. In contrast, BSB103 which contains the entire natural GAI tail (amino acids 152-161) and the entire region homologous to the starch-binding domains of several other enzymes (Svensson et al., 1989) has shown much reduced binding affinity ($K_{ad}=16$ ml/g starch) compared to BSB119. This suggests that the N-terminal 16 amino acid residues of the BSB119 may contribute to the starch binding function of the fusion protein by serving as a spacer between β -galactosidase and the starch binding domain. Further

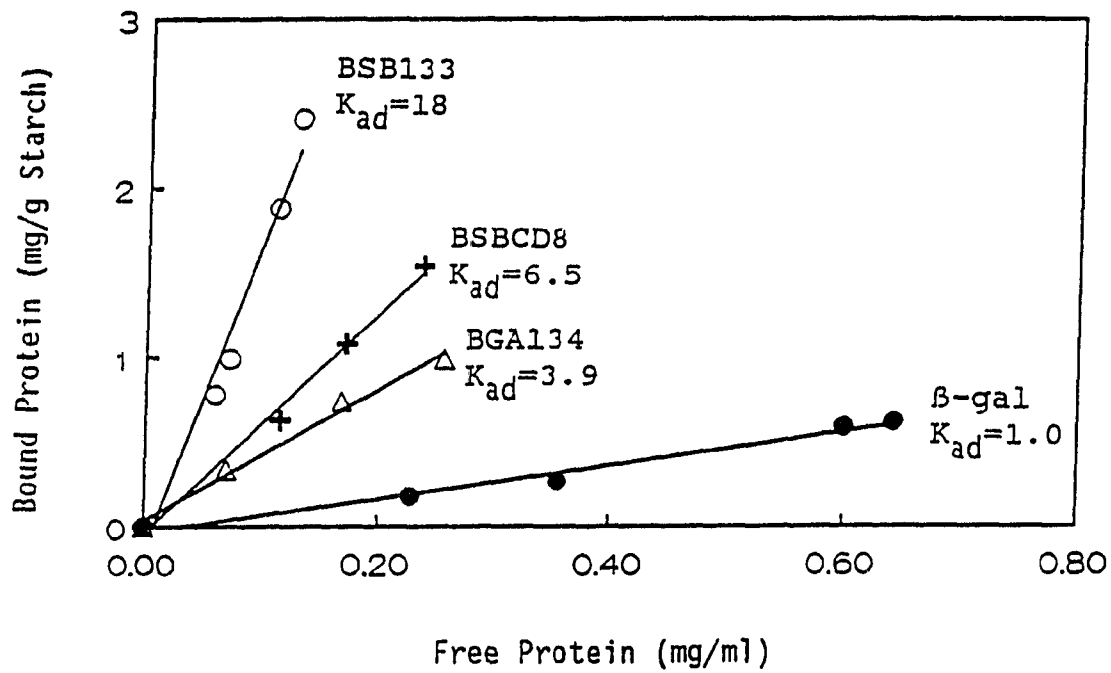


Fig. 12 Adsorption of purified fusion proteins BSB133, BSBCD8, sized-control BGA134 and control β -galactosidase to starch granules. Adsorption constants (K_{ad}) were calculated from the slopes of adsorption isotherms as described under Materials and Methods.

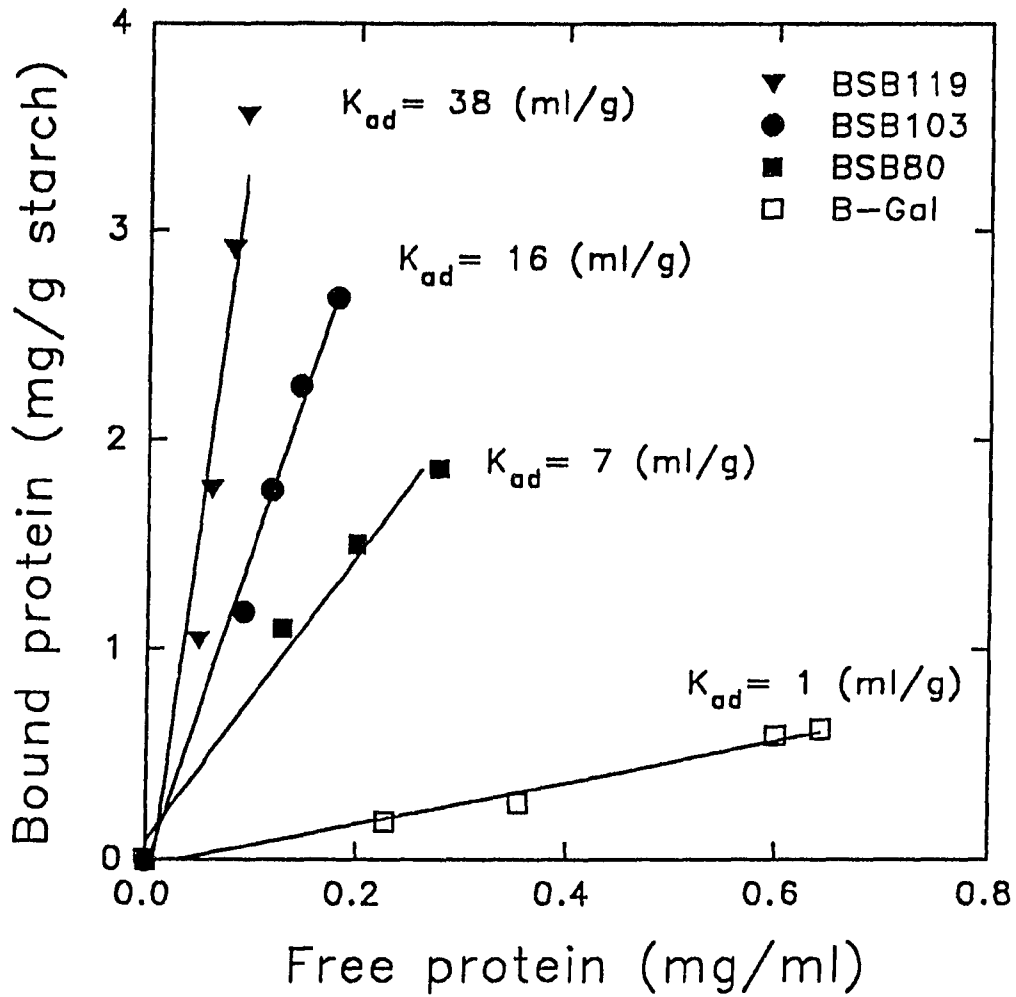


Fig. 13 Adsorption of purified fusion proteins BSB119, BSB103, BSB80 and control β -galactosidase to starch granules. Adsorption constants (K_{ad}) were calculated from the slopes of adsorption isotherms as described under Materials and Methods.

experiments need to be done in order to answer this question. Fusion protein BSB80 showed a large decrease in starch binding affinity ($K_{ad} = 7$ ml/g starch). This can be explained by the loss of 23 amino acid residues located in the starch-binding homologous region. Fusion protein BSBCD8 (with 8 amino acid residues deleted from the C-terminus of BSB133) showed a sharp decrease in starch binding. These results suggest that the C-terminal residues of the domain are essential for starch binding. This is consistent with the results obtained by chemical modification of glucoamylase Trp-615 suggesting this residue may be essential for starch binding (Svensson et al., 1986).

To determine the specificity of interaction between starch and the starch binding region, adsorption of the fusion protein to starch in the presence of crude soluble enzyme preparations was performed (Materials and Methods). Purified BSB133, BSB119, BSBCD8 and control β -galactosidase were added respectively to a crude enzyme extract from untransformed host cells and assayed for adsorption to starch (Fig.14). The adsorption constants for BSB133 ($K_{ad} = 14$ ml/g starch), BSB119 ($K_{ad} = 36$ ml/g starch), BSBCD8 ($K_{ad} = 1.9$ ml/g starch) and the β -galactosidase control ($K_{ad} = 0.96$ ml/g starch) in the presence of crude extract were similar to those obtained from the purified proteins. These results indicate a strong specificity of BSB133 and BSB119 to starch granules in the presence of background proteins and suggest the possible use of starch granules to purify fusion proteins containing the starch binding domain from crude extracts.

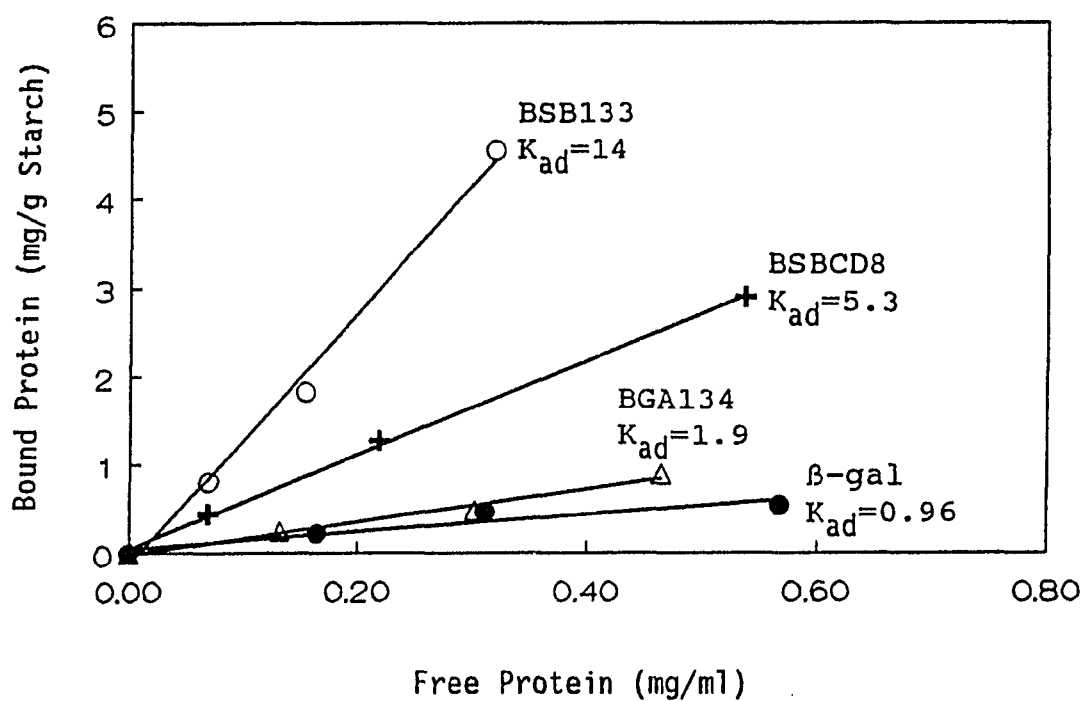


Fig. 14 Adsorption of fusion proteins BSB133, BSB119, BSBCD8, BGA134 and control β -galactosidase to starch in the presence of crude extracts. Adsorption constants (K_{ad}) were calculated from the slopes of adsorption isotherms as described under Materials and Methods.

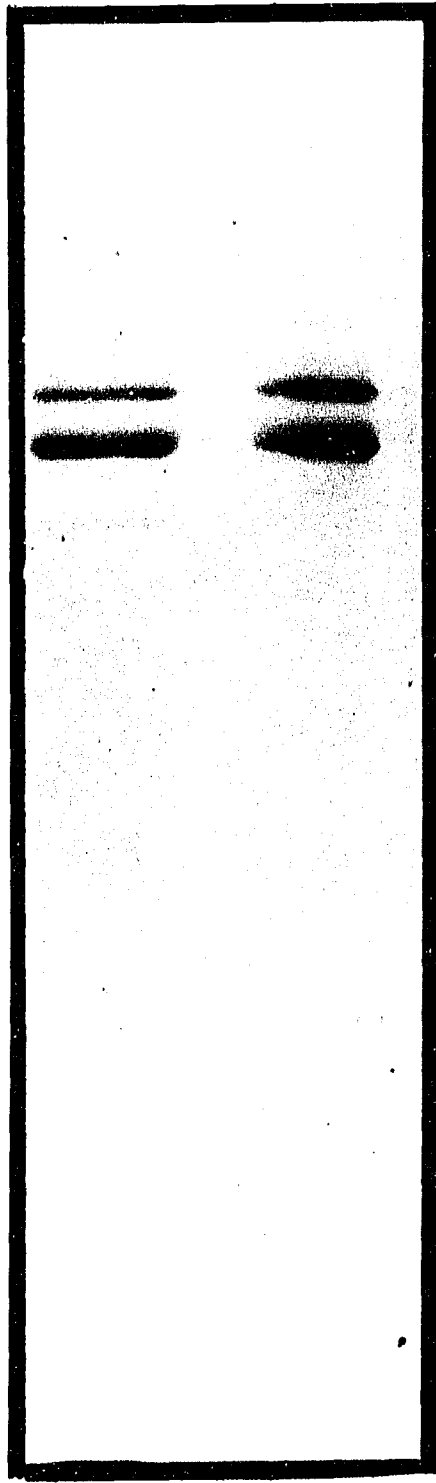
Elution of fusion proteins from starch

The elution of fusion proteins from starch granules was assayed (Materials and Methods). All of the fusion proteins were eluted from starch at very low yields (from 10 % to 17 %) using a boric acid-borax buffer. By contrast it has previously been reported that GAI from Aspergillus can be eluted by this method from starch at a yield of > 90% (Medda et al., 1982; Dalmia et al., 1990). The relatively poor elution of the fusion proteins was not surprising since β -galactosidase is a tetramer and conformation of β -galactosidase fusion proteins are different from that of native Aspergillus glucoamylase. Although the yield was not high, the purity of the fusion protein eluted from native starch had a similar purity to that achieved by affinity chromatography (Fig.15). Recently, Dalmia et al.(1991) reported BSB119 can be eluted from starch granules with high yield (80%) utilizing 10 mM β -cyclodextrin or 500 mM maltose as elutants.

Fig. 15 Purity of fusion protein BSB133 eluted from starch. Lane 1: BSB133 eluted from starch granules; Lane 2: BSB133 purified by affinity chromatography; Samples were electrophoresed in a 7.5% polyacrylamide gel containing 0.1% SDS, and were stained by Coomassie brilliant dye following the method of Laemmli (1970).

1

2



- 205

- 116

- 97

- 66

- 45

kDa

CONCLUSIONS

Using genetic engineering techniques, six β -galactosidase fusion proteins, containing different amounts of the C-terminal region of Aspergillus glucoamylase, have been constructed and expressed in E. coli. All the fusion proteins were tested for native starch binding and elution. The results showed that β -galactosidase fusion proteins containing 133 or 119 amino acids from the C-terminus of Aspergillus glucoamylase adsorbed to starch specifically with a strong affinity compared to either a β -Galactosidase or a similar sized fusion protein control. This confirms the presence of a strong starch binding region in the C-terminus of Aspergillus glucoamylase. Deletion of 8 amino acids from the C-terminus of the starch binding tail (BSBCD8) resulted in greatly decreased affinity, indicating that the C-terminal end of the tail was essential for strong starch binding activity. The results from further N-terminal deletion analysis of the starch binding region have shown that 119 amino acids is a near optimal functional length for the tail in β -galactosidase fusion proteins. Further studies by site - directed mutagenesis techniques can determine specific, functional important amino acid residue(s) involved in starch binding. Fusion proteins containing the C-terminal 133 and 119 amino acid residues from Aspergillus glucoamylase (BSB133) (BSB119) bind strongly and specifically on native starch granules in the presence of crude soluble cell extracts, and the bound BSB133 could be eluted from starch granules with similar purity to that achieved by affinity chromatography. These results suggest that it may be possible to use native starch as an

adsorbent to facilitate the purification or immobilization of fusion proteins containing the starch binding tail. Since β -galactosidase is the first target protein used in these studies, other proteins could be chosen to test the application of starch binding domain in protein purification.

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PAPER II: DELETION ANALYSIS OF THE STARCH BINDING REGION OF ASPERGILLUS
GLUCOAMYLASE SECRETED FROM SACCHAROMYCES CEREVISIAE

ABSTRACT

Glucoamylase (GA) from Aspergillus awamori (EC 3.2.1.3) exists in two forms: a large form (GAI) and a smaller form (GAII) which lacks the C-terminal 103 amino acid residues of GAI. Although both forms of glucoamylase can digest soluble starch, only GAI has the ability to bind to and therefore hydrolyze native starch granules. The C-terminal 103 amino acid region of GAI shows significant sequence similarity to putative starch binding domains of 7 other enzymes (Svensson et al., 1989), leading to the hypothesis that this region forms an independent starch binding domain. We have genetically constructed five glucoamylase mutants with extensive deletions within the C-terminal 103 amino acids of GAI. The mutated enzymes were expressed in Saccharomyces cerevisiae and were tested for starch binding and hydrolysis of native starch granules. Progressive loss of starch binding and starch-hydrolytic activity was observed upon removal of 8 and 25 amino acid residues from the C-terminus of the starch binding domain, or 21 and 52 amino acid residues from N-terminus. Removal of the entire tail of 103 amino acids resulted in nearly complete loss of starch binding and starch-hydrolytic ability. These deletions in the starch binding domain did not affect enzyme activity on soluble starch or thermostability of the enzyme, confirming the independence of the catalytic domain from the starch binding domain.

INTRODUCTION

Glucoamylase (1,4- α -D-glucan glucohydrolase, EC 3.2.1.3) catalyzes the release of glucose from the non-reducing ends of starch and related oligo- and polysaccharide molecules (Reilly, 1979). Glucoamylase from Aspergillus awamori exists in two forms: GAI, the larger form, is thought to consist of three functional regions: (1) a large catalytic domain from residues 1 to 470; (2) a heavily O-glycosylated serine- and threonine-rich region that overlaps the catalytic domain from residue 441 to 470 and continues to 512; (3) a carboxy-terminal region from residue 513 to 616, which is involved in native starch binding; GAII is identical to GAI but lacks the C-terminal starch-binding region. Recently, the crystal structure of glucoamylase-I' from Aspergillus awamori var kawachi has been determined (Honzatko et al., personal communication). The crystal structure of glucoamylase-I' shows 13 α -helices with limited regions of antiparallel β -strands in the vicinity of the active site. The N-terminal half of the glycosylated domain is in an extended conformation, wrapping around the catalytic domain. The entire O-glycosylated domain is probably in a extended conformation. Although GAII has similar ability to digest soluble starch as GAI, it has greatly reduced ability to bind to and hydrolyze native starch. Based on comparison of GAI and GAII, it has been postulated that the C-terminal region is involved in native starch binding (Svensson et al., 1982).

Comparison of the amino acid sequences of putative native starch binding domains from 8 different starch-degrading enzymes showed that the C-terminal sequence of GAI has significant homology (four areas of sequence similarity separated by short segments) to several other starch binding enzymes. Svensson et al.(1989) hypothesized that this C-terminal region constitutes a self-contained domain involved in native starch binding. Several specific amino acid residues were also identified that occurred in all 8 sequences. The strong consensus of these residues suggested that they might play a critical role in native starch binding. Recently, Dalmia et al. (1991) suggested that there is a large binding site including at least 5-6 subsites on the starch binding domain.

Hayashida et al. (1989) reported a GAI starch-binding site of consisting of 45 amino acids, from residue 471 to residue 515. This region must not play a large role in starch binding since GAII contains almost all of these residues and binds to native starch very poorly (Svensson et al., 1982; Dalmia et al., 1990). Libby et al. (1991) genetically engineered several internal deletions of the O-glycosylated region (from amino acid 460 to 512) of A. awamori glucoamylase secreted from Saccharomyces cerevisiae and found that partial deletions up to 30 amino acids did not affect native starch binding and digestion. These results are consistent with the hypothesis that the O-glycosylated region is not essential for starch binding, and that the catalytic and starch binding domains function independently in the hydrolysis of native starch granules. We have previously shown (Chen et al., 1991 a,

b) that a β -galactosidase fusion protein (BSB119) containing a C-terminal tail of 119 amino acids from the C-terminus of A. awamori glucoamylase is able to adsorb strongly to starch compared to a β -galactosidase control. Other β -galactosidase fusion proteins containing smaller segments of the C-terminal region of glucoamylase had decreased affinity to native starch, while a fusion protein with a larger fragment of this region (BSB133) showed increased proteolysis compared to BSB119. These results confirmed the presence of a native starch binding region at the C-terminus of GAI and suggested that a C-terminal tail of 119 amino acids is near the optimal size for starch binding when fused to β -galactosidase and expressed intracellularly in E. coli. Recently, Kusnadi et al.(1992) have fused various amounts of the starch binding region to the maltose binding protein (MBP) of E. coli. Functional fusion proteins were isolated containing 106, 122 or 136 amino acids from the C-terminus of GAI. They also were able to cleave the starch binding domain from these fusion proteins and showed that these independent domains were functional. Since the starch binding domain may have an altered conformation when fused to target proteins and because of lack of glycosylation in E. coli, the starch binding results obtained from bacterially expressed fusion proteins (β -galactosidase and maltose) may not be very conclusive. To further study the structure and function of the GAI starch binding domain, five mutations within the C-terminal 103 amino acids of GAI were made and expressed in Saccharomyces cerevisiae. Our starch binding results with the mutated enzyme confirm that the C-terminal 103 amino acid residues of glucoamylase are required

for fully functional native starch binding. These results are consistent with those obtained by protein fusion studies (Chen et al., 1991), chemical modification (Svensson et al., 1986a) and sequence comparisons (Svensson et al., 1989).

MATERIALS AND METHODS

Strains and plasmids

The Saccharomyces cerevisiae laboratory strain C468 (α , leu2-3, leu2-112, his3-11, his3-15, mal-) (Innis et al., 1985) was a generous gift from Cetus Corporation.

Vector pRE1 (Evans et al., 1990) was used for the construction of mutants as was the yeast expression Vector YEpPM18 (Cole et al., 1988) (Fig.1). Vector YEpPM18 also was the generous gift from Cetus Corporation.

Construction of the mutants

Plasmid YEpPM18 (Cole et al., 1988) (Fig.1) encoding the wild-type A. awomari glucoamylase gene was used as a basis for construction of five mutant plasmids (pGAMD21, pGAMD52, pGACD8, pGACD25 and pGACD513). All DNA manipulations were done according to standard procedures (Sambrook et al., 1989). Plasmid pGAMD21 which encodes mutated glucoamylase GAMD21 was constructed first by replacing a 66 bp BstXI-ClaI fragment from wild-type glucoamylase gene of pRE1 (Evans et al., 1990) with the adaptor (5' AGATCTT/3'GCGGTCTAGAAGC). Then a 0.49 Kb BamHI-HindIII fragment was excised from the mutated pRE1 and exchanged with the wild-type glucoamylase BamHI-HindIII fragment of YEpPM18. A similar strategy was used for the construction of plasmid pGAMD52 which encodes enzyme GAMD52. First an N-terminal deletion of the starch binding region was made, originating upstream of the domain and

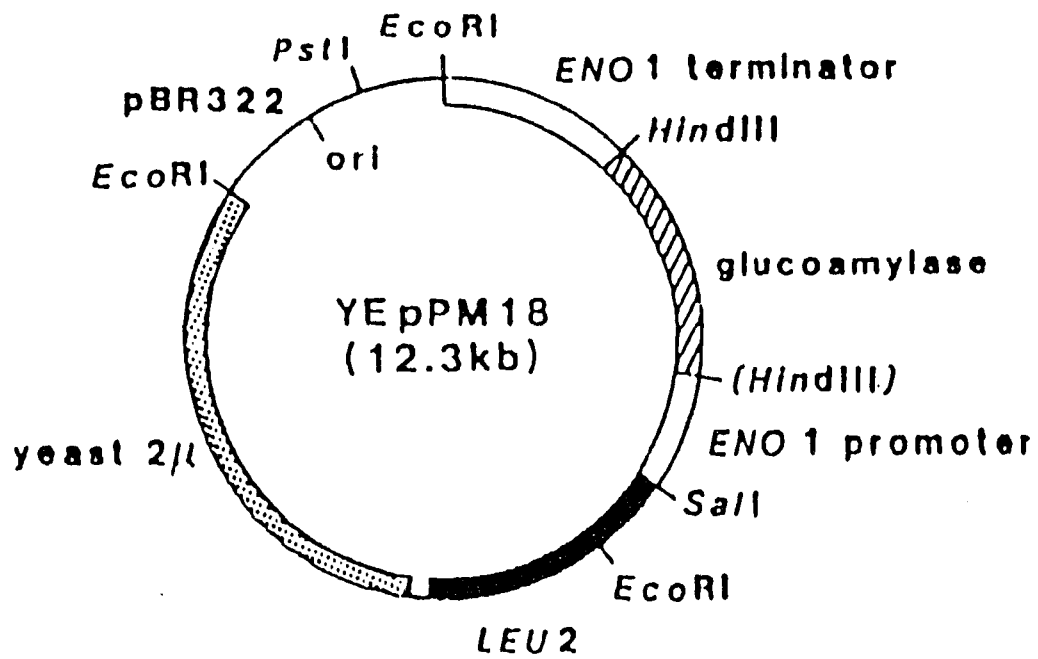


Fig. 1 The plasmid YE pPM18 received from Cetus corporation (Cole et al., 1988).

terminating at the nucleotides encoding Val-567. The deletion was made using exonuclease III and S1 Nuclease (Guo et al., 1983). A BglII linker (5'CAGATCTG) (New England Biolabs) was then ligated to the blunt terminus of the deletion. A second step of pGAMD52 construction involved replacing a 66 bp BstXI-ClaI fragment from the wild-type glucoamylase gene on pRE1 (Evans et al., 1990) with the adaptor (5'ATAGATCTG/3'GCGGTATCTAGAC) and joining it to the N-terminally deleted glucoamylase gene fragment made by the first step via this BstXI-BglII adaptor. The last step of pGAMD52 construction was an exchange of the 0.3Kb BamHI-HindIII fragment containing the deletion with the BamHI-HindIII fragment of the glucoamylase gene on plasmid YEppM18. Both adaptors in pGAMD21 and pGAMD52 were synthesized at Iowa State University Nucleic Acid Facility. The nucleotide sequences in the regions of modification for both mutants were verified by DNA sequencing at the Iowa State University Nucleic Acid Facility using an M13 universal primer. Plasmid pGACD8 encodes the mutated enzyme GACD8 which has an 8 amino acid deletion at the glucoamylase C-terminus. pGACD8 was constructed by exchanging a 0.55 kb BamHI-HindIII fragment from plasmid pLC2 containing the 8 amino acid deletion (Chen et al., 1991) with that of plasmid YEppM18. Similar to pGACD8, plasmid pGACD25 (encoding GACD25) and plasmid pGACD103 (encoding GACD103) were also constructed respectively by inserting a SpeI linker containing stop codons in three frames (5'-CTAGACTAGTCTAG, New England Biolabs). Plasmid pGACD25 was made by first inserting a SpeI linker into the glucoamylase gene of pRE1 at position Ser-591 using exonuclease III and S1 Nuclease. Then a 0.27

kb BamHI-HindIII fragment containing the inserted SpeI linker was excised and used to replace the wild-type BamHI-HindIII fragment of that of plasmid YEpPM18. The resultant plasmid pGACD25 expressed glucoamylase truncated at residue Ser-591, 25 amino acids shorter than the wild-type glucoamylase. Plasmid pGACD103 which encodes a phenocopy of GAI1 was made by exchanging the 0.55 kb BamHI-HindIII glucoamylase fragment of pRE513 (Evans et al., 1990) with the wild-type glucoamylase BamHI-HindIII fragment on plasmid YEpPM18. None of the adaptors or linkers added more than four additional residues to the original glucoamylase.

Expression and purification of mutated glucoamylases

Plasmids were transformed into Saccharomyces cerevisiae C468 using the Lithium Acetate method of Ito et al. (1983). The wild-type and mutants were grown separately in SD + his medium to saturation in shaking flasks at 30°C for five days. Culture supernatants were collected by centrifugation at 9820 xg for 10 min. For preparation of purified enzymes, the supernatants were collected and concentrated using an Amicon ultrafiltration system (Cartridge Model S1Y10, 10,000 MW Cut-off) and then loaded onto an acarbose-Sepharose affinity column (Clarke et al. 1984). After loading, the column was rinsed with 0.5 M NaCl-0.1 M NaoAC buffer at pH 4.4. The bound enzymes were eluted with a 1.7 M Tris-HCl buffer, pH 7.6. The eluted enzymes then were dialyzed against 0.05 M NaoAC-HAC solution (pH4.5). Protein concentrations were determined using the method of Bradford (1976).

Glucoamylase assay

Glucoamylase activity was determined at 35°C using 1.8% soluble starch as substrate in 0.05 M NaOAc-HAc buffer, pH 4.5. A unit of activity was defined as the amount of enzyme that produces 1 μ mol of glucose per minute under the assay conditions. The amount of glucose was determined using a glucose oxidase-peroxidase/O-dianisidine assay (Sigma Chemical, St. Louis, MO).

Starch clearing plate assay

Yeast cells (strain C468) containing YEPM18 (encoding wild-type glucoamylase), pGAMD52, pGAMD21, pGACD25, pGACD8, pGACD103 and pAC1 (no glucoamylase gene) were tested for their ability to hydrolyze starch in a plate assay. Cells were grown to log phase in SD + his (1.7 mg/ml yeast nitrogen base without amino acids or ammonium sulfate (Difco) / 5 mg/ml ammonium sulfate / 2% glucose / 0.1 mg/ml L-histidine) liquid medium to $A_{600} = 0.4$. Samples of 10 μ l of each culture were inoculated onto petri plates of SD + his medium containing 1% (W/V) soluble starch. The plates were incubated at 28°C for three days and then overnight at 50°C to develop halos of enzyme activity in the starch. Plates were stained with iodine vapor to detect halos.

Starch binding assay

Adsorption of purified enzymes to native starch was assayed by a procedure slightly modified from Medda et al. (1982). Native starch was washed 2 times with 0.05 M NaOAc-HAc buffer (pH4.5). Purified enzymes

were added to 0.1 g washed starch in a 1 ml total volume with enzyme concentrations ranging from 0.1 mg/ml to 0.6 mg/ml. The enzyme-starch mixture was shaken for 1 hour at 4°C. After centrifugation at 17,400 xg for 20 minutes, the protein concentration of the supernatant was assayed and the amount of adsorbed enzyme was calculated by subtraction.

Adsorption constants (K_{ad}) were derived from the slopes of the linear adsorption isotherms and averaged from four initial concentrations per purified enzyme. Adsorption of commercial *A. awamori* GAI and GAI1 to native starch was also assayed.

Starch hydrolysis assay

Hydrolysis of native starch was assayed using a procedure modified from Svensson et al. (1982). A suspension of 2% native starch in 0.05 M NaOAc-HAc buffer (pH 4.5) was preincubated at 30°C for 5 minutes. Purified enzyme (0.05 units) was added to the starch solution to a final volume of 2 ml. The starch-enzyme mixture was then incubated at 30°C with shaking. At suitable time intervals, 80 μ l of the reaction mixture was removed from incubation, the reaction was stopped by adding 270 μ l of 1.5 M Tris-HCl buffer (pH 7.0) and glucoamylase activity was determined.

Thermostability assay

Both wild-type and mutated glucoamylases were assayed for thermostability using the method of Svensson et al. 1986(a). One ml aliquots of enzyme (10 μ M in 0.05 M NaOAc-HAC buffer) were incubated for

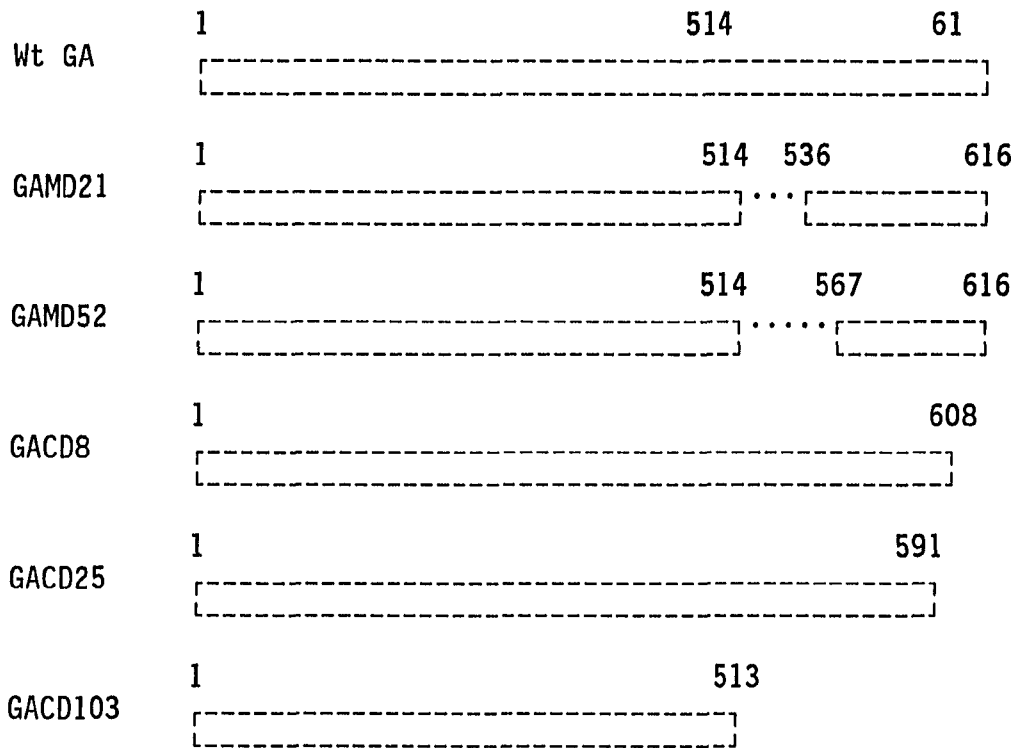
5 minutes at different temperatures ranging from 50°C to 80°C, followed by quick freezing in dry-ice/ethanol. All samples were thawed and then glucoamylase activity was assayed at 35°C using 1.8% soluble starch as substrate.

RESULTS AND DISCUSSION

Construction of mutants

Five plasmids pGAMD21, pGAMD52, pGACD8, pGACD25 and pGACD103 were constructed to encode glucoamylases designated GAMD21, GAMD52, GACD8, GACD25 and GACD103, respectively, with deletions in the starch binding domain (Fig.2). The plasmids were constructed based on the glucoamylase expression vector YEpPM18 (Cole et al., 1988) and were transformed into the yeast *S. cerevisiae* for expression of the mutated enzymes. All the deletions were made within the 103 amino acid C-terminal domain (from amino acid 513 to 616) which is essential for strong GAI starch-binding activity (Svensson et al., 1982). This 103 amino acid region exists in GAI but is lost in GAII and it is highly homologous to the starch binding domains of other enzymes (Svensson et al., 1989). GAMD21 and GAMD52 were constructed with deletions of 21 amino acids (from Val-515 to Gly-535) and 52 amino acids (from Val-515 to Thr-566) residues, respectively, from the N-terminus of the 104 amino acid domain. GACD8 and GACD25 were constructed with truncations of 8 amino acids (at position Thr-608) and 25 amino acid residues (at position Glu-591), respectively, from the C-terminus of wild-type glucoamylase. GACD103 (truncated at Val-515) was constructed to produce a phenocopy of natural GAII (Svensson et al., 1986b) and served as a negative control.

Fig. 2 Construction of mutated glucoamylase. A. Schematic of mutant glucoamylases. Numbers indicate glucoamylase amino acid positions. Dotted lines indicate amino acid deleted from wild-type glucoamylase (wt GA). Sequence information for wild-type glucoamylase is from Nunberg et al. (1984). B. Nucleotide and amino acid sequences at the deletion junctions of the glucoamylase mutants. Bold-type amino acid are encoded by inserted adaptors or linkers. The remaining amino acid residues are part of the wild-type glucoamylase sequence. The numbers indicate glucoamylase amino acid position.



B.

GAMD21 ...ACT CCC ACC GCC AGA TCT TCG ATC TCT...
 ...Thr Pro Thr Ala Arg Ser Ser Ile Ser...
 514 536

GAMD52 ...CCC ACC GCC ATA GAT CTG GTG ACT CTG...
 ...Pro Thr Ala Ile Asp Leu Val Thr Leu...
 514 567

GACD8 ...ACG TCG ACT AGA CTA GTC TAG
...Thr Ser Thr Arg Leu Val STOP
608

GACD25 ...GAG TGG GAG ACT AGA CTA GTC TAG
...Glu Trp Glu Thr Arg Leu Val stop
591

GACD103 ...ACC ACT CCC ACC TAG
...Thr Thr Pro Thr Stop
513

Characterization of mutants

Wild-type glucoamylase and the five mutant enzymes were expressed in S. cerevisiae and purified by affinity chromatography (Materials and Methods). Specific activities on soluble starch for each of the mutants (Table 1) were similar to that of wild-type glucoamylase from yeast and of commercial Aspergillus GAI. These results suggest that deletion of part or all of the native starch binding region does not affect the ability of the catalytic domain to hydrolyze soluble starch and are consistent with the observation that A. awamori GAI and GAII have similar ability to digest soluble starch (Svensson et al., 1982).

SDS-PAGE of mutated glucoamylase and wild-type glucoamylase encoded by YEPM18 prepared from culture supernatants is shown in Fig.3. GAI from YEPM18 and GAII (encoded by GA103) had an apparent M_r of approx. 97 and 65 kDa respectively, with the mutants having intermediate mobilities. GAI expressed in yeast has a slower mobility than GAI from Aspergillus, apparently due to hyperglycosylation of the enzyme in yeast (Innis et al., 1985).

Starch-clearing plate assay

To visualize activity of mutated glucoamylase on soluble starch, a starch-clearing assay was performed (Materials and Methods). Halos were produced by cells containing YEPM18, pGAMD21, pGAMD52, pGACD8, pGACD25 and pGACD103. No halo was observed for negative control cells containing pAC1 which has no glucoamylase gene (Fig.4). These results confirm that neither deletion of some amino acid residues within the C-

Table 1. Specific activity of glucoamylases

Enzyme	Units/ μ mol
Commercial <u>A. awamori</u> GAI	590
Commercial <u>A. awamori</u> GAII	463
Wt GA (YEpPM18)	569
GAMD21	407
GAMD52	476
GACD8	530
GACD25	412
GACD103	497

Note: Glucoamylase activity was determined at 35°C using 1.8% soluble starch as substrate in 0.05 M NaOAc-HAc buffer, pH 4.5. A unit of activity was defined as the amount of enzyme that produces 1 μ mol of glucose per minute under the assay conditions. The data from duplicate assays were averaged. In all cases, duplicate data points deviated less than 5% from each other. The molarity of the enzyme (μ mol) was calculated based on protein concentrations (determined by the method of Bradford, 1976) and the deduced M_r of each mutated glucoamylase.

Fig. 3 SDS-PAGE of culture supernatants. Concentrated culture supernatants (50ug protein per lane) were electrophoresed in a 7.5% polyacrylamide gel containing 0.1% SDS and stained by Coomassie Brilliant Blue dye according to the method of Laemmli (1970). Lane 1: molecular weight standards. Lane 2: commercial GAI (from Aspergillus). Lane 3: pAC1 which does not contain a glucoamylase gene. Lane 4: wt GA expressed by YEpPM18. Lane 5: GAMD21. Lane 6: GAMD52. Lane 7: GACD8. Lane 8: GACD25 and lane 9: GACD103. The positions of wild-type glucoamylase and mutated enzymes were indicated.

1 2 3 4 5 6 7 8 9

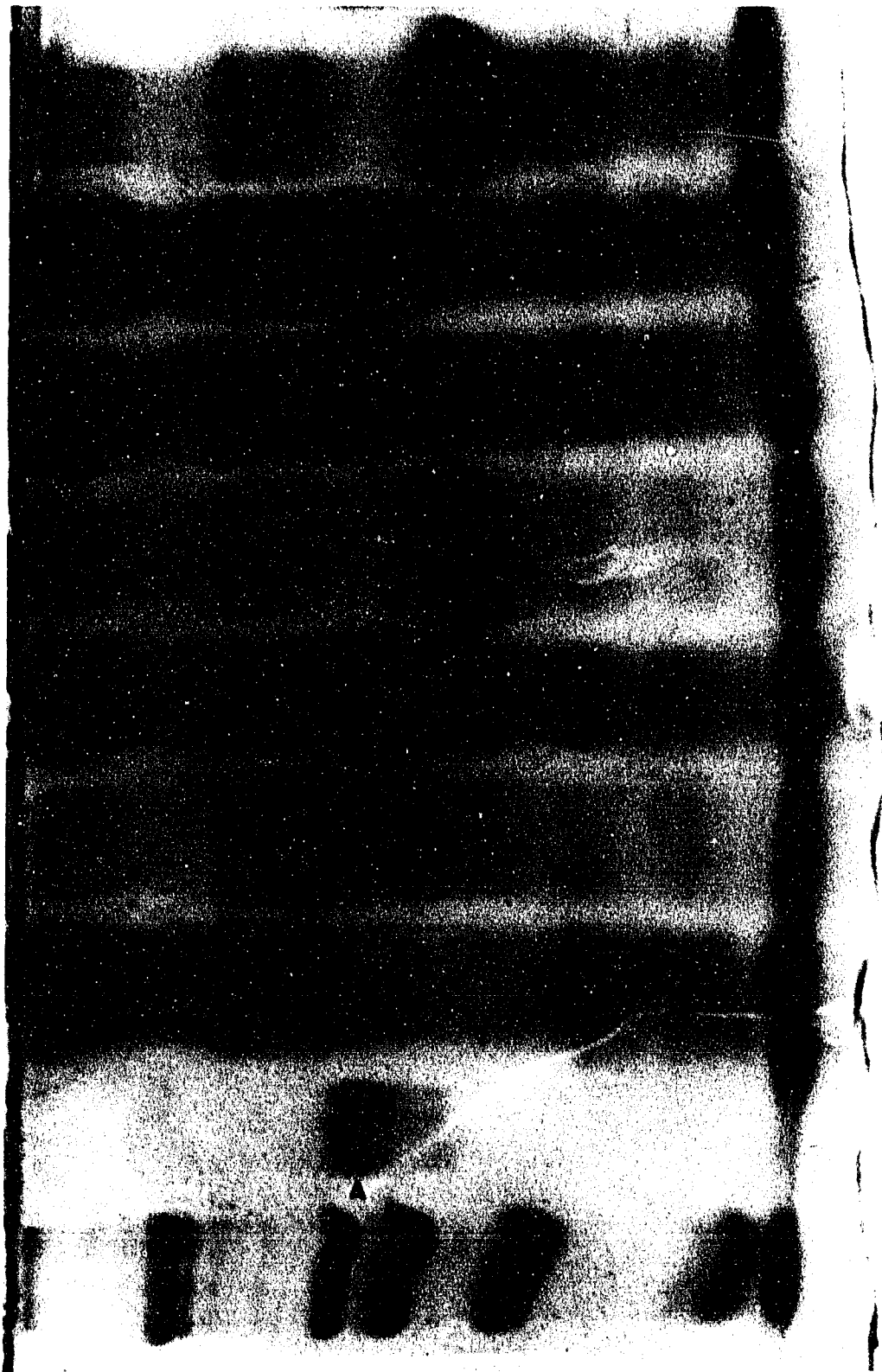


Fig. 4 Starch-clearing plate assay. Yeast cells containing YEpPM18 (encoding wild-type GAI), pGAMD21, pGAMD52, pGACD8, pGACD25, pGACD103 and control plasmid pACI (no glucoamylase gene) were tested for the ability to hydrolyze soluble starch in a plate assay.

GACD103

GAI

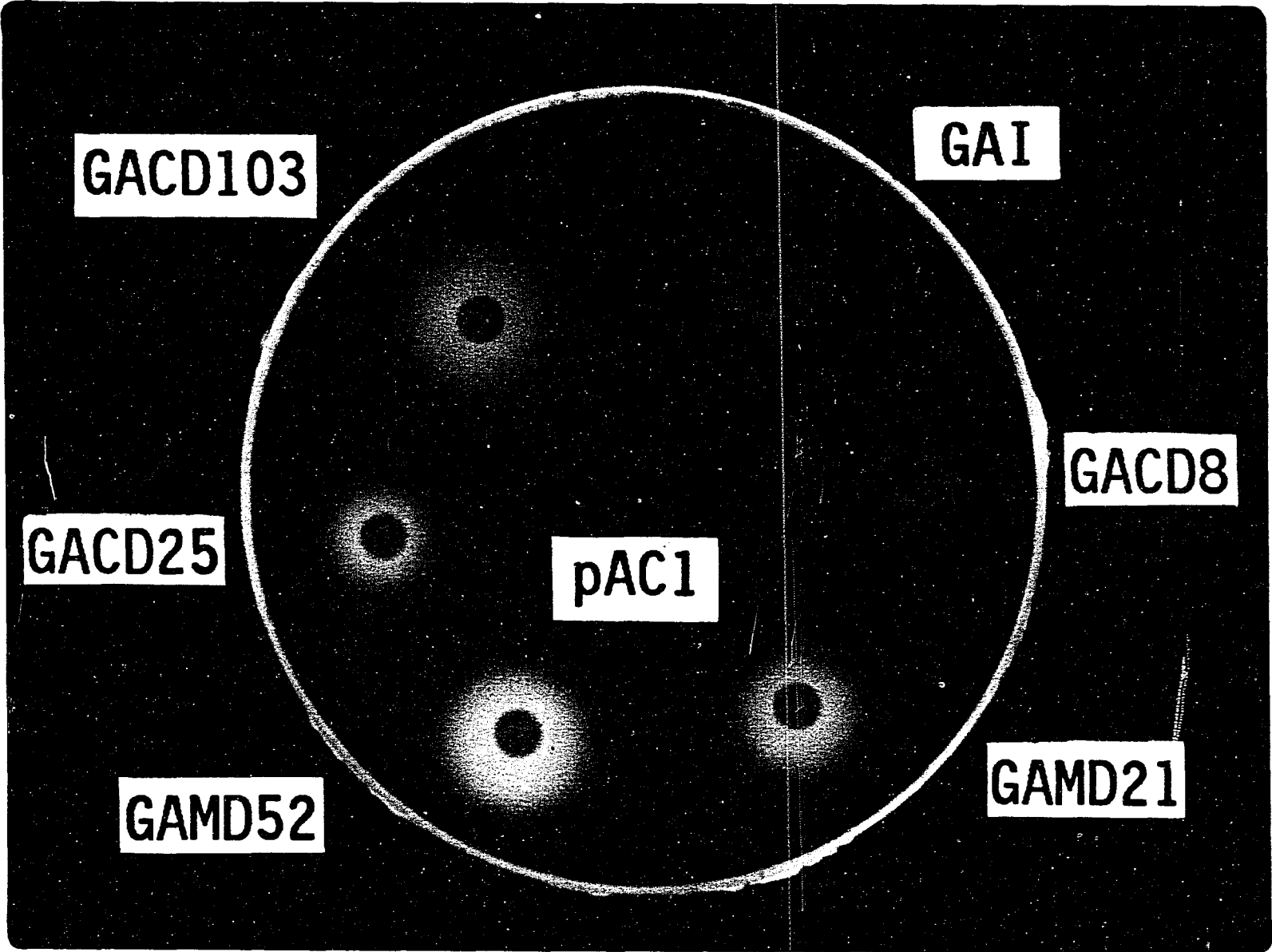
GACD8

GACD25

pAC1

GAMD21

GAMD52



terminal starch binding tail nor complete removal of the C-terminal tail (GACD103) affects enzyme activity on soluble starch. This result is consistent with that obtained by assaying activity of purified enzymes (Table 1). Larger halos were produced by colonies expressing GACD103, GAMD54 and GAMD23 than were produced by colonies expressing wild-type, GACD25 or GACD8. Since all of the enzymes have similar catalytic activity to wild-type glucoamylase on soluble substrate, larger halos are interpreted to result from increased diffusion due to changes in conformation of the mutants.

Adsorption to native starch

Adsorption to native starch of purified wild-type and mutant glucoamylases was assayed (Fig.5). Adsorption isotherms indicated that deletion of amino acid residues from either the N-terminus or C-terminus of the starch binding domain resulted in decreased ability of the enzymes to adsorb to native starch. Wild-type glucoamylase secreted from yeast adsorbed to native starch ($K_{ad} = 33 \text{ ml/g starch}$) with an affinity 20-fold higher than that of GAI03 ($K_{ad} = 1.6 \text{ ml/g starch}$) or commercial Aspergillus GAI ($K_{ad} = 1.1 \text{ ml/g starch}$). The affinity of commercial A. awamori GAI to native starch ($K_{ad} = 86 \text{ ml/g starch}$) is 2.5-fold higher than that of wild-type GAI secreted from yeast. This may be the result of hyperglycosylation of the enzyme in yeast. GAMD21 showed only about 1/3 the adsorption to starch of GAI ($K_{ad} = 12.6 \text{ ml/g starch}$). The deletion in GAMD21 eliminates part of homology region I including the highly conserved residues Thr-525 and Gly-528 (Svensson

Raw Starch Binding Assay

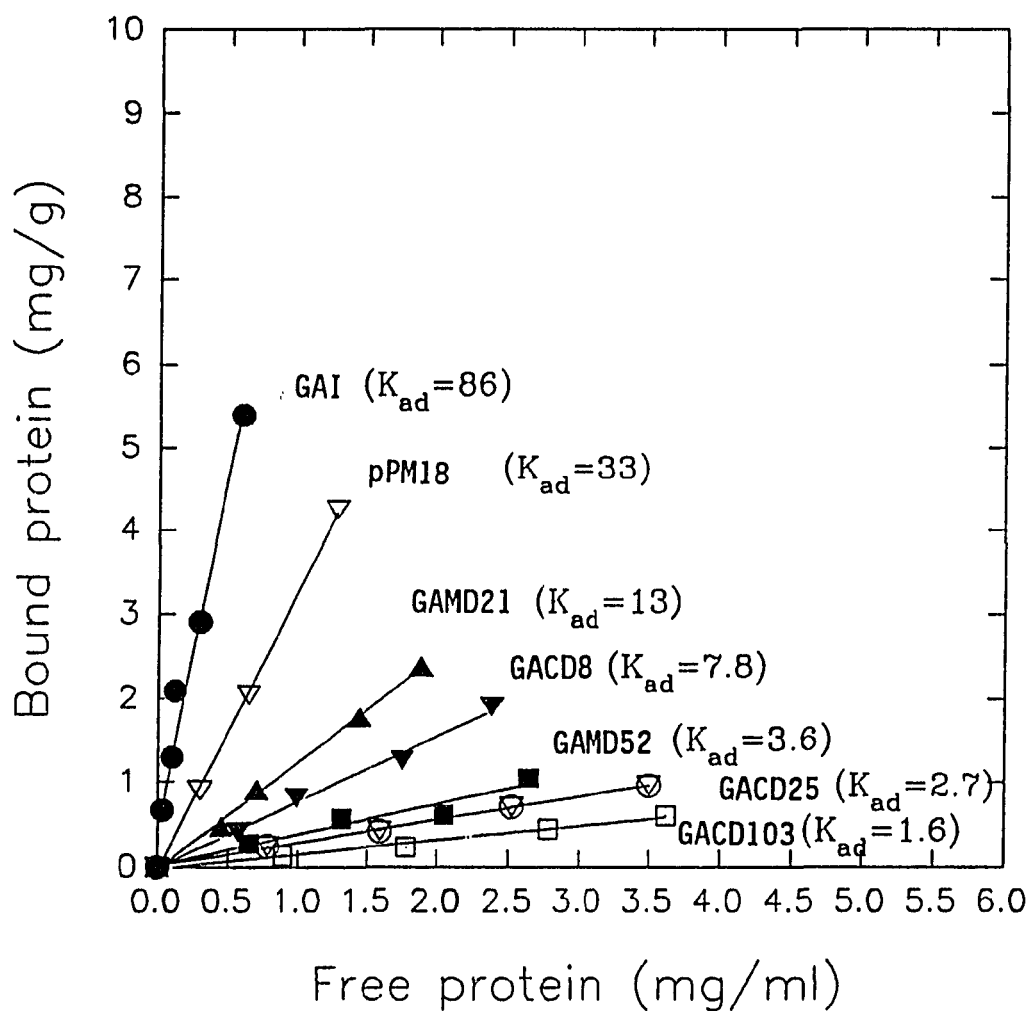


Fig. 5 Adsorption of purified enzymes to native starch. Adsorption constants (K_{ad}) were calculated from the slopes of adsorption isotherms. The data from duplicate assays were averaged, and duplicate data points deviated less than 5% from each other.

et al., 1989). By comparison, GAM52 showed greatly reduced adsorption to starch ($K_{ad} = 3.6$ ml/g starch). The deletion in GAM52 involves the entire homology region I and one more highly conserved residue Trp-543. These highly conserved residues may play important roles in starch binding. GACD8 ($K_{ad} = 7.8$ ml/g starch) with a C-terminal deletion of 8 amino acids including Trp-615 showed sharply decreased starch binding ability compared to GAI. This is consistent with the results obtained by chemical modification (Svensson et al., 1986a) which suggested that Trp-615 may be essential for starch binding. The lower K_{ad} value of GACD25 ($K_{ad} = 2.7$ mg/g starch) compared to GACD8 was expected, because this mutant contains a further deletion of 16 amino acid residues extending to homology region III and including the entire homology region IV (Svensson et al., 1989). Also, the further deleted 16 amino acid residues contain Cys604 which forms a disulfide bond with Cys509 (Svensson, personal communication). The formation of disulfide bonds may be necessary for proper enzyme folding and function.

Hydrolysis of native starch

Hydrolysis of native starch by the mutated glucoamylases and wild-type glucoamylase was assayed (Fig. 6). In general, the results paralleled the results obtained in the starch binding assay. GAI from YEpPM18 expressed in yeast showed a much greater ability to hydrolyze native starch than did the GAII phenocopy GACD103. This is consistent with the observation that GAI of *A. awamori* has a much greater ability to hydrolyze native starch than does GAII (Svensson et al., 1982). The

mutated enzymes GAMD21, GAMD52, GACD8 and GACD25 had decreased activities compared to GAI with GAMD52 (containing the largest deletion) showing the lowest activity (Fig.6). This was expected because all the deletions included sequences that are conserved (Svensson et al., 1989). The loss of activity of the mutated enzymes on native starch appears to be closely associated with their decreased native starch binding ability. Svensson et al. (1982) reported that A. niger GAI binds to and digests native starch, however GAII which lacks the starch binding domain basically does not bind to or digest native starch. Adsorption to native starch does seem to be a pre-requisite for its hydrolysis (Dalmia et al., 1990). Our data supported this. GAI from several sources adsorbs more strongly onto native starch and is able to digest native starch much better than the corresponding GAII from the same organisms that adsorbs only weakly (Miah et al., 1977 a, b; Yoshino, 1978; Saha et al., 1979).

Thermostability

The thermal stability of wild-type GAI, and five mutated glucoamylases were tested (Fig.7). All the enzymes remained stable at temperatures below 60°C and showed similar inactivation profiles with the midpoint of inactivation at approx 70°C. These were consistent with those obtained from other deletions made from amino acid 460 to amino acid 513 of glucoamylase (Libby, 1991). These results suggested that deletions of amino acid residues from either end of the starch binding domain and even extending to the glycosylated region (Libby, 1991) did

Raw Starch hydrolysis

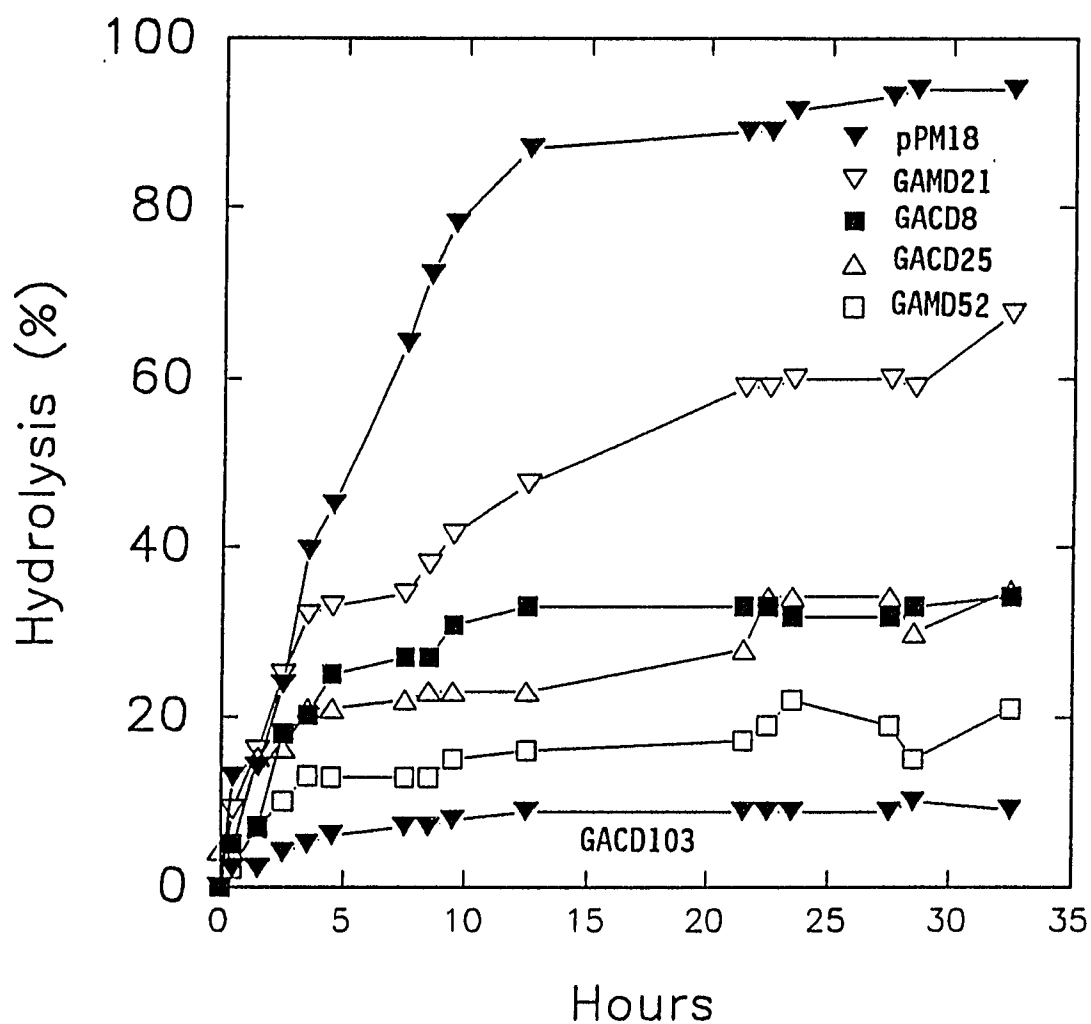


Fig. 6 Hydrolysis of native starch by purified enzymes. Reaction mixtures contained 2% native starch, 0.05 M NaOAc buffer (pH 4.5) and 0.05 units of enzyme in a final volume of 2.0 ml. The reaction was incubated at 30°C.

Thermostability Assay

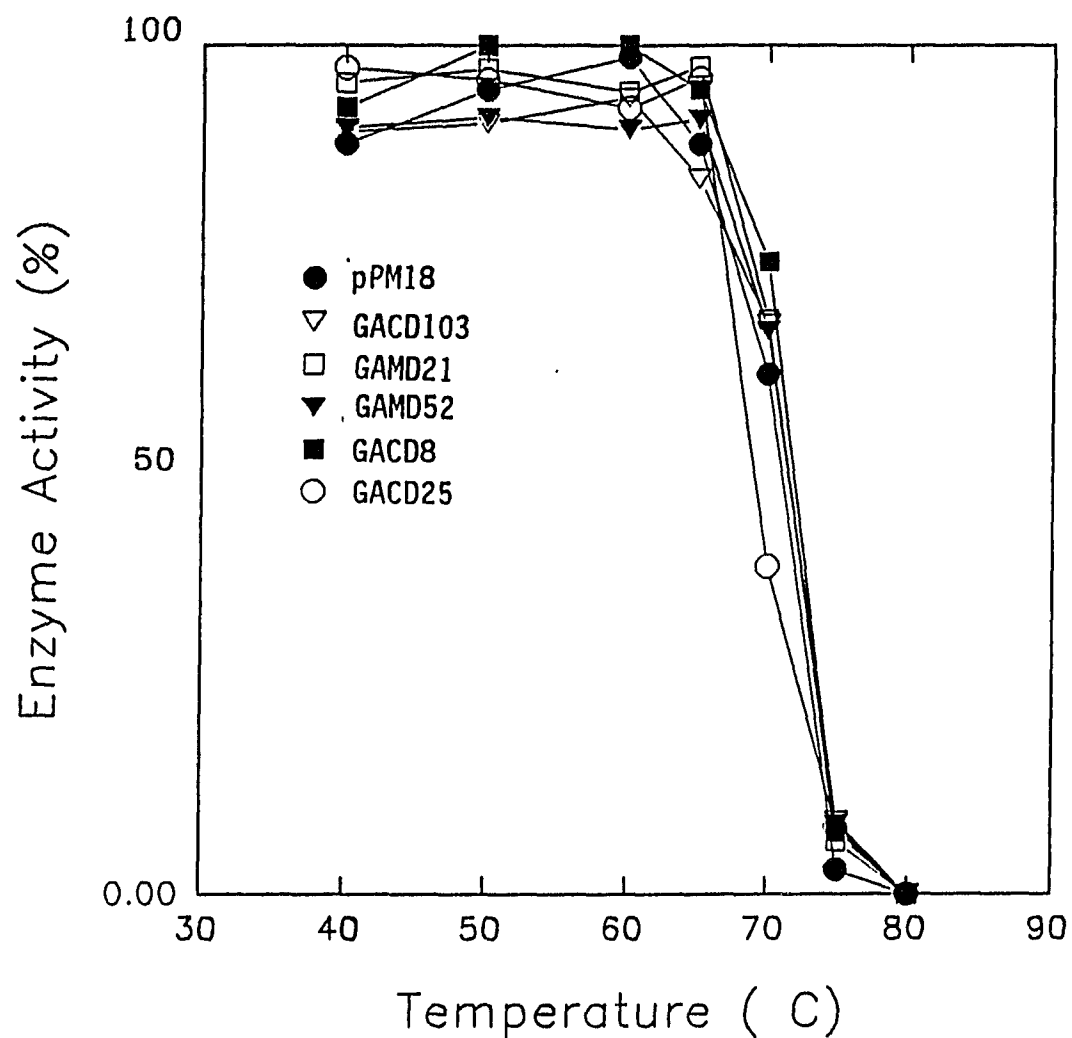


Fig.7 Thermostability Assay. Purified enzymes (10 μ M) were incubated at varying temperatures in 0.05 M NaOAc-HAc buffer, pH 4.5 for 5 min, followed by quick freezing in dry-ice/ethanol. After all samples were thawed, glucoamylase activity was assayed at 35°C using 1.8% soluble starch as substrate.

not have much effect on thermal stability of GAI. Since thermal stability was measured by activity on soluble starch, requiring only the catalytic domain, these results are consistent with the hypothesis that the catalytic domain functions independently of the starch binding domain (Hayashida et al., 1982; Ueda et al., 1982; Dalmia et al., 1991).

CONCLUSIONS

We have created five mutations at the C-terminus of A. awamori glucoamylase by genetic engineering techniques. All the deletions resulted in diminution or loss of the ability of the enzyme to bind to and digest native starch when compared to wild-type glucoamylase. The removal of amino acid residues from both ends of the native starch binding domain did not have much effect on either soluble starch hydrolysis or thermal stability of the enzyme, supporting the hypothesis that the starch binding domain is separate and independent from the catalytic domain. These results confirm that the C-terminal 103 amino acid residues of A. awamori glucoamylase are involved in native starch adsorption and indicated that the deleted amino acid residues are required for full function of the starch binding domain. These results will be useful for further structure and function studies of the starch binding domain of Aspergillus glucoamylase.

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GENERAL SUMMARY

Glucoamylase (GA) from Aspergillus exists in two forms: GAI and GAII. The larger form (GAI) is thought to consist of three functional regions: (1) a large catalytic domain from amino acid residue 1 to 470; (2) a heavily O-glycosylated region that overlaps the catalytic domain from amino acid residue 441 to 512; and (3) a carboxy-terminal region from amino acid residues 513 to 616, which is involved in native starch binding. GAI and GAII are both able to digest soluble starch, but only GAI has the ability to bind to and hydrolyze native starch granules. Based on sequence comparison of GAI and GAII, it has been suggested that the carboxy tail (involving residues 513-616) of glucoamylase is involved in native starch binding. This is also consistent with results obtained by chemical modification. Svensson et al., (1989) found significant amino acid sequence homology between the C-terminal domain of GAI and starch binding domains of several other putative starch binding enzymes. However, the boundaries of the starch binding domain of GAI have not been defined.

In paper 1 of the thesis, the construction of six β -galactosidase fusion proteins containing C-terminal fragments of GAI was described. All the fusion proteins were tested for native starch binding. It was found that the C-terminal 119 amino acid residues of GAI is near the optimum functional size for fusion proteins expressed in E. coli. Fusion proteins containing the starch binding domain can specifically and strongly bind to native starch granules and can be eluted with

similar purity to that achieved by affinity chromatography, These results suggest the potential application of native starch as an adsorbent to facilitate the purification or immobilization of fusion proteins containing the starch binding domain. Starch granules are very stable, non-toxic and inexpensive, and their use as an adsorbent could lower the cost of protein purification in downstream processing.

β -galactosidase is the first target protein we tested and severe degradation of fusion proteins was observed. Other proteins could be chosen to make fusion proteins.

In paper 2, five glucoamylase mutants with extensive deletions within the C-terminal 103 amino acids of GAI were constructed. The deletions in the starch binding domain did not affect enzyme activity on soluble starch or thermostability of the enzyme, but progressive loss of starch binding and starch hydrolytic activity were observed upon removal of different amounts of amino acid residues. This suggested that the 103 amino acid residues at the C-terminus of GAI are necessary for starch binding function and confirmed the independence of the catalytic domain from the starch binding domain.

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APPENDIX A. PUBLICATION 1

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