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# Characterization of the starch-binding domain of *Aspergillus* glucoamylase produced in *Escherichia coli*

by

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Signature was redacted for privacy. Signature was redacted for privacy.

For the Major Department

For the Graduate College

Iowa State University
Ames, Iowa

1992

To my parents, husband, and son

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# GENERAL INTRODUCTION

We are interested in studying the interaction of granular starch with the starch-binding domain of Aspergillus glucoamylase I and the possible application of the starch-binding domain as an affinity tail for recovery or enzymatic immobilization using granular starch as an adsorbent. There are several advantages of using starch as an adsorbent: it is abundant, cheap, stable, generally recognized as safe, and easy to recover.

Starch-binding domain of Aspergillus glucoamylase I was functional when produced in Escherichia coli as a fusion protein with ß-galactosidase (Chen et al., 1991a,b). The ß-galactosidase fusion proteins containing different lengths of starch-binding domain adsorbed to granular starch due to the presence of the starch-binding domain. However, a further comparison and biochemical characterization of the starch-binding fragments in the fusion proteins was impossible because partial proteolysis of the purified ß-galactosidase fusion tetramer resulted in a mixed population of fusion proteins containing between 30 and 50% of full-sized tetramer (Chen et al., 1991a,b; Dalmia and Nikolov, submitted). Functional starch-binding fragments were recently isolated from a protease digest of native glucoamylase I using a tedious and time consuming procedure (45 times of ion exchange chromatography) (Belshaw and Williamson, 1990; Williamson et

al., 1992).

This dissertation addresses attempts to produce functional starch-binding fragments in *Escherichia coli* in a three-step process. The interaction of the isolated starch-binding fragments with starch-like molecules was also investigated by using ultraviolet difference spectroscopy.

**Explanation of dissertation format**

This dissertation contains three papers. Preceding them is a literature review of the performed studies. The three papers are written in the form of research papers. The first paper is a part of a published paper in collaboration with Dr. L. J. Chen, Dr. C. F. Ford, and Dr. Z. L. Nikolov. Additional information, which was not reported in the published paper was also included in paper 1. In this paper we discuss the large scale production and granular starch adsorption of β-galactosidase and two β-galactosidase fusion proteins possessing starch-binding fragments. The second paper has been submitted to Gene for reviewing. In this paper we report the production of a functional starch-binding domain of *Aspergillus* glucoamylase I in *Escherichia coli*. In the third paper we discuss the characterization of the produced starch-binding domain. The three papers are followed by a general conclusions and bibliography listing references cited in the General Introduction, Literature Review, and General Conclusions sections.

# LITERATURE REVIEW

## Glucoamylase

Glucoamylase (α-1,4-glucan glucohydrolase, E.C. 3.2.1.3) is an exohydrolase which catalyzes the release of ß-D-glucose from the non-reducing ends of starch and related oligo- and polysaccharides (Hiromi et al., 1966; Hayashida, 1975; McCleavy and Anderson, 1980; Saha and Zeikus, 1989). Glucoamylase cleaves mainly α-1,4 and α-1,6 glucosidic bonds of starch (Figure 1). It can also break α-1,3, α-1,2 and α,ß-linkages of disaccharides although at a much slower rate (Meagher et al., 1989).

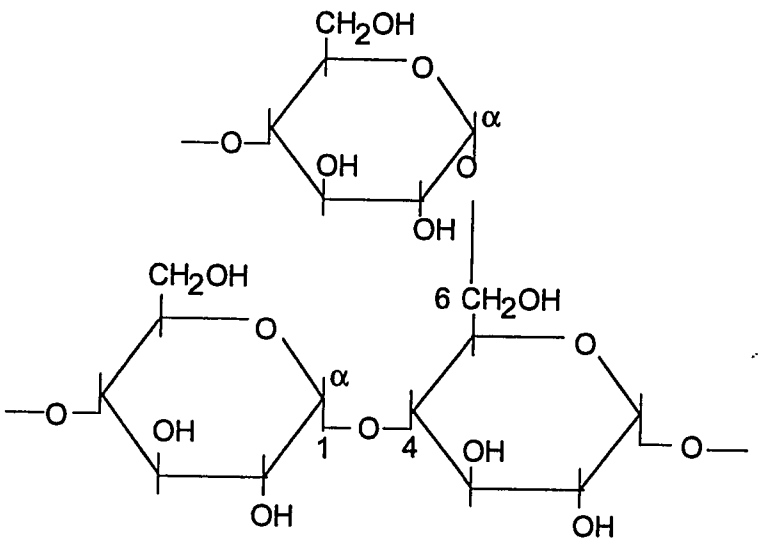


Figure 1. α-1,4 and α-1,6 linkages of starch (Saha and Zeikus, 1989).

For industrial purposes, glucoamylase from Aspergillus and Rhizopus species are the most widely used. Glucoamylase is one of the key enzymes in the production of glucose and high fructose corn syrups, which are mainly consumed by food and beverage industry (Saha and Zeikus, 1989; Toldra et al., 1992). Another important use of glucoamylase is in alcohol fermentation. Figure 2 summarizes the use of glucoamylase in starch bioprocessing. Because of its industrial importance glucoamylase has been and is currently studied extensively.

**Distribution and multiple molecular forms of glucoamylase**

Glucoamylase has been found in numerous microorganisms. The microbial glucoamylase is produced by several species of Aspergillus, Rhizopus, Candida, and Endomyces (Manjunath et al., 1983; Saha and Zeikus, 1989).

The number of glucoamylases per strain ranges between one and five. Single forms of glucoamylase (no multiple molecular forms) were isolated from A. awamori, R. delemar, Endomycopsis fibuligera (Pazur and Okada 1967; Kato et al., 1976; Yamasaki et al., 1977; Ueda and Saha, 1983). The multiple molecular forms of glucoamylase was first observed in the enzyme preparations from black-koji mold (A. awamori var. kawachi) (Ueda, 1957). Since then, many researchers have reported the multiplicity of glucoamylase preparations. Glucoamylase of Aspergillus species, such as A. awamori, A. niger, A. saitoi, A. foetidus and A. phoenicis, and Rhizopus (Manjunath et al.,

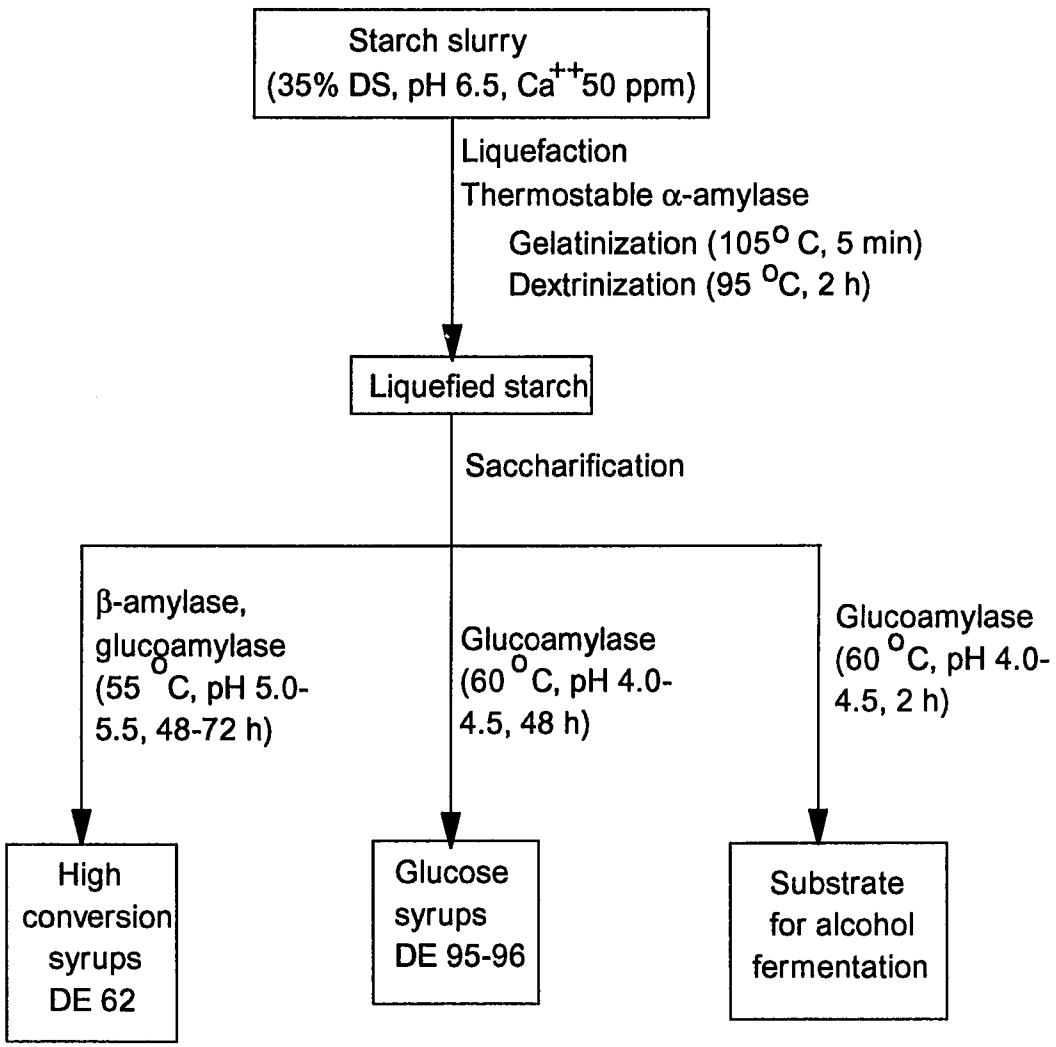


Figure 2. Starch bioprocessing using glucoamylase (Saha and Zeikus, 1989).

1983; Saha and Zeikus, 1989) exist in two active forms. In some cases, more than two forms of glucoamylase from A. niger, A. oryzae, and R. niveus have been reported. The production of glucoamylase and its multiple molecular forms was affected by medium composition and culture conditions of the microorganisms. Hayashida et al. (1976) and Hayashida and Yoshino (1978) reported that multiple molecular forms of glucoamylase may be formed by stepwise degradation of native glucoamylase by proteases and glycosidases during cultivation and purification. A. awamori var. kawachi mutant (protease-negative and glycosidase-negative) produced only one form of glucoamylase (Hayashida and Flor, 1981). A protease might play a major role in the formation of multiple molecular forms of glucoamylase from Rhizopus sp. (Takahashi et al., 1982). A mRNA splicing (Boel et al., 1984) or a post-translational modification (limited proteolysis at the carboxyl terminal) (Svensson et al., 1986) was suggested as a mechanism for the formation of two forms of A. niger glucoamylase.

**Domain organization of Aspergillus glucoamylase**

Glucoamylase from A. niger exists in two major molecular forms: glucoamylase I and glucoamylase II (Lineback et al., 1969, Pazur et al., 1971; Svensson et al., 1982). The primary structure of glucoamylase I (Figure 3) was identified by Svensson et al. (1983). The amino acid sequence of glucoamylase II is identical to that of glucoamylase I from

```
1    ATLDSWLSNE ATVARTAILN NIGADGAWVS GADSGIVVAS PSTDNPDYFY

51   TWTRDSGLVL 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 RIESDDSVEW ESDPNREYTV
     __________ __________ __________ __________ __________

601  PQACGTSTAT VTDTWR
     __________ ______
```

Figure 3. The one-letter amino acid sequence of A. niger glucoamylase (Svensson et al., 1983). Symbols: * O-glycosylated residues, --- starch binding domain.

amino acids 1 to 512 (Svensson et al., 1986). Both glucoamylase I and glucoamylase II hydrolyze soluble substrates, but only glucoamylase I can bind to and hydrolyze granular starch (Dalmia and Nikolov, 1990).

Glucoamylase I from Aspergillus sp. has three functional domains: a catalytic domain, a highly O-glycosylated domain,

and a starch-binding domain (Svensson et al., 1983; Gunnarsson et al., 1984). The catalytic domain comprises residues 1-440, and has a strong sequence homology to glucoamylase from Saccharomyces, Rhizopus, and Saccharomycopsis (Tanaka et al., 1986; Itoh et al., 1987). The highly O-glycosylated domain of glucoamylase I consists of residues 441-512, and is rich in Ser and Thr. This domain stabilizes glucoamylase I (Pazur et al., 1971; Evans et al., 1990) and seems to be in a predominantly extended conformation (Williamson et al., 1992b). One of the roles of O-glycosylated domain is to maintain the catalytic domain and the starch-binding domain apart at a fixed distance (Aleshin et al., 1992; Williamson et al., 1992a). The carbohydrate moieties (mannose residues) were important in the digestion of granular starch (Fukuda et al., 1992), but not in the actual adsorption to granular starch as has been suggested before by Hayashida et al. (1989b). Svensson et al. (1982) hypothesized that the starch-binding domain comprises residues 513-616 which allows glucoamylase I to adsorb to and hydrolyze granular starch. A similar domain organization was also found in some cellulases (Ong et al., 1989).

**Identification of the presence of starch-binding domain**

Ueda (1957) reported that glucoamylase can digest raw starch. However, only the largest form of glucoamylase multiple molecular forms (referred to as glucoamylase I) had the

ability to adsorb to raw starch and digest it (Pazur et al., 1971; Ueda et al., 1974; Hayashida, 1975; Miah and Ueda, 1977; Medda et al., 1982a,b, Svensson et al., 1982; Takahashi et al., 1985).

Hayashida et al. (1976) reported that the raw starch digestibility of glucoamylase I required the intact structure of the enzyme. For the first time, the importance of a part other than the catalytic site of glucoamylase I on granular starch digestibility was suggested. Glucoamylase I digestion of granular starch was linked to its ability to adsorb to starch granules (Ueda, 1981). By studying the granular starch adsorption and elution of glucoamylase of black Aspergillus and Endomycopsis fibuligera, Medda et al. (1982) and Ueda and Saha (1983) suggested that the catalytic domain and the starch-binding domain are different regions of the enzyme molecule. Subtilisin digested-glucoamylase I lost its ability to adsorb to and to digest granular starch, and the resulting glycopeptide (Gp-1) preserved its granular starch adsorbability, but not its digestibility (Hayashida et al., 1982). This glycopeptide (Gp-1) which was part of glucoamylase I, was named by Hayashida et al. (1982) as the "raw-starch affinity site". Later, the same group determined that the "raw-starch affinity site" was located between Ala-471 and Val-514 (Hayashida et al., 1989a, c). The primary structure of this 45 amino acid long Gp-1 is highly homologous to the O-glycosylated region in the primary structures of A.

awamori, A. niger, and R. oryzae (Hayashida et al., 1989a).

Takahashi et al. (1985) reported that the starch-binding domain of Rhizopus sp. glucoamylase is also different from its catalytic site, and it is located at the N-terminus region (residues 27-134). In the case of Aspergillus glucoamylase, the starch-binding domain is thought to be located at the C-terminus region, and it involves Trp-590 and Trp-615 (Svensson et al., 1986a).

By comparing primary structures of different starch-degrading enzymes, Svensson et al. (1989) found a common feature among these enzymes in their terminal sequence motif, which appeared as a self-contained domain important in adsorption to granular starch. This terminal sequence motif was observed at the C-terminus of A. niger glucoamylase and Bacillus macerans cyclodextrin glucanosyltransferase, two highly homologous α-amylases, two exo-α-amylases, and a ß-amylase (Figure 4). The compared C-terminal sequences consist of 93 to 103 amino acid residues containing four areas of homology (21-44%) separated by short segments varying in length (Figure 4). The conserved Trp residues are likely to participate in binding of glucosyl residues through hydrophobic or hydrogen bonding forces, as reported for other protein-carbohydrate complexes (Quicho, 1986; Jhonson et al., 1988). The existence of a starch-binding domain separate from the catalytic domain was further confirmed by Chen et al. (1991a, b) and Svensson and Sierks (1992).

```
                          I                          II
gaRh      9-vqldsynydgstfsg-kiyv-27 45-dnwnnngntiaasysapisgsnyeywtf- 72
αSli    442-QTSASFHVNATTAWGENIYVTGDQAALGNWDPARALKL-------DPAAYPVWKL-489
BCth    420-IPVTFTINNATTYYGQNVYIVGSTSDLGNWNTTYARGP------ASCPNYPTWTI-469
gaAN    514-AVAVTFDLTATTTYGENIYLVGSISQLGDWETSDGIALSAD---KYTSSDPLWYV-565
MαBs    581-SVVFTVKSAPPTNLGDKIYLTGNIPELGNWSTDTSGAVNNAQGPLLAPNYPDWFY-635
MTPs    430-VSVSFRCDNGATQMGDSVYAVGNVSQLGNWSPAAALRL------TDTSGYPTWKG-478
CAIB    587-VTVRFVINNATTALGQNVFLTGNVSELGNWDPNNAIGPMYNQ---VVYQYPTWYY-638
CKpn    530-QSINFTCNNGYTISGQSVYIIGNIPQLGGWDLTKAVKI-------SPTQYPQWSA-577
Consensus              T  G      G    LG W                    P W

                    II          III          IV
gaRh     73-sasingikefyikyev-------sgktyydnnnsanvqvst-104
αSli    490-DVPLAAGTPFQYKYLRKDAA---GKAVWESGANRTATVGTT---GALTLNDTWRG*538
BCth    470-TLNLLPGEQIQFKAVKIDSS---GNVTWEGGSNHTYTVPTS---GTGSVTITWQN*519
gaAN    566-TVTLPAGESFEYKFIRIESD---DSVEWESDPNREYTVPQACGTSTATVTDTWR*616
MαBs    636-VFSVPAGKTIQFKFFIKRAD---GTIQWENGSNHVATTPTG---ATGNITVTWQN*684
MTPs    479-SIALPAGQNEEWKCLIRNEANATQVRQWQGGANNSLTPSE-----GATTVGRL*526
CAIB    639-DVSVPAGQTIEFKFLKKQG-----STVTWEGGANRTFTTPTS---GTATVNVNWQP*626
CKpn    578-SLELPSDLNVEWKCVKRNETNPTANVEWQSGANNQFNSND-----TQTTNGSF*625
Consensus              K              W    N
```

Figure 4. Comparison of C-terminal sequences of amylases and cyclodextrin gluconotransferases. --- indicates gaps, and * indicates the C-terminal residues of the protein. gaRh, glucoamylase from Rhizopus oryzae; αSli, α-amylase from Streptomyces limosus; ßCth, ß-amylase from Clostridium thermosulfurogenes; gaAn, glucoamylase from Aspergillus niger; MαBS, maltogenic α-amylase from Bacillus stearothermophilus; MTPs, maltotetraose-forming amylase from Pseudomonas stutzeri; CAlB; cyclodextrin glucanotranferase from alkalophilic Bacillus sp. strain; and CKpn, cyclodextrin glucanotransferase from Klebsiella pneumoniae.

Previous studies reviewed above showed the existence of starch-binding domain at the C-terminus of Aspergillus sp., in contradiction to that suggested by Hayashida et al. (1989b, c). To determine the optimal size of the starch-binding

domain, six different lengths of starch-binding fragments were fused to ß-galactosidase by means of recombinant DNA techniques (Chen et al., 1991a, b). The six different fragments investigated included amino acid 380-513, 484-608, 484-616, 498-616, 514-616, and 537-616 of A. awamori glucoamylase I. The near optimal size of starch-binding fragment for granular starch adsorption was found to be 119 amino acid long (amino acid 498-616). Furthermore, removal of eight amino acids (amino acids 608-616 of glucoamylase I) from the C-terminus of one of the ß-galactosidase fusion proteins diminished the granular starch-binding ability of the ß-galactosidase fusion protein, suggesting the importance of the eight C-terminus residues (Chen et al., 1991a). The additional deletion analysis of the C-terminus of glucoamylase I suggested that 103-amino-acid fragment from the C-terminus is important in facilitating granular starch adsorption of the enzyme (Chen, 1992). These studies supported the previous hypothesis that the starch-binding domain of Aspergillus glucoamylase I was located at its C-terminus.

Three different sizes of starch-binding fragments of A. niger glucoamylase I (amino acid 471-616, amino acid 499-616, and amino acid 509-616) were isolated from a protease VIII digest of glucoamylase I (Belshaw and Williamson, 1990; Williamson et al. 1992a). The three fragments could adsorb to granular starch and bind ß-cyclodextrin, a specific ligand for the starch-binding domain (Savel'ev et al., 1990). However,

the peptide consisting of amino acid 499-616 was found to be of an optimal size for the formation of a functional starch-binding domain (Williamson et al., 1992a, b) as has been shown previously (Chen et al., 1991b).

**Adsorption to starch and starch-like molecules**

Glucoamylase I of black Aspergillus adsorbed to granular starch almost at the same level in the absence (99% of 0.475 mg/ml was bound) and presence (82%) of an amylase inhibitor which specifically binds to the catalytic site of glucoamylase I (Medda et al., 1982a). Hayashida et al. (1982) reported that Gp-1 fragment isolated from the subtilisin digest of glucoamylase I adsorbed to chitin, granular starch, and alkali-treated cell wall of Aspergillus with surface concentration of 0.9, 0.4, and 0.8 mg per mg of adsorbent, respectively. The dissociation constant ($K_d$) for Rhizopus sp. glucoamylase I binding to granular starch was estimated at 8.3 $\mu$M (Takahashi et al., 1985). The latter group investigated inhibition of glucoamylase I binding to granular starch by using different saccharides at pH 5 and 37°C (Table 1). The granular starch inhibitory effect of the saccharides tested increased with their increase in size. Maltose, for example, had 30 times higher inhibition effect than glucose. It was suggested that all the tested saccharides, regardless of their size, affected granular starch binding through steric hindrance.

Table 1. $K_m$ or $K_i$ values of inhibition of raw starch binding of Rhizopus glucoamylase I

| Saccharide | $K_m$ ($K_i$) |
|---|---|
| Glycogen | 0.004% |
| Soluble Starch | 0.005% |
| Maltose | 3.1 mM |
| Methyl α-D-glucoside | 61.9 mM |
| Glucose | 98.5mM |

Savel'ev et al., (1990) investigated the adsorption of various ligands to the starch-binding domain of glucoamylase I from A. awamori using the equilibrium gel filtration method. The glucoamylase I adsorbed the strongest to ß-cyclodextrin (a cyclic heptamer of glucose) with a ratio of one to one and $K_d$ of 5.6 μM. For α-cyclodextrin (a cyclic hexamer of glucose) (Figure 5) the $K_d$ value was ten times higher. Svensson and Sierks (1992) reported a much higher value of $K_d$ for ß-cyclodextrin (19 μM) and a similar value for α-cyclodextrin (35 μM). The $K_d$ values for the adsorption of maltose and maltoheptaose to the starch-binding domain of glucoamylase were estimated to be 8.8 and 0.5 mM, respectively (Savel,ev et al., unpublished data). Increasing the degree of polymerization of amylose (from 17 to 40), decreased the dissociation constant from 38 to 10 μM (Savel'ev et al., 1990). Thus, longer ligands showed stronger interaction with the starch binding domain of glucoamylase I.

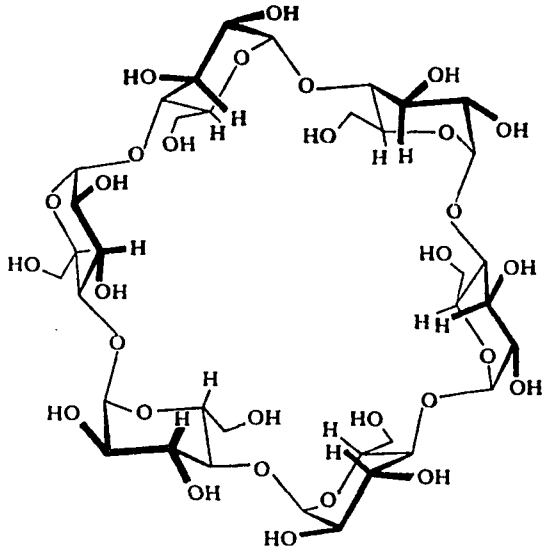

Figure 5. Structure of α-cyclodextrin.

A. niger glucoamylase I adsorption to granular starch and its adsorption inhibition was extensively characterized (Dalmia, 1990; Dalmia and Nikolov, 1991). At pH 3.5 and 0.35 M ionic strength, glucoamylase I adsorbed to granular starch with $K_d$ value of 0.2 μM, and maximum adsorption capacity ($Q_{max}$) of 3.5 mg/g. The presence of various saccharides did not affect the maximum adsorption capacity of glucoamylase I. Maltose inhibited glucoamylase I adsorption to granular starch 30 times stronger than glucose, as was reported for glucoamylase I from Rhizopus sp. (Takahashi et al., 1985). Furthermore, oligosaccharides with a degree of polymerization

of six or seven had the strongest inhibition of glucoamylase I adsorption to granular starch.

ß-galactosidase fusion proteins possessing different length of starch-binding fragments of glucoamylase I adsorbed to granular starch because of the presence of starch-binding domain (Chen et al., 1991a, b). However, ß-galactosidase fusion protein possessing amino acids 498-616 of glucoamylase I adsorbed stronger to granular starch compared to the one possessing amino acids 380-513, amino acids 484-616, amino acids 514-616, amino acids 537-616, or amino acids 484-608. These results indicating that the starch-binding domain was located within amino acids 498-616. Moreover, granular starch adsorption studies performed on Aspergillus glucoamylase I with five different deletions at its C-terminus revealed that amino acids 514-616 was required for full function of starch-binding domain (Chen, 1992).

The three fragments that were isolated (amino acids 471-616, amino acids 499-616, and amino acids 509-616) by Belshaw and Williamson (1991) and Williamson et al. (1992) adsorbed to granular starch with $Q_{max}$ value of 27 mg peptide/g starch and $K_d$ values between 12.7 and 19.6 $\mu$M which were higher than those reported for glucoamylase I (Dalmia and Nikolov, 1991). These peptides seemingly bound ß-cyclodextrin with a stoichiometry of one to two in contrast to one to one stoichiometry in the case of glucoamylase I (Savel'ev et al., 1990). The $K_d$ values for ß-cyclodextrin binding to the three fragments were between

1.7 and 3.3 μM (Williamson et al., 1992a).

## Expression and stability of foreign proteins in Escherichia coli

E. coli is the most widely used microorganism for the production of commercially important recombinant proteins because it presents several advantages as a host for protein expression. Its genetics are well understood, it can be grown to high cell density on inexpensive fermentation broth, and fermentation scale-up is straightforward (Georgiou, 1988). A major problem associated with the expression of foreign DNA sequences in E. coli is proteolytic degradation because foreign polypeptides are often recognized as abnormal and are subject to extensive degradation by the host proteolytic system. Proteolytic degradation of the expressed protein by the host proteases causes low yield and complicates purification procedure.

### Factors affecting stability of a foreign protein in E. coli

**Putative protease cleavage sites** In Table 2 some of the E. coli proteases, their recognition sequence, pH optima, location and inhibitors are listed. A protein that has a recognition sequence for any of the E. coli proteases will probably be hydrolyzed, and if the recognition sequence is exposed on the surface of the protein, the proteolysis is more likely to occur. A recognition site may be accessible to

Table 2. Some of E. coli proteases

| Protease | Location | Specificity | pH optimum | Inhibitors |
|---|---|---|---|---|
| Re | Cytoplasm | Ala-Gly<br>Val-Glu<br>Tyr-Leu<br>Val-Cys | 7.0-8.5 | 2 mM TPCK<br>10 mM PMSF<br>10 mM DFP<br>0.1 mM $ZnCl_2$ |
| La | Cytoplasm | | | DFP, EDTA,<br>N-ethylmaleimide |
| IV | Inner membrane | L-Ala<br>L-Leu | 8.1-8.4 | 1 mM PMSF<br>0.4 mM AAPCK<br>5 mM antipain |
| I | Periplasm | Phe-Tyr<br>Phe-Phe<br>Ser-His<br>Leu-Tyr | 7.5 | 0.1 mM DFP |
| III (Pi) | Periplasm membrane | Tyr-Leu<br>Phe-Tyr | 7.4 | 1 mM EDTA<br>10 mM DTT<br>10 mM 2-ME |
| VII | Outer | Arg-Arg | 6.0 | 0.5 mM $ZnCl_2$ |
| (OmpT) | membrane | Lys-Arg<br>Arg-Lys<br>Lys-Lys | | 0.5 mM $CuCl_2$<br>5 mM Benzamidine |

2-ME: 2-mercaptoethanol; AAPCK: Acetyl-L-alanine-L-phenylalanine chloromethyl ketone; DTT: Dithiothreitol; DFP: p-diisopropyl fluorophosphate; EDTA: Ethylenediamine tetraacetate; PMSF: phenylmethyl-sulfonyl fluoride; TPCK: N-α-p-tosyl-L-phenylalanine chloromethyl ketone.

proteases in the native protein or could be exposed on the protein surface as a result of improper folding. Proper folding is affected by several factors. There are at least two factors that could cause an improper folding. First, the formation of disulfide bonds of cytoplasmic protein could be

prevented because of the reducing environment of cytoplasm (Fahey et al., 1977). And, second, the lack of folding catalysts (certain cellular enzymes and chaperonins) could result in accumulation of partially folded protein intermediates, which are more likely to be cleaved by proteases.

**N-terminus amino acid** N-terminus amino acid residue was shown to be important in determining proteolytic stability of a protein (Bachmair et al., 1986; Bachmair and Varshavsky, 1989). In a significant fraction of E. coli proteins, the N-terminus Met residue which is incorporated during the translation initiation step is subsequently excised by methionine aminopeptidase. The efficiency of the removal of N-terminus Met depends on the amino acid following the Met residue. Hirel et al. (1989) showed that Met is preferentially removed when the second amino acid has a small side chain length (e.g. Gly, Ala, Ser, Cys, Pro or Thr). Therefore, proteins containing a destabilizing amino acid may be protected from degradation by the inefficient removal of the N-terminus Met by methionine aminopeptidase.

**Protein size** In E. coli, shorter foreign polypeptide chains are degraded faster than larger proteins (Tamaldge and Gilbert, 1982). These shorter polypeptides could be stabilized by fusion to native E. coli proteins. Insulin

(Goeddel et al., 1979), ß-endorphin (Shine et al., 1980), and human fibroblast interferon (Taniguchi et al., 1980) were fused to ß-galactosidase to increase their stability.

**Post-translational modifications** Oxidation of Cys, His, Met, Trp and Tyr can increase protein degradation (Jori and Spikes, 1984) because of conformational changes sufficient to expose protease cleavage site. Robinson et al. (1970, 1974) postulated that the deamidation of Asn and Gln residues increased the proteolytic susceptibility of a protein by disrupting its structure. This hypothesis was supported by the existence of an inverse correlation between Asn and Gln content and the half-life of proteins (Robinson et al., 1974; Rogers and Rechsteiner, 1988). Other post translational modifications, such as phosphorylation of serine or threonine residues, the acetylation and carbamylation of Lys residues, the formation of mixed disulfides and the methylation of deamidated Asn residues, may also signal degradation (Rivett, 1986; Stadtman, 1990).

## Strategies to minimize proteolytic degradation

**Protein engineering** Foreign proteins stabilization could be achieved by modifying amino acid residues recognized by proteases through in vitro mutagenesis. This approach requires information on the three-dimensional structure and the importance of particular amino acid on the stability of

the folded molecule. Generally, a number of mutants have to be constructed before a biologically active and stable protein can be isolated. Hellebust et al. (1988) showed that a cytoplasmic fusion protein of Protein A and ß-galactosidase is rapidly degraded at a Lys-Arg bond located in the linker region between the two domains. Substitution of this region with a new linker lacking this protease sensitive site eliminated degradation.

Amino and carboxyl terminal extensions also have been investigated as means to increase stability of proteins expressed in E. coli. By inserting a six to seven amino acid long hydrophilic homooligopeptide linker between the first five amino acids of ß-galactosidase and the beginning of the mature human proinsulin, production of human proinsulin was increased (Sung et al., 1987). Hammarberg et al. (1989) have constructed a stable Human insulin- like growth factor II (IGF-II) fused to a truncated Protein A domain as a leader sequence, and B1B2 domain of protein G fused to the C-terminal of IGF-II.

**Cellular localization** One way to stabilize a foreign protein is by secreting it to the periplasmic space because some proteins are degraded less rapidly in the periplasmic space compared to the cytoplasm (Talmadge and Gilbert, 1982). An important advantage of secretion is that a product with a N-terminus identical to the authentic protein is obtained if

the signal peptide is correctly processed. Furthermore, periplasmic proteins represent only 4% of the total cellular proteins (Nossal and Heppel, 1966), which simplifies the purification of the target protein.

Transport of cloned gene products to the periplasmic space can be achieved by fusing the protein of interest to the signal sequence of a readily exported protein. The signal sequences of secreted E. coli proteins such as OmpA, maltose-binding protein (the malE gene product), alkaline phosphatase (the phoA gene product), and ß-lactamase (the bla gene product) have been used successfully to produce eukaryotic proteins in the periplasmic space (Ghrayeb et al., 1984; Bedouelle and Duplay, 1988; Maina et al., 1988; Oka et al., 1985; Villa-Komaroff et al., 1978).

Fusing the protein of interest to maltose-binding protein gives several advantages. (i) maltose-binding protein does not have any Cys residues that could interfere with disulfide bond formation within the protein of interest, (ii) the fusion protein can be isolated in a single affinity chromatography step under physiological condition, and (iii) the materials for affinity chromatography are inexpensive and easy to prepare, making large scale purification simple and feasible (Guan et al., 1988). Considering these advantages, the starch binding domain of A. niger glucoamylase I fused to maltose-binding protein was used in this work.

Since E. coli does not excrete proteases under normal

conditions, another approach to increase the stability of a foreign protein is by excreting it to the growth medium. This approach introduces another level of complexity because the protein has to translocate through the inner membrane as well as transverse the outer membrane, and isolation of the protein of interest from the fermentation broth is complicated. Nevertheless, a number of successful approaches for protein excretion have been reported (Georgiou, 1988).

**Formation of inclusion bodies** The expression of foreign and native proteins at significantly higher level can result in the formation of dense, amorphous protein aggregates known as inclusion bodies (Marston, 1986; Kane and Hartley, 1988; Georgiou and Bowden, 1990). These aggregates result from improper folding and consist mainly of reversibly denatured polypeptides. Kitano et al. (1987) showed that formation of inclusion bodies increased the stability of recombinant interleukin-2 and interferon-c. Short and highly unstable polypeptides, such as human insulin (Goeddel et al., 1979), ß-endorphin (Shine et al., 1980) and the yeast cell division cycle (the CDC28 gene product), have also been expressed at high levels by fusing them to the cytoplasmic E. coli enzyme ß-galactosidase. These proteins were isolated from the insoluble fraction of the cells; it is likely that the formation of inclusion bodies effectively protected the polypeptides from proteolytic degradation.

Inclusion bodies not only protect unstable soluble proteins from degradation, but also permit the intracellular production of polypeptides that may be toxic for the cell. Many important products such as bovine somatotropin, urokinase and interleukin-2 and antibody fragments (Hutson et al., 1988) are obtained from E. coli inclusion bodies.

The main problem associated with the production of aggregated proteins is the recovery of biologically active products following solubilization and renaturation. The refolding of small polypeptides that contain few disulfide bonds is generally more efficient than that of larger and complex proteins. In addition, some proteases may aggregate together with the desired polypeptide and cause extensive degradation during solubilization and refolding.

## Measuring protein-ligand interaction

A common event in living systems is the interaction between proteins and one or more molecules. A molecule bound to a macromolecule is defined as a ligand (Freifelder, 1982). Specific interactions of proteins with other molecules generally involve a binding step as the major event. In order to characterize such molecular recognition, one needs to quantify the stoichiometry of binding and affinity of a binding site for the ligand concerned (Bagshaw and Harris, 1987).

There are two principal methods in measuring binding:

direct methods, and indirect methods (Freifelder, 1982; Oberfelder and Lee, 1985). In the direct methods, the macromolecules and ligands are first mixed and the bound ligands are separated from the free ligands. The ligand-protein complex is detected by directly determining the difference in ligand concentration (Oberfelder and Lee, 1985). This is commonly done by equilibrium dialysis. In the indirect methods, binding is observed by measuring a change in a physical property of the macromolecule or ligand induced by binding. Indirect approaches can be performed by utilizing spectroscopy.

**Equilibrium dialysis**

In equilibrium dialysis, a solution of macromolecules is placed inside a dialysis bag, and suspended in a medium containing a certain concentration of a ligand. At equilibrium, the concentration of the unbound ligand outside the dialysis bag and the bound ligand inside the dialysis bag can be determined. The disadvantages of this procedure is that the concentration range to be used must be determined in advance, results are obtained hours or days later which can cause denaturation of the macromolecule or the ligand, and the success depends on membrane permeability and the rate of separation of the free and bound ligand. Equilibrium dialysis has been used to measure the binding of glucoamylase I to α- and ß-cyclodextrin (Savel'ev et al., 1989).

**Spectroscopy**

Spectral changes are easily detected whenever the spectrum of the macromolecule or the ligand changes significantly after binding. The spectral changes observed might be a shift in the absorption maximum, an increase or decrease of absorbance, a change in intensity of fluorescence, or an increase or decrease in band width in NMR spectra (Cantor and Schimmel, 1980; Freifelder, 1982; Oberfelder and Lee, 1985; Bagshaw and Harris, 1987). A classical example is the measurement of oxygen binding to haemoglobin. The spectroscopy method offers several advantages. Firstly, results may be obtained instantly in such that the progress of an experiment can be modified accordingly. Secondly, ligand and protein are allowed to equilibrate and are measured in one vessel, so that membrane permeability problems and rapid separation of the free and bound ligand are avoided. The main disadvantage of measuring spectral changes upon binding is that the optical signal is used empirically.

**Fluorescence spectroscopy** Formation of a ligand-protein complex may result in alteration in the fluorescence intensity of the aromatic amino acids of the protein, extrinsic probe or the ligand. Fluorescence intensities are proportional to concentrations over a narrow range of optical densities. This method has been used widely in determining the binding of various ligand to glucoamylase. Hydrolysis of

maltodextrin by R. niveus glucoamylase I was studied by fluorescence stopped-flow method (Hiromi et al., 1974). The interaction of Rhizopus glucoamylase I with glucose and gluconolactone (Ohnishi and Hiromi, 1976; Ohnishi et al., 1977), and Aspergillus glucoamylase I with maltose, gluconolactone, and acarbose (Clarke and Svensson, 1984) was studied using fluorescence spectroscopy. However, the interaction of Aspergillus glucoamylase I and a glucoamylase I fragment with ß-cyclodextrin could not be studied using this technique, because no decrease in fluorescence was observed upon binding (Belshaw and Williamson, 1991).

**Ultraviolet difference spectroscopy** In difference spectroscopy, binding of a ligand to a protein may result in a change in the environment surrounding either the ligand or amino acid residues in the protein. Such a change perturbs the electronic interactions of the ligand or the residues in the protein producing a shift in the absorbance spectrum. The difference between the unperturbed and the perturbed spectrum is termed the difference spectrum (Figure 6).

Perturbation of a protein can easily be detected in the 250- to 300-nm range mainly due to Phe, Tyr, and Trp perturbation. These residues when perturbed have difference spectra which are easily recognized (Figure 7). The perturbation may result as a direct interaction of the ligand in or around the binding site, conformational change distant

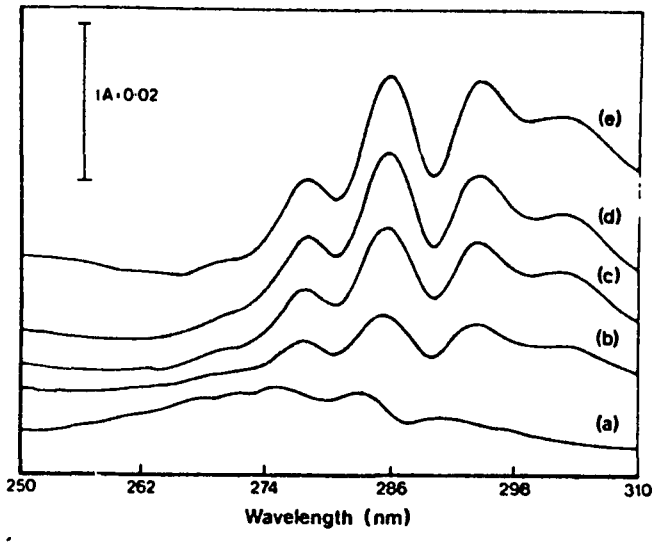


Figure 6. Ultraviolet difference spectra of a glucoamylase I fragment perturbed by ß-cyclodextrin (Belshaw and Williamson, 1991). (a) 0 $\mu$M, (b) 18.1 $\mu$M, (c) 42.3 $\mu$M, (d) 66.5 $\mu$M, and (e) 121 $\mu$M ß-cyclodextrin

from the binding site, association and dissociation of the ligand-protein complex, a ligand transfer from an aqueous environment to a hydrophobic one upon binding to the protein, or the change of ionization state of the protein due to ligand binding.

This technique has been used extensively to study the interaction of glucoamylase with various ligands. The interaction of Rhizopus glucoamylase with maltose (Ohnishi and Hiromi, 1976), maltotriose and maltodextrin (Ohnishi and Hiromi, 1978), Streptomyces trehalase inhibitor and gluconolactone (Tanaka et al., 1982) have been studied by

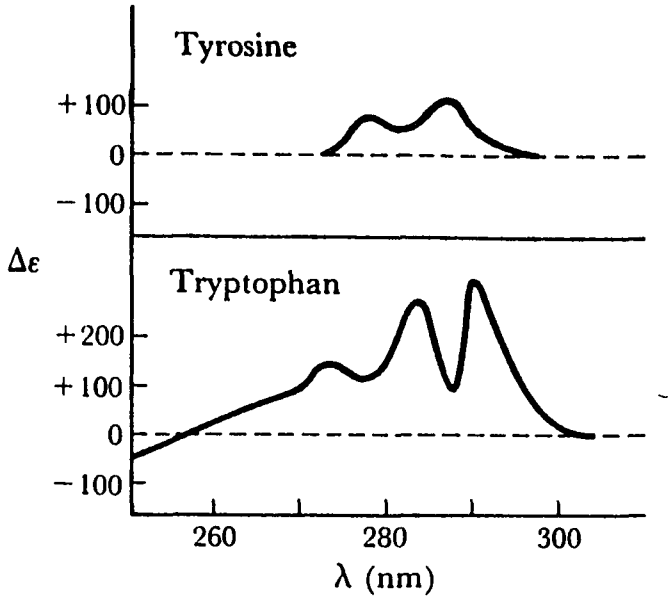


Figure 7. Difference spectra for tyrosine and tryptophan in 80% $H_2O$, 20% ethylene glycol

ultraviolet different spectroscopy. The interaction of Aspergillus glucoamylase with gluconolactone (Clarke and Svensson,1984), acarbose, methyl acarviosinide, D-gluco derivative, L-ido derivative, 1-deoxynojirimycin, miglitol, and emiglitate (Svensson and Sierks, 1992), and 6'-S-α-D-glucopyranosyl-6'-thiomaltose (Cottaz et al., 1992). Belshaw and Williamson (1991) used difference spectroscopy to estimate the $K_d$ values for adsorption of Aspergillus glucoamylase I starch-binding domain.

**Treatment of binding data**

$$P + L \rightleftharpoons PL \qquad (1)$$

The dissociation constant ($K_d$) of the protein ligand complex (PL) is given as:

$$K_d = e.l/b \qquad (2)$$

where e, l and b are the concentrations of the free protein, free ligand, and bound ligand at equilibrium, respectively.

**Semi-quantitative determination of dissociation constant and number of binding sites** Figure 8 shows theoretical spectroscopic titration curves. If a protein binds a ligand strongly in such that the initial protein concentration ($e_0$) is more than ten times greater than the $K_d$ value, $K_d$ and the number of binding sites (n) can be estimated from the plot of the response versus the initial ligand concentration (Figure 8, curve a). The $K_d$ value is estimated from the intersection point of the initial rise and the plateau of the response, and read on the abscissa of the titration plot. If a proteinbound relatively weakly to a ligand in such that the initial protein concentration is less than 1/10 of the $K_d$ value, very little ligand is bound in the initial stages of titration. Thus, the initial ligand concentration is about the same as the initial

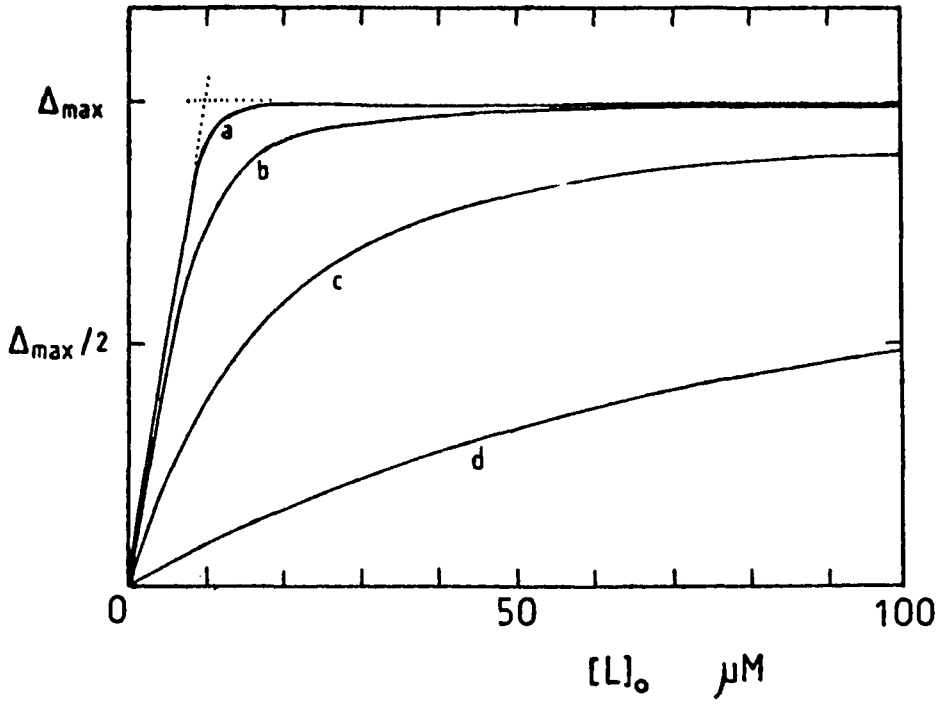


Figure 8. Theoretical spectroscopic titration curves. The initial protein concentration was taken as 10 $\mu$M. The dependence of change in signal ($\Delta$) on initial ligand concentration ($[L]_0$) is shown for values of $K_d$ (a) 0.1 $\mu$M, (b) 1 $\mu$M, (c) 10 $\mu$M, and (d) 100 $\mu$M.

enzyme concentration, and the free ligand concentration is about the same as the initial ligand concentration. In this case, the dissociation constant value can be estimated from the ligand concentration that gives half of maximal response ($K_d = l_{1/2}$) as shown by curve d in Figure 8. However, a dissociation constant value will be overestimated by the latter method if binding is stronger ($K_d$ values are lower), because the initial ligand concentration is higher than the free ligand concentration (Figure 8, curves b and c).

**Graphical determination of $K_d$ and n** In determining $K_d$ and n the conditions should be chosen so that a significant percentage, but not all of the added ligand is bound (Bagshaw and Harris, 1987). And, it is assumed that there is only one noninteracting ligand-binding site, or all the existing ligand-binding sites are uniform. If a fraction of the protein sites ($\alpha$) is occupied by the ligand at equilibrium, then:

$$e = e_0 (1-\alpha)$$

$$b = \alpha e_0$$

$$l = l_0 - b = l_0 - \alpha e_0$$

Substituting e, b, and l values to Equation 2 gives

$$l_0/\alpha = [K_d/(1-\alpha)] + e_0 \quad (3)$$

$e_0$ in this case is the concentration of ligand binding sites on the protein. In a spectrophotometric titration of a protein with a varying concentrations of ligand, $\alpha$ at different initial ligand concentrations can be calculated from Equation 4.

$$\alpha = \Delta/\Delta_{max} \quad (4)$$

where $\Delta$ and $\Delta_{max}$ are the optical change measured, and maximum optical change, respectively. $K_d$ and $e_0$ can be estimated from the slope, and the intercept at $l_0/\alpha$, respectively, from a plot of $l_0/\alpha$ versus $1/(1-\alpha)$. The number of binding sites can be calculated by dividing $e_0$ by the actual protein concentration used. Another graphical method to determine $K_d$ and n is by using Equation 5 (Bagshaw and Harris, 1987), which is based on the Scatchard equation (Scatchard, 1949). At equilibrium, the free protein concentration is given by Equation 5.

$$e = e_0 - b \qquad (5)$$

Substituting this e value in Equation 2 gives Equation 6.

$$b/l = -b/K_d + e_0/K_d \qquad (6)$$

A plot of bound ligand concentration divided by free ligand concentration (b/l) versus the concentration of bound ligand (b) gives a slope of $(-1/K_d)$, an intercept on the abscissa equal to $e_0$. Deviations from linearity in the plots mentioned above, could be used to determine the presence of cooperativity in binding or of a mixture of binding sites with different affinity.

PAPER 1: LARGE SCALE PURIFICATION AND GRANULAR STARCH ADSORPTION OF β-GALACTOSIDASE FUSION PROTEINS CONTAINING THE STARCH BINDING DOMAIN OF *ASPERGILLUS* GLUCOAMYLASE I

## ABSTRACT

A ß-galactosidase and two ß-galactosidase fusion proteins containing amino acids 498-616 (BSB119), and amino acids 537-616 (BSB80) of Aspergillus glucoamylase I were purified on a large scale. The yields of ß-galactosidase, BSB80, and BSB119 were 8.8, 3.4, and 3.2 mg/l, respectively. Maintaining dissolved oxygen at 80% in the fermenter doubled the yield of BSB119. Adsorption isotherms of the three proteins were of the Langmuirian type. Analysis of the granular starch adsorption data by using the Scatchard plot indicated the presence of homogeneous binding sites on the starch surface. BSB119 has a maximum binding capacity ($Q_{max}$) of 36.3 mg/g starch, which is about 10 times higher than that of BSB80 and ß-galactosidase. The apparent association constant ($K_a$) is three times higher than that of BSB80 and ß-galactosidase.

# INTRODUCTION

Glucoamylase (EC 3.2.1.3, 1,4-α-D-glucan glucohydrolase) catalyses the release of ß-D-glucose from the non-reducing ends of starch and related oligo- and polysaccharides (Reilly, 1979). Glucoamylase from Aspergillus niger mainly exists in two forms, glucoamylase I and glucoamylase II (Lineback et al., 1969; Pazur et al., 1971; Svensson et al., 1982). Both glucoamylase I and glucoamylase II hydrolyze soluble substrates, but only glucoamylase I binds to and hydrolyses granular starch (Svensson et al., 1982). Glucoamylase I (amino acids 1-616) consists of two discrete functional domains: the catalytic domain (amino acids 1-440) and the starch-binding domain (amino acids 513-616), which are separated by a highly glycosylated region rich in Ser and Thr (amino acids 441-512) (Gunnarson et al., 1989, Svensson et a., 1989; Williamson et al., 1992). Glucoamylase II (amino acids 1-512) lacks the starch-binding domain and hence cannot adsorb to and hydrolyze granular starch and has 30 times lower affinity to granular starch compared to glucoamylase I (Dalmia, 1990).

Five ß-galactosidase fusion proteins containing different sizes of glucoamylase I starch-binding domain were constructed (Chen, 1991a, b). Their purification on a small scale (1-l shake flask) and granular starch adsorptivity (with initial protein concentrations of 0.1-0.5 mg/ml) were investigated.

Since the granular starch adsorption isotherms for these fusion proteins were determined over a narrow range of initial protein concentrations, their maximum binding capacity could not be determined. In this paper we report a large scale preparation of ß-galactosidase and two ß-galactosidase fusion proteins (BSB80 and BSB119), and their adsorption to granular starch over a wide range of initial protein concentrations.

## MATERIALS AND METHODS

**Preparation of ß-galactosidase, BSB80, and BSB119**

**Fermentation** Three Escherichia coli Y1089-1 cultures, each possessing plasmids pUR290, pLC4, and pLC6, for the expression of ß-galactosidase, BSB119 and BSB80, respectively, were provided by Dr. Ford of the Department of Food Science and Human Nutrition at Iowa State University (Chen et al., 1991a,b). BSB119 and BSB80 are ß-galactosidase fusion proteins containing 119 and 80 amino acid from the N-terminus of Aspergillus awamori glucoamylase I, respectively (Figure 1).

The E. coli strains harboring each of the three plasmids were preserved as follows. Each culture was grown in LB liquid medium (Sambrook et al., 1989) overnight at 37°C, and shaken at 200 rpm. Sterile glycerol was added to each culture to a final concentration of 15%. The cell culture containing glycerol was transferred into 1.5-ml eppendorf tubes, and then frozen in liquid nitrogen. All inocula were prepared using these preserved cultures.

A 300-ml inoculum was prepared as follows. Into a 1-l flask containing 300 ml of TB medium (Sambrook et al., 1989) supplemented with 100 µg/ml ampicillin, 100 µl of a preserved E. coli culture harboring pUR290, pLC4, or pLC6 was added. The culture was then shaken over night at 200 rpm and 37°C. Into a 7.5-l Microferm fermenter (New Brunswick) containing

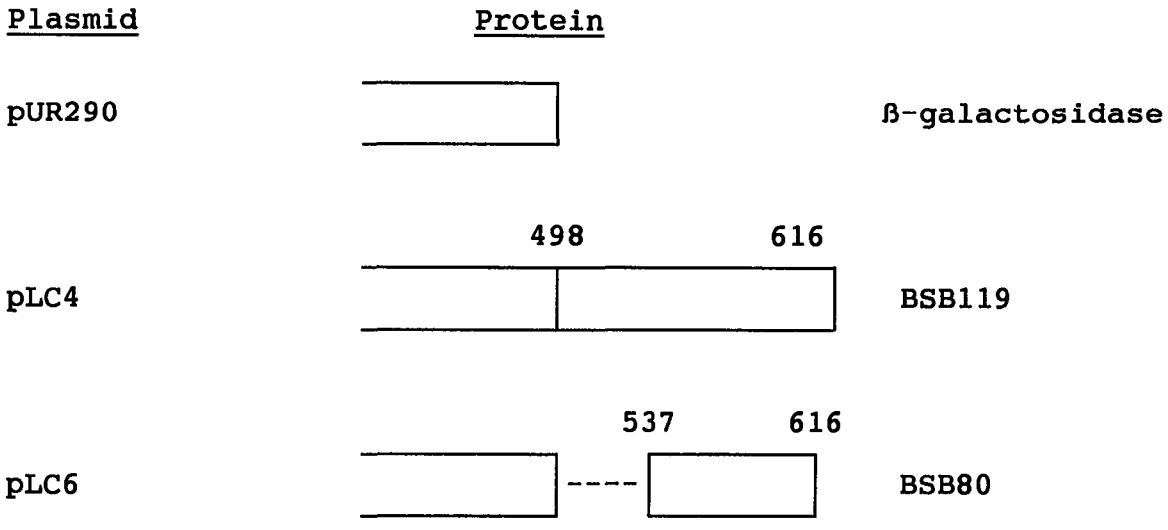


Figure 1. ß-Galactosidase fusion proteins containing glucoamylase starch-binding domain at the C-terminus. Numbers indicate glucoamylase I amino acid residues. ------ indicates deletion

5 l of TB medium (Sambrook et al., 1989) supplemented with 100 µg/ml ampicillin, 250 ml (5% v/v) inoculum was added. The fermenter was aerated and agitated at 200 rpm, and temperature was controlled at 37 °C.

**Protein purification** When the cell culture reached absorbance at 600 nm of 2-4, the cells were harvested by centrifugation at 5,000 x g for 15 minutes. The cells were suspended in buffer A which consisted of 20 mM Tris-HCl, pH 7.5, 1.2 M NaCl, 10 mM 2-mercaptoethanol, 1 mM ethylenediamine tetraacetic acid, 0.1 mM phenylmethanesulfonyl fluoride, and 1 mM $MgCl_2$. In addition to these components, the lysis buffer

for the purification of BSB80 consisted of 0.1% sodium dodecylsulfate and 1 M urea. The cells were broken by using a French press operated at 2000 psi and then centrifuged at 50,000 x g for 60 minutes. The cell-free extract was loaded onto a p-aminobenzyl 1-thio-ß-D-galactopyranoside-agarose column that had been equilibrated with buffer A without phenylmethanesulfonyl fluoride. The bound protein was eluted with 100 mM borax of pH 10 containing 10 mM 2-mercaptoethanol, and 10 mM $MgCl_2$ (Steers et al., 1971), and dialyzed against 10 mM sodium phosphate buffer at pH 7, 10 mM $MgCl_2$, and 10 mM 2-mercaptoethanol, followed by dialysis against 10 mM sodium phosphate buffer at pH 7, and 4°C. After dialysis, the purified protein was concentrated by ultrafiltration and quantified by the method of Bradford (1976) with bovine serum albumin as a standard. The purification procedure was followed by measuring ß-galactosidase activity according to Miller (1972).

To produce a larger amount of BSB119, E. coli cells harboring plasmid pLC4 were also grown in a 19-l Bioengineering-NLF 22 fermenter with a working volume of 15 l. The procedure was similar to the 5-l fermentation except for the dissolved oxygen concentration, harvesting, and homogenization. The dissolved oxygen in the cell culture was kept constant at 80% saturation. Because of the large volume of the cell culture, cells were harvested by using a hollow-fiber filter (Amicon DC10L) and homogenized by using a Manton-

Gulin homogenizer before clarification by centrifugation. The resulting cell-free extract was subjected to the same process mentioned above.

### Starch binding assay

Adsorption of purified fusion proteins and ß-galactosidase to granular starch was assayed by a slightly modified procedure of Dalmia and Nikolov (1991). Granular starch was washed twice with 10 mM sodium phosphate buffer (pH 7.0) containing 100 mM NaCl. Purified fusion proteins (in the washing buffer) with initial concentrations ranging from 0.02 to 11 mg/ml were added to 0.1 g of washed starch in a 1-ml of total volume. The mixtures with initial concentration ranging from 0.02 to 2 mg/ml were shaken for 30 minutes, and from 0.02 to 11 mg/ml were shaken for 60 minutes at 4°C to ensure that equilibration was reached. After equilibration the mixture was centrifuged at 4,000 x g. The protein concentration of the supernatant was assayed (Bradford, 1976), and the amount of adsorbed protein was calculated from the difference between the initial and the final protein concentrations in the supernatant. The adsorption data were fit to the equation given below:

$$Q^* = Q_{max} K_a C^* / (1 + K_a C^*) \qquad (1)$$

where,

$Q^*$ = solid phase concentration of protein at equilibrium

$Q_{max}$ = maximum adsorption capacity of the adsorbent

$K_a$ = association constant

$C^*$ = equilibrium protein concentration in bulk solution

The granular starch adsorption constants were determined by a nonlinear regression using the curve fit routine of Sigma Plot computer program (Jandel Scientific, Corte Madera, CA). The uniformity of binding sites on the starch granules was determined by fitting the granular starch adsorption data using the Scatchard equation (Scatchard, 1951):

$$Q^*/C^* = Q_{max}K_a - K_aQ^* \qquad (2)$$

To determine whether the active site of ß-galactosidase participated in the adsorption to starch, ß-galactosidase was adsorbed to starch in the presence of 0.3 M isopropyl-ß-D-thiogalactoside. The adsorption was performed as before. Isopropyl-ß-D-thiogalactoside was added to a final concentration of 0.3 M, and only one initial ß-galactosidase concentration was used (0.5 mg/ml). A parallel experiment without isopropyl-ß-D-thiogalactoside was performed as a control.

# RESULTS AND DISCUSSION

## Protein purification

Our observation that the yields of BSB80 and BSB119 were lower compared to that of ß-galactosidase prompted us to measure the distribution of ß-galactosidase activity between the cell free extract and the cell debris. We found that a significant amount of ß-galactosidase activity was retained by the cell debris after centrifugation. To improve the yield we investigated the possibility to extract the fusion proteins with mild denaturants (1% sodium dodecylsulfate and 1 M urea). By using 1% sodium dodecylsulfate or 1 M urea 40% and 17% of BSB80 and BSB119 were recovered, respectively. The fact that 1% sodium dodecylsulfate eluted more BSB80 than BSB119 suggested that perhaps BSB119 bound more strongly to the cell debris. We did not investigate stronger denaturants because they might affect the functionality of the fusion proteins. Sodium dodecylsulfate-polyacrylamide gel electrophoresis of the proteins recovered from the cell debris showed the same pattern as that from the cell free extract. As a standard procedure for the purification of BSB80 we used a combination of 0.1% sodium dodecylsulfate and 1 M urea in the lysis buffer, because 0.1% sodium dodecylsulfate did not precipitate at 4°C, and it is milder than 1% sodium dodecylsulfate. We estimated that approximately 8% of ß-galactosidase activity remained with the cell debris. For the purification of

BSB119, these denaturants were not included in the lysis buffer because only 17% could be extracted.

Table 1 summarizes the yield and specific activity of ß-galactosidase fusion proteins and ß-galactosidase produced in a 7.5-l fermenter. The yield of both BSB80 and BSB119 were approximately 2.5 times lower than that of ß-galactosidase. The lower yield observed in the case of BSB80 and BSB119 was probably due to proteolytic degradation. The specific activities of the two ß-galactosidase fusion proteins were between 30% and 50% lower than that of the ß-galactosidase. The lower specific activity of the fusion proteins compared to that of the ß-galactosidase might indicate that the fusion proteins suffered from proteolysis during their production in the E. coli cytoplasm. It could also indicate some conformational changes in the ß-galactosidase molecule when the starch-binding domain was fused. The specific activity of BSB119 was about 30% higher than that of BSB80 suggesting that the 80 amino acid long starch-binding fragment was more susceptible to proteolysis than the 119 amino acid long starch-binding fragment.

To improve the production of BSB119, E. coli cells harboring pLC4 were grown in a 19-l Bioengineering-NLF 22 fermenter with a working volume of 15 l. The yield of the purified BSB119 was higher (7.1 mg/l medium) in comparison to the 5-l fermentation. This higher yield of BSB119 might be the result of healthier E. coli cells grown under controlled

Table 1. Yield and specific activity of ß-galactosidase fusion proteins and ß-galactosidase purified on p-aminobenzyl 1-thio-ß-D-galactopyranoside-agarose.

| Protein | Yield (%) | Recovered (mg) | Specific activity (U/nmole) |
|---|---|---|---|
| ß-gal[b] | 67 ± 18 | 8.8 ± 4.3 | 119 ± 44 |
| BSB80[a] | 67 ± 5 | 3.4 ± 0.6 | 57 ± 13 |
| BSB119[a] | 75 ± 15 | 3.2 ± 0.8 | 90 ± 43 |

ß-gal: ß-galactosidase

[a]Average of three purifications

[b]Average of five purifications

dissolved oxygen concentration (kept constant at 80%) .

Sodium dodecylsulfate-polyacrylamide gel electrophoresis analysis of the purified BSB80 and BSB119 showed two protein bands as observed before by Chen et al. (1991b), which hybridized with antibody against ß-galactosidase. The high molecular weight protein band corresponded to the full-size monomer of ß-galactosidase fusion protein (125-130 kDa). The low molecular weight protein band corresponded to the ß-galactosidase monomer (116 kDa). The observed ß-galactosidase was the result of proteolysis of ß-galactosidase fusion protein tetramer possessing 1, 2, 3, or 4 "tail(s)" at the junction of ß-galactosidase and starch-binding domain. The ratio of the two bands was estimated by image analysis to be one to one (Chen et al., 1991b). Since no method has been

developed to separate the full-size ß-galactosidase fusion protein from the partially degraded fusion proteins containing 1, 2, 3, or 4 starch-binding fragments, the granular starch adsorption analysis of BSB80 and BSB119 was performed using their mixture.

## Granular starch adsorption

Adsorption isotherms of ß-galactosidase, BSB80, and BSB119 resembled the Langmuirian-type adsorption isotherm (Figure 2), which is defined by Equation 1. The adsorption data were also analyzed using the Scatchard equation (Equation 2) in order to observe any deviation from the Langmuirian behavior. Linearity of the Scatchard plot tests the identity and independence of binding sites (Edsall and Gutfreund, 1983). The Scatchard plots of granular starch adsorption data of ß-galactosidase, BSB80, and BSB119 were linear (Figure 3) suggesting the absence of heterogeneous binding sites on the starch surface.

Both BSB80 and ß-galactosidase had similar adsorption affinities (measured by $K_a$ values) and maximum binding capacities (measured by $Q_{max}$ values) (Table 2). This data indicate that the 80 amino acid at the C-terminus of glucoamylase I (amino acids 537-616, glucoamylase I amino acid numbering) had no effect on the adsorption of BSB80 to granular starch. The affinity of the adsorption of BSB119 (2.4 ml/g) was almost three times higher than that of ß-

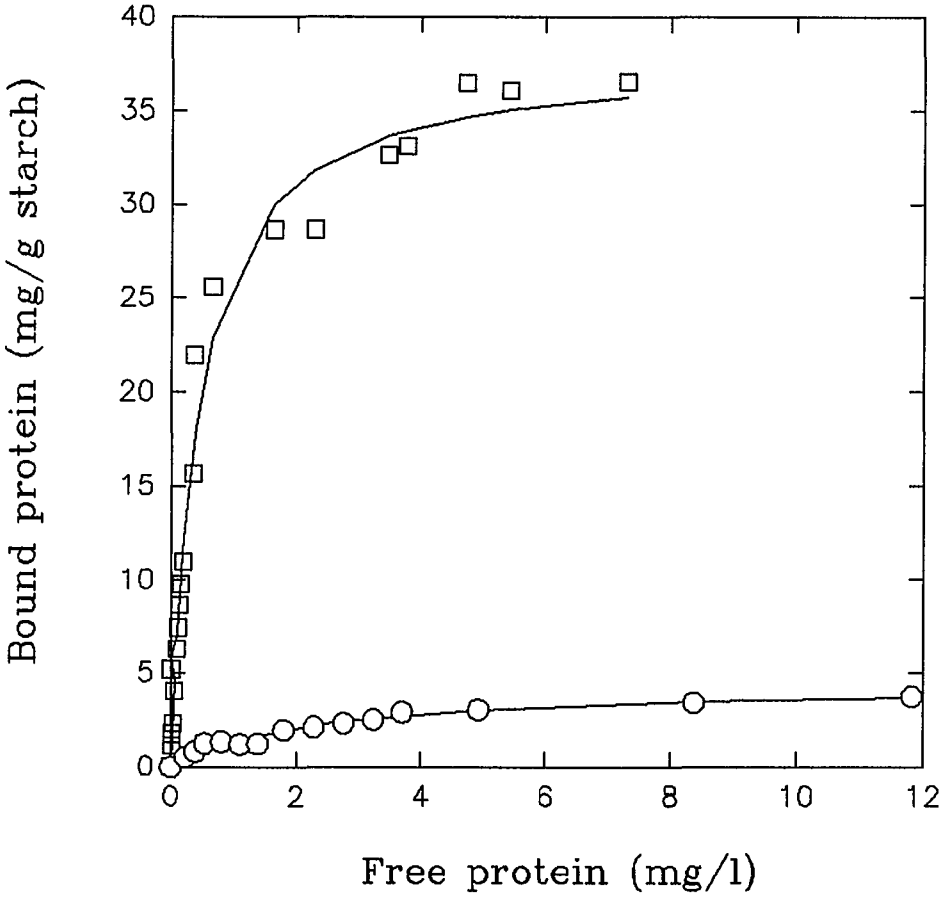


Figure 2. Adsorption isotherm of purified fusion protein BSB119 (□) compared to control ß-galactosidase (o). Solid lines indicate Langmuir model fits

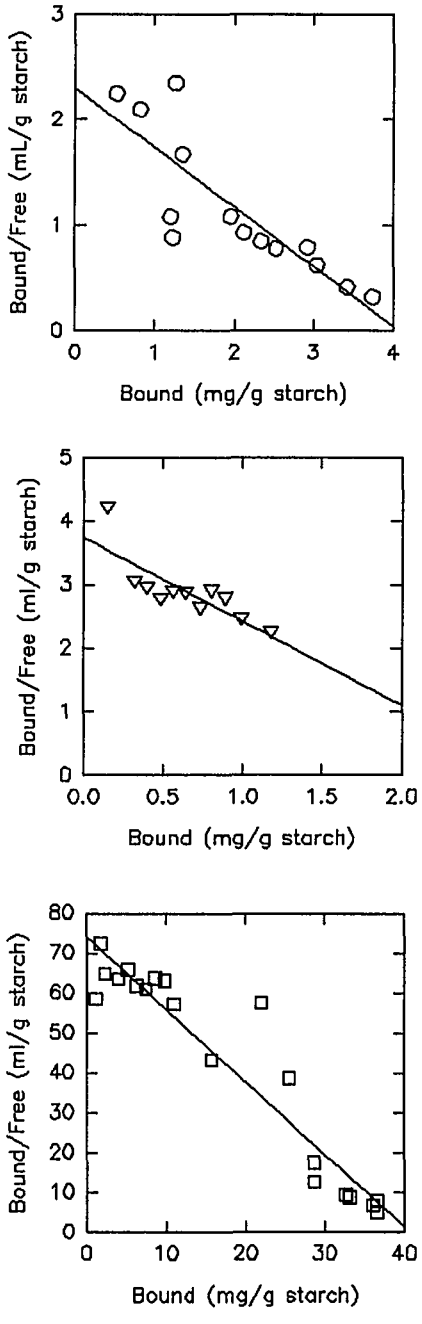


Figure 3. Scatchard plot of granular starch adsorption of ß-galactosidase (o), BSB80 (▽), and BSB119 (□)

galactosidase (0.9 ml/g), and its maximum binding capacity was 11 times higher (36, 3.8, and 4 mg/g starch, respectively forBSB119, BSB80, and ß-galactosidase) (Table 2). Due to the proteolysis of the fusion proteins, the calculated granular starch adsorption parameters are probably underestimated. Nevertheless, as much as 6 times more BSB119 can adsorb to starch granules than glucoamylase II can (5.6 mg/g starch). The higher $Q_{max}$ value of BSB119 was not due to the adsorption of ß-galactosidase active site to the starch molecule. The adsorption experiments in the presence of up to 0.3 M isopropyl-ß-D-thiogalactoside indicated that ß-galactosidase (0.5 mg/ml) adsorbed to granular starch at the same level (1.2 mg/g starch) as before (Figure 2).

Table 2. Granular starch adsorption constants of ß-galactosidase and ß-galactosidase fusion proteins determined using a nonlinear regression

| Proteins | $K_a$ (ml/g) | $Q_{max}$ (mg/g) |
|---|---|---|
| ß-galactosidase | 1.4 ± 0.3 | 3.1 ± 0.4 |
| | 0.4 ± 0.1 | 4.4 ± 0.3 |
| BSB80 | 2.2 ± 0.3 | 2.9 ± 0.2 |
| | 0.9 ± 0.2 | 5.1 ± 0.6 |
| BSB119 | 2.5 ± 0.6 | 33 ± 3.7 |
| | 2.3 ± 0.2 | 38 ± 0.9 |

The difference between granular starch adsorption of glucoamylase I and a ß-galactosidase fusion protein containing starch-binding domain is not known, but could be the result of: (i) Conformational differences of the starch-binding domain in the native glucoamylase I and in the ß-galactosidase fusion protein. Moreover, the tripeptide (Gly-Arg-Pro) which links the C-terminus of ß-galactosidase with the N-terminus of the starch-binding domain may have affected the folding pattern of the starch-binding domain. (ii) The contribution of glucoamylase I catalytic domain to granular starch binding (Dalmia and Nikolov, 1991). (iii) Difference in size between glucoamylase I (80 kDa) and the tetramer ß-galactosidase fusion proteins (4 x 125-130 kDa).

Our results suggest that the starch-binding domain is functional in BSB119, and that residues 498-536 of glucoamylase I are important for the functioning of starch-binding domain.

# CONCLUSIONS

Addition of sodium dodecylsulfate and urea into the lysis buffer increased the yield of BSB80. Controlling dissolved oxygen level at 80% in the fermenter during fermentation seemed to increase the yield of BSB119. BSB119 had a stronger adsorptivity and higher maximum adsorption capacity on granular starch compared to BSB80, implying that amino acid 498-616 of glucoamylase I are crucial in granular starch adsorption. The functionality of BSB119 in granular starch adsorption suggesting that the starch-binding domain can be used for purification or immobilization of protein of interest.

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PAPER 2: PRODUCTION OF A FUNCTIONAL STARCH-BINDING DOMAIN OF *ASPERGILLUS* GLUCOAMYLASE I IN *ESCHERICHIA COLI*

## ABSTRACT

We have previously shown that the C-terminal starch-binding domain of Aspergillus glucoamylase I is functional when fused to the C-terminal of E. coli ß-galactosidase and produced intracellularly in E. coli (Chen et al., 1991a,b). We have now fused three different lengths of starch-binding fragments encoding glucoamylase I residues 511-616, 495-616, and 481-616 to the C-terminal of the E. coli maltose-binding protein in vectors for both periplasmic and cytoplasmic expression systems. The produced fusion proteins were purified by affinity chromatography. Factor $X_a$ digestion of the fusion proteins resulted in the release of functional starch-binding domain fragments that were purified from maltose-binding protein on the basis of differential affinity for cross-linked amylose. Clones were also constructed for the independent production of the three different starch-binding domain fragments ($SBD_{515-616}$, $SBD_{497-616}$, and $SBD_{483-616}$) in E. coli using the intracellular expression vector pKK233-2. The three starch-binding domain fragments were produced at very low levels. $SBD_{483-616}$ was produced at a higher level than either $SBD_{515-616}$ or $SBD_{483-616}$, which were almost undetectable by immunoblotting. The independent production of starch-binding domain in E. coli supports our conclusion that the starch-binding domain of Aspergillus glucoamylase I is in fact an independent domain.

# INTRODUCTION

Glucoamylase I from Aspergillus sp. has two discrete functional domains: a catalytic domain (residues 1-440) and a C-terminal starch-binding domain (residues 513-616) (Svensson et al., 1983). The two domains are linked by a Ser/Thr-rich highly O-glycosylated region (residues 441-512) (Gunnarson et al., 1984), which likely exist in a predominantly extended conformation and may function to maintain the two domains apart at a fixed distance (Aleshin et al., 1992; Williamson et al., 1992a). A similar domain organization has also been found in some cellulases (Ong et al., 1989).

No three-dimensional structure has been defined yet for the starch-binding domain, and the exact boundaries of this domain are unknown. A natural proteolytic product of glucoamylase I, designated as glucoamylase II, is cleaved at residue 513 and lacks the C-terminal 104 residues of glucoamylase I. Because glucoamylase II lacks the ability to adsorb strongly to granular starch, the starch-binding domain may be included in or overlapping the C-terminal 104 residues of glucoamylase I. Chen (1992) have recently shown that deletions within the C-terminal 104 residues of glucoamylase I lead to reduced starch binding, and thus indicated that this entire region is necessary for starch-binding function.

The potential role of the O-glycosylated region in starch binding has also been studied. Hayashida et al. (1989a,b)

have isolated a 45 amino acid glucoamylase I fragment (residues 470-514), which contains a large portion of the O-glycosylated linker, and have identified the fragment as a "raw-starch-affinity-site". This identification implies the involvement of carbohydrate moieties in adsorption to native starch. Williamson et al. (1992a) assessed the role of O-glycosylation in glucoamylase binding to starch in three proteolytically produced fragments of glucoamylase I corresponding to residues 471-616, 499-616, and 509-616, containing different proportions of O-glycosylated linker. Because the three fragments exhibited similar affinity for starch, Williamson et al. (1992a) have concluded that O-glycosylated residues did not contribute significantly to starch adsorption.

To define the functional size of the starch-binding domain and to test its effectiveness when produced in E. coli, we previously constructed ß-galactosidase fusion proteins containing 103, 119, and 133 residues from the C-terminus of glucoamylase I (Chen et al., 1991 a,b). Although all the fusion proteins showed significant increases in starch-binding activity compared with a ß-galactosidase control, the 119 residue fusion exhibited the highest activity. There was a high level (50-70%) of proteolysis in all the constructs, which resulted in loss of the starch-binding domain from the fusion proteins. The 133 residue fusion showed a twofold higher proteolytic loss of starch-binding domain than did the

119 residue fusion, and this twofold loss of starch-binding domain corresponded to a twofold decrease of granular starch adsorption. The 103 residue fusion, however, was not less stable than the 119 residue fusion, yet still showed a greater than twofold lower level of starch adsorption. These results suggested that the extra amino acids in the 119 residue fusion were necessary as either a bridge between ß-galactosidase and the starch-binding domain or were a functional part of the starch-binding domain.

To clarify the questions about the functional size, potential independence, and proteolytic stability of the starch-binding domain raised by the ß-galactosidase fusion work, we have now constructed a new set of starch-binding domain fusion proteins with the E. coli maltose-binding protein, which contains a factor $X_a$ proteolytic-specific site at the fusion junction for in vitro cleavage of the starch-binding domain. These constructs have made possible the isolation of starch-binding fragments of different sizes as functionally independent polypeptides. In this work, the starch-binding domain was fused to maltose-binding protein because the produced protein can be targeted to periplasm as well as cytoplasm, and, most importantly, maltose-binding protein does not have any cysteine residue that can interfere with the formation of disulfide bonds in starch-binding domain (Guan et al., 1988). Two different expression systems (cytoplasmic and periplasmic) were chosen in this work to

compare their effects on the stability and yield in E. coli. The potential of the different starch-binding domain fragments to function as independent polypeptides has also been tested by direct production in E. coli.

# MATERIALS AND METHODS

**Construction of malE-Starch binding domain gene fusions.**

Plasmids pMAL-SBD136p, pMAL-SBD122p and pMAL-SBD106p (encoding MBP-SBD136, MBP-SBD122 and MBP-SBD106 fusion proteins, respectively, in the periplasm) were constructed by cloning respectively 583 bp BamHI-HindIII, 541 bp NheI-HindIII, and 488 bp BstXI-HindIII fragments of plasmid pGAC9 (gene coding for Aspergillus glucoamylase I) at the 3' end of the E. coli malE gene (coding for maltose-binding protein) of plasmid pMAL-p expression vector (New England Biolabs, Beverly,MA). Plasmids pMAL-SBD136c, pMAL-SBD122c, and pMAL-SBD106c (encoding MBP-SBD136, MBP-SBD122, and MBP-SBD106 fusion proteins in the cytoplasm) were constructed using the same pGAC9 fragments, except that the expression vector used was pMAL-c (New England Biolabs, Beverly, MA). All the cloning procedures were done using standard gene manipulation techniques (Sambrook et al., 1989).

**Preparation of vector** All restriction enzyme digestions were performed using conditions recommended by the manufacturer. One μg of plasmid pMAL-p and one μg of pMAL-c were digested with StuI (New England Biolabs, MA) and HindIII (Promega, Madison,WI). The completion of digestion was checked by running 4 μl of reaction mixture on a 0.8% agarose gel in 1X TAE buffer at a constant voltage of 70 volts for 45

minutes. The 1X TAE buffer was made from 20X TAE stock pH 8.2, containing 96.8 g of Tris base, 54.4 g of NaOAc, and 7.44 g of ethylene diamine tetraacetic acid per liter of buffer. Electrophoresis was performed using a submarine mini gel (6 cm x 8 cm x 0.3 cm). After electrophoresis the gel was stained for 10-15 minutes in 5 μg/ml ethidium bromide solution, and then washed several times with deionized water. The DNA bands were visualized using ultraviolet light. The presence of a 6.22 kb DNA band confirmed that the restriction enzymes digestion was complete. The rest of the reaction mixture was electrophoresed on a 0.8% preparative agarose gel. The 6.22 kb DNA band was excised from the gel and the DNA was extracted using a Gene Clean II kit (Bio 101, La Jolla, CA).

**Preparation of insert** All DNA fragments of pGAC9 were derived from plasmid pAK1, which was constructed by cloning a 583 bp BamHI-HindIII fragment of pRE1 (Evans, 1990) into the BamHI/HindIII site of plasmid $pBS^+$ (Stratagene, La Jolla, CA). Ten μg of $pBS^+$ and pRE1 were digested with BamHI (Promega, Madison, WI). The completion of digestion was checked by electrophoresis as mentioned above. The BamHI digested $pBS^+$ and pRE1 were then digested with HindIII. The double digested $pBS^+$ and pRE1 were electrophoresed on a 1% preparative agarose gel and the 3.2 kb and 583 kb DNA fragments, respectively from BamHI/HindIII digested $pBS^+$ and pRE1, were excised and extracted from the agarose gel as mentioned above. Fifty ng

of the BamHI/HindIII fragment of pRE1 was ligated with the 3.2 kb BamHI/HindIII fragment of plasmid $pBS^+$ using T4 DNA ligase (Life Technologies, Inc., Gaithersburg, MD) at 16°C overnight. The ligation product was transformed into TG-1 competent cells (SupE hsdΔ5 thiΔ (lac-proAB) F'[traD36 $proAB^+$ $lacI^q$ lacZΔM15]) (Gibson, 1984) which were prepared by the $CaCl_2$ precipitation method. Ten μl of ligation mixture was transformed into 200 μl of competent E. coli TG-1 cells, and was kept on ice for 30 minutes. The mixture was heat-shocked at 42°C for 2 minutes, and 0.8 ml of LB medium was added. The LB medium was prepared by autoclaving 10 g of bacto tryptone (Difco Laboratories, Detroit, MI) 5 of g bacto yeast extract (Difco Laboratories, Detroit, MI), and 10 g of NaCl per liter of medium. The heat-shocked reaction mixture was incubated at 37°C for one hour to allow the ampicillin resistance gene to be expressed. Then 200 μl of the reaction mixture was spread onto a 1.5% LB agar plate containing 50 μg ampicillin/ml. The plate was incubated at 37°C overnight. Several colonies were transferred into individual tubes with 2 ml of 2X YT medium containing 100 μg ampicillin/ml. The 2X YT medium contained 16 g of bacto tryptone, 10 g of yeast extract, and 5 g of NaCl per liter. The cultures were shaken overnight at 37°C. Plasmid DNA was isolated from the culture using the alkaline DNA mini preparation method (Sambrook et al., 1989). BamHI/HindIII restriction digestion was performed to check for the presence of a 583 bp BamHI-HindIII fragment.

The clone that carried plasmid pAK1 was grown in 500 ml of 2X YT medium containing 100 μg ampicillin/ml at 37°C overnight. From the 500 ml culture, 25 ml was withdrawn and sterile glycerol was added to a final concentration of 15% and then stored frozen at -70°C to serve as a cell stock. Stocks of all other clones constructed later were made and stored in the same way. The rest of the culture was used to isolate the mutant DNA by using the alkaline method (Sambrook et al., 1989). To confirm that no mutation occurred, plasmid pAK1 was sequenced using the universal M13 primer by the dideoxy method. All DNA sequencing was performed by Iowa State University Nucleic Acid Facility (Ames, Iowa).

Fifty μg of pAK1 was digested with BamHI and HindIII. The 583 bp BamHI-HindIII fragment from BamHI/HindIII digested pAK1 was isolated from a 1% agarose gel as mentioned above. The same amount of pAK1 was also digested with NheI/HindIII and BstXI/HindIII for the isolation of 541 bp NheI/HindIII and 488 bp BstXI/HindIII fragments.

**Construction of plasmids pMAL-SBD** Plasmid pMal-SBD122p was constructed by ligating the 541 bp NheI-HindIII fragment to StuI/HindIII digested pMAL-p and adaptor 5 (5'-GCGACTG-3'/3'-CGCTGACCATC-5'). Adaptor 5 was made by annealing 5'-GCGACTG-3' with 3'-CGGTGACCATC-5' in the presence of annealing buffer. The oligonucleotides were heated at 60°C for 2 minutes, followed by gradual cooling of reaction mixture in an aluminum

block to room temperature.

The ligation mixture was transformed into competent TB-1 E. coli cells ($F^-$ araΔ(lac$^-$proAB) rpsL 80 lacZΔM15 hsdR ($rK^-$, $mK^+$)) (Focus, 1984) and plated on an LB agar plate containing 100 μg ampicillin/ml and incubated at 37°C overnight. Several colonies were transferred into 2 ml of 2X YT medium containing 100 μg ampicillin/ml, and grown overnight at 37°C. Plasmid DNA was isolated from these cultures, and the presence of the correct insert in the correct orientation was determined by digesting the DNA with NcoI (Promega, Madison, WI). The presence of the 541 bp fragment showed that the insert was the correct size, and was in the correct orientation. The resulting plasmid pMAL-SBD122p was sequenced using pMAL primer (New England Biolabs, Beverly,MA).

Plasmids pMAL-SBD136p and pMAL-SBD106p were constructed using the same procedure. For plasmid pMAL-SBD136p, the 583 base-pair BamHI/HindIII fragment of pAK1 and Adaptor 4 (5'-CCCACTG-3'/3'-GGGTGACCTAG-5') were used. For plasmid pMAL-SBD106p, the 488 base-pair BstXI/HindIII fragment of pAK1 and Adaptor 6 (5'-ACTCCCACCGCC-3'/3'-TGAGGGTG-5') were used.

Plasmid pMAL-SBD136c, pMAL-SBD122c,and pMAL-SBD106c (coding for MBP-SBD136, MBP-SBD122, and MBP-SBD106 fusion proteins, respectively, expressed in the cytoplasm) were constructed with the same procedure, except that pMAL-c was used as the expression vector instead of pMAL-p.

**Checking for the production of the fusion proteins**

Ten ml of cultures containing E. coli cells transformed with each plasmid were grown in 2X YT medium supplemented with 100 μg ampicillin/ml at 37°C. When $OD_{600}$ reached 0.3-0.4, isopropyl ß-D-thiogalactopyranoside was added to a final concentration of 0.3 mM, and the culture was incubated for two more hours. A one ml sample was taken before adding isopropyl ß-D-thiogalactopyranoside, and a 0.5 ml sample was taken two hours after the addition of isopropyl ß-D-thiogalactopyranoside. These samples were microcentrifuged for 2 minutes, the supernatant was discarded, and the cell pellets were suspended in 50 μl and 100 μl of sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample treatment buffer. The samples were then boiled for 5 minutes, and 10 μl of the treated samples were electrophoresed on a 10% SDS-polyacrylamide gel, followed by immunoblotting. The membrane was hybridized with polyclonal antibody against maltose-binding protein (New England Biolabs, Beverly, MA) as well as polyclonal antibody against glucoamylase I (made by the Iowa State University Hybridoma Service, Ames, IA).

**Production of MBP-SBD fusion proteins**

**Inoculation and induction** Ten ml of an overnight culture of cells containing the fusion plasmid was transferred into a flask containing 1 liter of rich medium (10 g of bacto tryptone, 5 g of bacto yeast extract, 5 g of NaCl and 2 g of

glucose) supplemented with 100 μg ampicillin/ml. The cell culture was incubated at 37°C, and shaken at 200 rpm until $OD_{600}$ reached 0.4-0.6. Expression of the malE-SBD gene fusion was induced by adding 0.3 mM isopropyl ß-D-thiogalactopyranoside.

**Harvesting, cold osmotic shock and lysis of the cells** Two hours after induction with isopropyl ß-D-thiogalactopyranoside the cells were harvested by centrifugation at 5,000 x g for 10 minutes.

For periplasmic fusion proteins, the cell pellet was osmotically shocked to release the periplasmic proteins according to the method of Neu and Heppel (1965). The cell pellet was suspended in 80 ml of 30 mM Tris-HCl, 20% sucrose, pH 8.0 per gram of cell pellet. Ethylenediamine tetraacetic acid was added to a final concentration of 1 mM and incubated with stirring at room temperature for 10 minutes. The solution was centrifuged at 8,000 x g for 20 minutes at 4°C. The supernatant was discarded and the cell pellet was osmotically shocked by resuspension in the same volume of ice-cold 5 mM $MgSO_4$ followed by incubation in an ice bath with stirring for 10 minutes. The solution was centrifuged at 8,000 x g for 20 minutes at 4°C. The resulting supernatant is the cold osmotic shock fluid containing the periplasmic proteins.

For cytoplasmic fusion proteins, the cell pellet was suspended in 10 ml of lysis buffer per gram of cell pellet.

The lysis buffer consisted of 10 mM sodium phosphate, 30 mM NaCl, 0.25% Tween-20, 10 mM 2-mercaptoethanol and 10 mM ethylenediamine tetraacetic acid. The cells were broken using an SLM Aminco French pressure cell (SLM Instruments, Inc.) operated at 2,000 psi. Phenylmethyl-sulfonyl fluoride was added to a final concentration of 0.1 mM before and after cell breakage. The lysed cells were centrifuged at 9,000 x g for 30 minutes. The resulting supernatant is the cell-free extract containing total cellular proteins. Ammonium sulfate was added slowly to the cell free extract to give a concentration of 60%. The solution was stirred at 4°C temperature for 1-2 hours, and centrifuged at 9,000 x g for 30 minutes. The supernatant was discarded, and the pellet was suspended with high-salt-column buffer (10 mM sodium phosphate, 0.5 M NaCl, 1 mM $NaN_3$, and 1 mM ethylenediamine tetraacetic acid, pH 7.2) and dialyzed extensively against the same buffer to remove the ammonium sulfate.

**Affinity chromatography on cross-linked amylose** The cold osmotic shock fluid was applied to a cross-linked amylose column preequilibrated with low-salt-column buffer consisting of 10 mM sodium phosphate, 30 mM NaCl, 1 mM $NaN_3$, and 1 mM ethylenediamine tetraacetic acid, pH 7.2. After loading the sample, the column was washed with 3-5 volumes of low salt column buffer. The maltose-binding protein was eluted with 10 mM maltose in the same buffer, and after elution of maltose-

binding protein the fusion protein was eluted with 10 mM ß-cyclodextrin. To purify MBP-SBD fusion proteins from the E. coli cytoplasm, dialyzed ammonium sulfate precipitated proteins (0-60% cut-off) were loaded on to the cross-linked amylose column. The buffer used was high salt column buffer.

The purity and identity of the eluted fusion protein was checked by sodium dodecylsulfate-polyacrylamide gel electrophoresis and immunoblotting.

**Efficiency of targeting the fusion proteins to the periplasmic space** A 500 ml culture was grown as mentioned earlier. Cells were harvested and osmotically shocked to recover the periplasmic proteins. After removal of periplasmic proteins, the cell pellet was broken using a French pressure cell. The resultant cell free extract was subjected to a cross-linked amylose column to purify the MBP-SBD fusion protein. MBP-SBD fusion protein was also purified from the cold osmotic shock fluid containing periplasmic proteins. The purification procedures were the same as mentioned earlier. The yield of MBP-SBD fusion protein purified from the cold osmotic shock fluid (p) was compared to that from cell free extract (c). The percentage of produced MBP-SBD fusion proteins exported to the periplasmic space was calculated as [p/(c+p)] x 100%.

## Preparation of starch-binding domain

In order to obtain the starch-binding domain, the purified fusion protein was digested with factor $X_a$ (New England Biolabs, Beverly, MA). The purified fusion protein was first concentrated by ultrafiltration using a YM10 membrane (Amicon, Beverly, MA), and then dialyzed extensively against factor Xa buffer which consisted of 20 mM Tris-HCl pH 8.0, 100 mM NaCl, 2 mM $CaCl_2$ and 1 mM $NaN_3$. The concentrated and dialyzed fusion protein was digested with 1 unit factor $X_a$ per 50 $\mu$g fusion protein at room temperature for 10-12 hours. The digested fusion protein was then applied to a cross-linked amylose column preequilibrated with low salt column buffer. The maltose-binding protein and starch-binding domain that resulted from factor $X_a$ digestion were separated by performing step wise elution as described before. The starch-binding domain was eluted with 0.5 M maltose after eluting maltose-binding protein with 10 mM maltose.

## Analysis of the purified MBP-SBD fusion proteins and starch-binding fragments

The purity and identity of the purified fusion proteins and starch-binding domain fragments were determined by sodium dodecylsulfate-polyacrylamide gel electrophoresis and immunoblotting.

**Protein determination** Protein concentration was determined by the Bradford procedure (Bradford, 1986), as follows. Bovine serum albumin (Bio-Rad, Richmond, CA) was diluted with $H_2O$ to make 800 $\mu$l standards containing 0 (blank), 3, 7, 10, 13, and 16 $\mu$g bovine serum albumin. Protein samples were diluted with $H_2O$ to be within the range of the standards, in a total volume of 800 $\mu$l. Into each of the standard protein samples, 200 $\mu$l of concentrated Bio-Rad Dye (Bio-Rad) was added and vortexed well. The color reaction was allowed to proceed at room temperature for 20-30 minutes. The absorbance of all samples was determined at 595 nm using Beckman DU50 spectrophotometer. The r value of the standard curve was greater than 0.990.

**Sodium dodecylsulfate-polyacrylamide gel electrophoresis** Sodium dodecylsulfate-polyacrylamide gel electrophoresis was performed according to the method of Laemmli (Laemmli, 1970). The gel consisted of a separating gel of 10% or 15% polyacrylamide at pH 8.8, and a stacking gel of 4% polyacrylamide at pH 6.8. Protein samples were diluted 1:1 with sample treatment buffer, which consisted of 0.125 M Tris-HCl pH 6.8, 4% sodium dodecylsulfate, 20% glycerol, 10% 2-mercaptoethanol, and 0.02% bromphenol blue. The treated samples were boiled for 2-5 minutes. Protein samples used were 0.2-1 $\mu$g in a maximum loading volume of 20 $\mu$l. The gel was run at a constant current of 10 mAmp per gel until the

tracking dye front reached the bottom of the gel (90-120 minutes). After electrophoresis, the proteins were fixed by immersing the gel in 50% methanol for 1 hour. The proteins were visualized by silver staining (Wray et al., 1981).

**Immunoblotting** Following sodium dodecylsulfate-polyacrylamide gel electrophoresis, gels were soaked for 10 minutes in 1X Bjerrum and Schafer-Nielsen transfer buffer (Bjerrum and Schafer-Nielsen, 1986). The transfer buffer was made as a 10X stock solution and consisted of 58.2 g of TRIS base, 29.3 g of glycine ,and 0.375 g of sodium dodecylsulfate per liter. The 1X transfer buffer was made by diluting 100 ml of the 10X buffer with 200 ml methanol and 700 ml $H_2O$ and stored at 4°C. The gel was put on top of a piece of 0.2 $\mu$ nitrocellulose membrane (BioRad, Richmond, CA) presoaked in 1X transfer buffer. The acrylamide gel and the nitrocellulose membrane were sandwiched with filter paper and put on a semidry-electroblotting apparatus (Bio-Rad, Richmond,CA) and the blotting was performed at constant voltage of 10 volts (the current limit was set at 1 amp) at room temperature for 20-30 minutes. After electroblotting, the nitrocellulose sheet was incubated in 10% milk diluent solution (Kirkegaard and Perry Laboratories, Inc. Gaithersburg, MD) in PBS-T at room temperature to block the membrane. The PBS-T solution consisted of 6.7 gram NaCl, 2.3 g $Na_2HPO_4$, 5.4 g $KH_2PO_4$ and 0.5 ml of Tween-20 per liter. A polyclonal antibody raised

against Aspergillus niger glucoamylase I at concentration of 10 μg/ml in blocking solution was used to incubate the membrane overnight at room temperature. A duplicate membrane was incubated with 1/10,000 dilution of anti-MBP (New England Biolabs, Beverly, MA). The next day, the excess antibody was removed by washing the membrane 4 times with PBS-T; each washing was 5 minutes. Then the membrane was incubated for 1-4 hours (until pinkish protein bands were visible) with Protein-A gold solution (Bio-Rad, Richmond, CA). If the protein bands were not visible after a prolonged incubation (more than 12 hours) with Protein-A gold solution, the protein bands were visualized using the gold enhancement kit (BioRad, Richmond, CA). The membrane was then rinsed with $H_2O$ and air dried.

**Cloning and expression of the starch-binding domain in E. coli**

**Cloning strategy** All DNA fragments encoding different lengths of starch-binding fragments were derived from plasmid pAK1. Plasmid $pSBD_{483-616}$ was constructed by cloning a 575 base-pair NcoI fragment of pAK2 (constructed by inserting adaptor 5'-AATTCGGCCATGG-3'/3'-GCCGGTACCCTAG-5' to the BamHI/EcoRI site of the plasmid pAK1) into plasmid pKK233-2 (Pharmacia LKB Biotechnology, Pistacaway, NJ) at the NcoI site. Plasmid $pSBD_{497-616}$ was constructed by cloning a 532 base-pair NcoI fragment of pAK4 (constructed by inserting adaptor 5'-AATTCGGCCATGG-3'/3'-GCCGGTACCGATC-5'at the NheI/EcorI site of

pAK1) into the NcoI site of pKK233-2. Plasmid $pSBD_{515-616}$ was constructed by cloning the 479 base-pair NcoI fragment of pAK6 (constructed by digesting plasmid pAK1 with BstX1, blunt-ending the protruding end, EcoRI digestion, and inserting adaptor 5'-AATTCGCCATG-3'/3'-GCGGTAC-5') into the NcoI site of pKK233-2. All oligonucleotides were synthesized by the Iowa State University Nucleic Acid Facility. Plasmids $pSBD_{515-616}$, $pSBD_{497-616}$, and $pSBD_{483-616}$ were transformed into E. coli JM105 (Yanisch-Perron et al., 1985) for the production of $SBD_{515-616}$, $SBD_{497-616}$, and $SBD_{483-616}$.

**Production of the starch-binding domain** Cells bearing plasmids pKK233-2 (negative control), $pSBD_{515-616}$, $pSBD_{497-616}$, and $pSBD_{483-616}$ were grown at 37°C in 250 ml TB medium (Sambrook et al., 1989) supplemented with 100 μg/ml ampicilin to an $OD_{600}$ of 0.4-0.6 and induced by addition of isopropyl-ß-D-thiogalactopyranoside to a final concentration of 1 mM. The cultures were harvested 6 hours after induction. A French pressure cell was used to disrupt the cells in 15 ml of 10 mM sodium phosphate pH 6.5, 1 mM benzamidine, 5 mM dithiothreitol, 5 mM ethylenediamine tetraacetic acid, 0.02% Triton X-100, and 1 mM phenylmethyl-sulfonyl fluoride. The presence of the starch-binding fragments in the cell-free extract was determined by immunoblotting.

# RESULTS AND DISCUSSION

## Cloning of starch-binding domain into pMAL-p and pMAL-c expression vectors

The six plasmids (pMAL-SBD106c, pMAL-SBD122c, pMAL-SBD136c, pMAL-SBD106p, pMAL-SBD122p, and pMAL-SBD136p) that code for fusion proteins consisting of the maltose-binding protein and 106, 122, and 136 residues from the C-terminus of A. awamori glucoamylase I are outlined in Figure 1. Plasmids pMAL-SBD106c, pMAL-SBD122c, and pMAL-SBD136c were used to produce cytoplasmic MBP-SBD fusion proteins , whereas plasmids pMAL-SBD106p, pMAL-SBD122p, and pMAL-SBD136p were used to produce periplasmic MBP-SBD fusion proteins in E. coli. MBP-SBD106, MBP-SBD122, and MBP-SBD136 contain amino acid 511-616, 495-616, and 481-616 of glucoamylase I, respectively. These fusion proteins include amino acid 513-616 of glucoamylase I, which are important for the binding of GA to granular starch (Svensson et al., 1989). The different lengths of starch-binding fragment (136, 122, and 106 amino acid long) were fused to the C terminus of maltose-binding protein separated by a factor $X_a$ cleavage site at the junction of the two polypeptides (Figure 1). The cloning strategy was designed in such a way that after digestion of the fusion proteins with factor $X_a$ no vector derived sequence would be attached to the starch-binding fragment. Figure 2 depicts the main steps that were used to construct pMAL-SBD122p. All

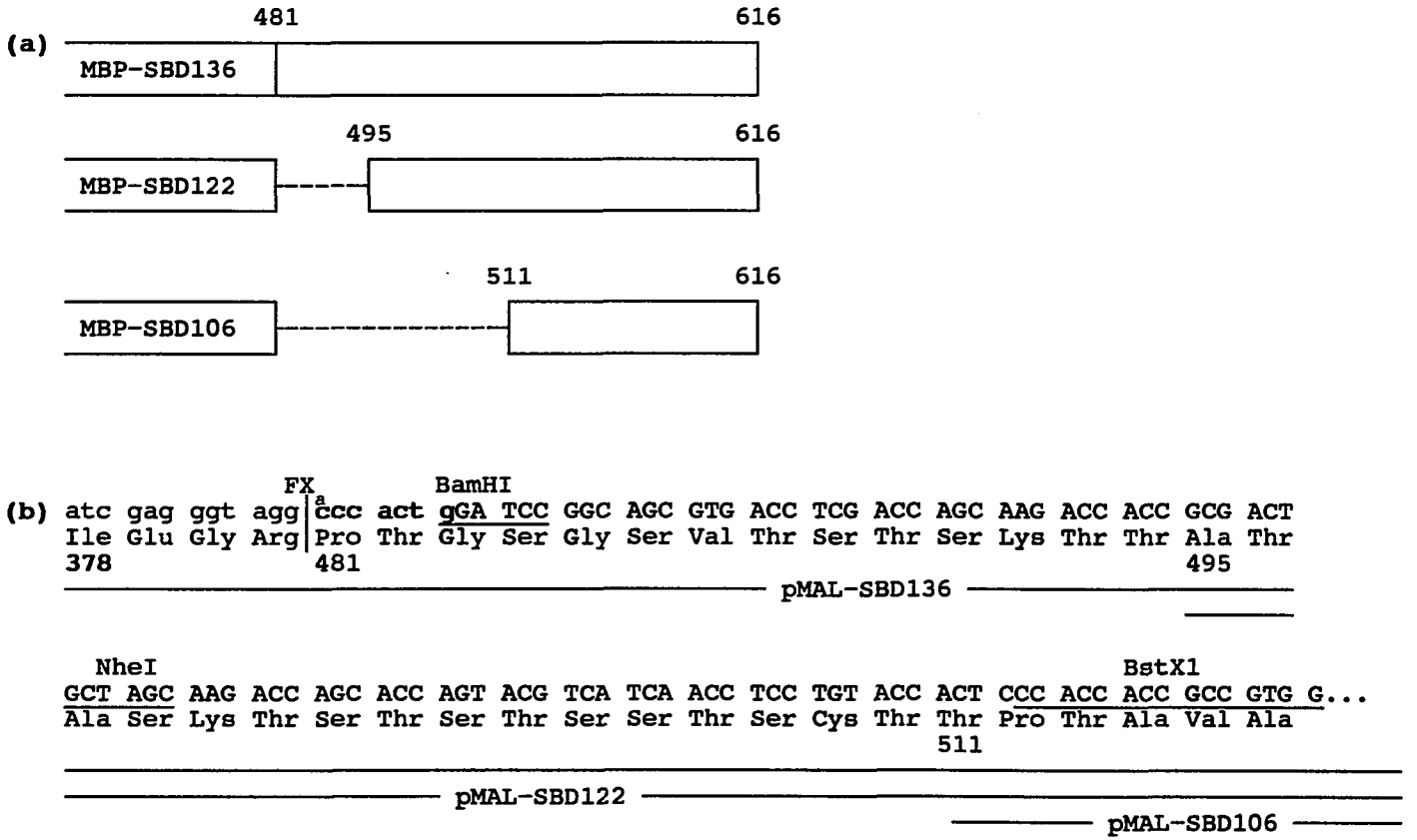



Figure 1. Construction of MBP-SBD fusion proteins. (a) MBP fusion proteins containing starch-binding domain fragments of different lengths at the C-terminus. Numbers above the box indicate amino acid of glucoamylase I. Broken-lines indicate deleted sequence of glucoamylase I. (b) Construction of MBP-SBD fusion protein

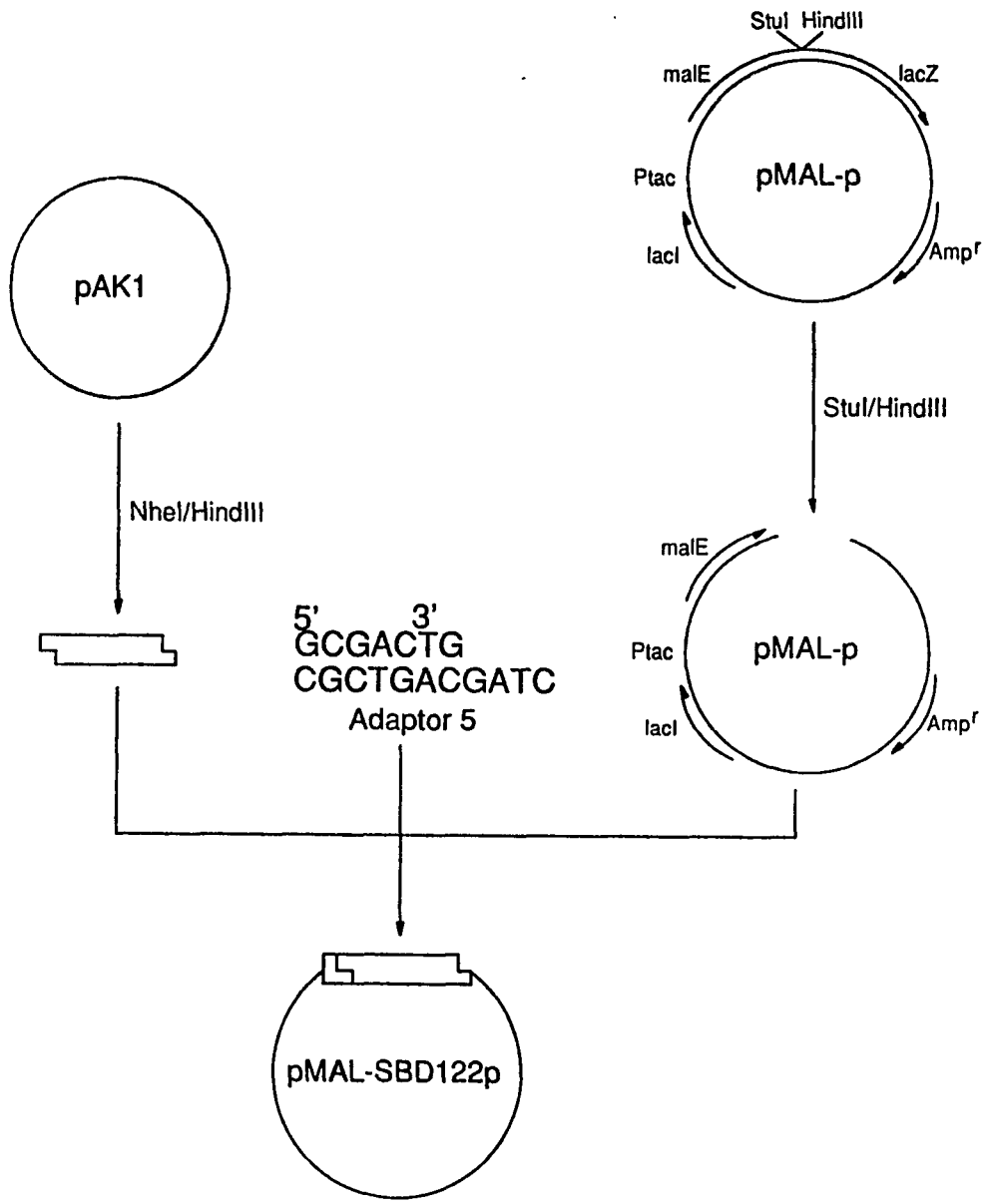



Figure 2. Schematic representation of cloning strategy for the construction of pMAL-SBD122p

other clones were constructed using the same procedure. All six plasmids constructed were digested with restriction enzyme NcoI to check whether or not the plasmids possessed the gene coding for starch-binding domain. The presence of a 772 bp, 730 bp or 682 bp DNA fragment depending on the size of starch-binding domain confirmed the presence of starch-binding domain. Figure 3 shows a DNA agarose gel of the NcoI digested pMAL-SBD122p. The DNA sequences at the junction of all six constructed plasmids were sequenced to confirm that no mutation, deletion or insertion had occurred during the construction.

**Purification of the MBP-SBD fusion proteins**

The production of periplasmic MBP-SBD122 was first checked in a small scale fermentation (10-ml). The immunoblot showed that the fusion protein was produced one hour after the induction. Maltose-binding protein was also produced by proteolysis of the fusion protein. Based on this result, all cell cultures were harvested two hours after induction.

The fusion proteins were produced in E. coli and isolated from the periplasm or the cytoplasm. The image analysis of immunoblots (Figure 4) indicated less than 25% proteolysis for the different fusion proteins. This level of proteolysis is much lower than that observed for the ß-galactosidase fusions (50-70%) (Chen et al., 1991b). When probed with anti-maltose-binding protein antibody, the immunoblots revealed the

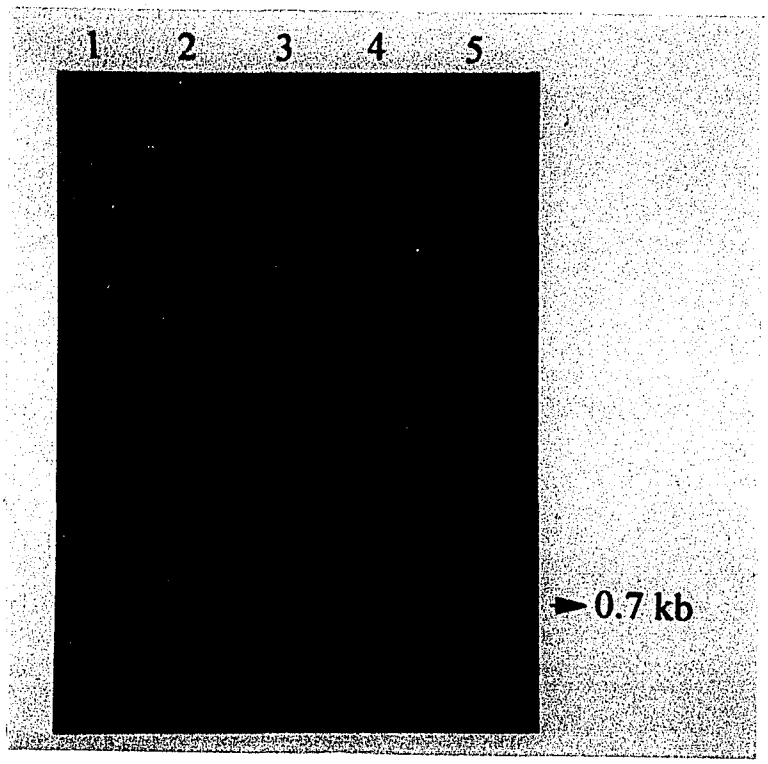



Figure 3. Agarose gel of NcoI digest of pMAL-SBD122p. Lanes: (1) λ/HindIII DNA ladder; (2) and (4) pMAL-SBD122p; (3) and (5) NcoI digest of pMAL-SBD122p. The presence of DNA bands were visualized under ultraviolet light after staining with ethidium bromide

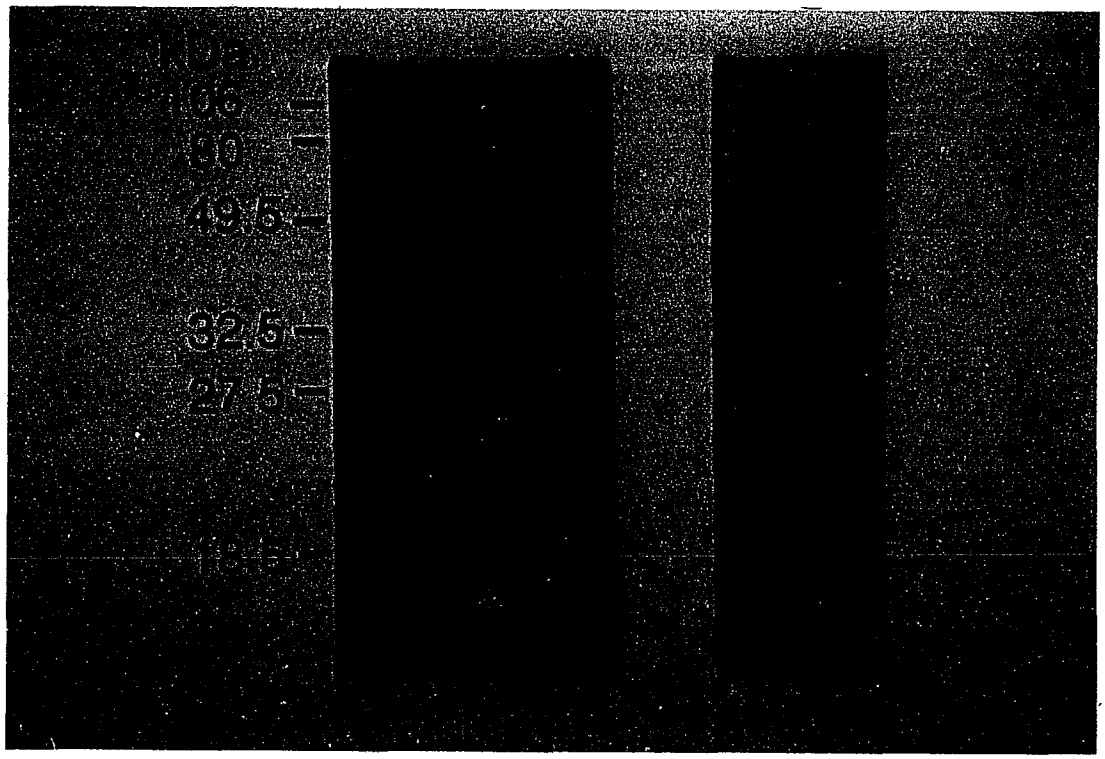



Figure 4. Immunoblot of a whole cell extract of an isopropyl-ß-D-thiogalactoside-induced culture (two hours after induction). Ten µl of the treated sample was electrophoresed on a 0.1% sodium dodecylsulfate, 15% polyacrylamide gel and then electroblotted. The presence of MBP-SBD fusion protein and maltose-binding protein were visualized by hybridization with anti-glucoamylase I or anti-maltose-binding protein antibody, followed by gold conjugated Protein-A

presence of a distinct degradation product approximately the same size as native maltose-binding protein. When probed with anti-glucoamylase I antibody, the immunoblots showed only the full-sized fusion protein. These results suggested that the starch-binding fragment of the fusion protein had been cleaved off proteolytically and subsequently degraded, possibly as a result of misfolding.

Both starch-binding domain and maltose-binding protein in MBP-SBD fusion proteins adsorbed to cross-linked amylose, but with different affinities. Maltose-binding protein was eluted from this matrix at a low concentration of maltose (10 mM) (Maina et al., 1988), whereas elution of functional starch-binding fragments required 500 mM maltose or 10 mM ß-cyclodextrin, which is a specific ligand for the starch-binding domain (Savel'ev et al., 1990; Belshaw and Williamson, 1991; Svensson and Sierks, 1992). The high maltose concentration needed to elute the MBP-SBD fusion proteins was probably due to the higher affinity of the starch-binding domain for amylose than for maltose (Savel'ev et al., 1990; Svensson and Sierks, 1992). The maltose-binding protein binds maltose specifically with $K_d$ of about 3-4 $\mu$M at 4°C (Schwartz et al., 1976), and maltoheptaose with $K_d$ of 1 $\mu$M (Martineau et al., 1990). It is expected that maltose-binding protein binds amylose with about the same strength as maltoheptaose. This explains the effectiveness of eluting maltose-binding protein from cross-linked amylose with 10 mM maltose. During the

stepwise elution from the amylose column, both MBP-size degradation products of MBP-SBD fusions and/or full-size fusions with a nonfunctional starch-binding domain were eluted with 10 mM maltose (Figure 5a, peak 1). Functional MBP-SBD fusion proteins were eluted with 10 mM ß-cyclodextrin or 500 mM maltose (Figure 5a, peak 2). Elution from the column with 10 mM ß-cyclodextrin (or 500 mM maltose) thus served as a test of starch-binding domain functionality throughout this study. Maltose binds to maltose-binding protein and elutes the maltose-binding protein part of the fusion protein, but starch-binding domain remains bound to cross-linked amylose due to 200-400 times higher affinity for amylose than maltose (Savel'ev et al., 1990; Williamson et al., 1991; Svensson and Sierks, 1992). This observation suggests that in MBP-SBD fusion protein molecule, starch-binding domain is a separate domain which folds properly in such a way that it retains its functionality as in the native starch-binding domain in glucoamylase I molecule.

Purification of MBP-SBD122 produced using the periplasmic system was also attempted using QAE Sephadex (Diethyl[2-hydroxypropyl]aminoethyl sephadex) ion exchange chromatography (Kellermann and Ferenci 1990). The bound maltose-binding protein and periplasmic MBP-SBD122 were separated using an NaCl gradient (0 to 300 mM). The maltose-binding protein was eluted first, followed by the fusion protein. Even though this procedure gave a comparable result as chromatography on

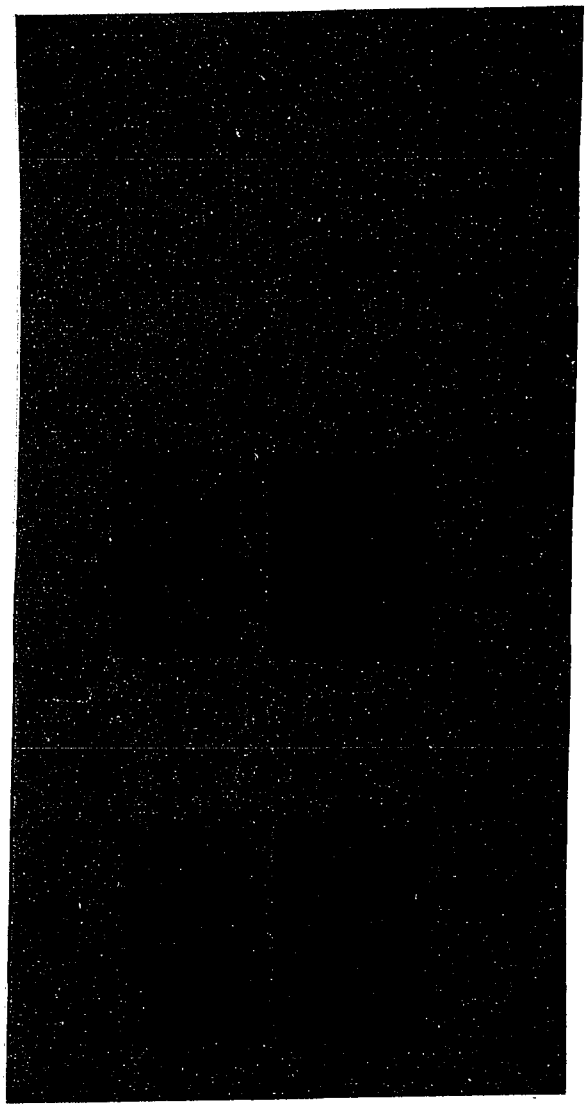


Figure 5. (a) Schematic representation of the stepwise elution of a MBP-SBD preparation, (b) Immunoblot analysis of a cytoplasmic MBP-SBD fusion protein. (c) Immunoblot analysis of a periplasmic MBP-SBD fusion protein. Peak 1 represents the protein fraction eluted with 10 mM maltose, and Peak 2 represents the fraction eluted with 10 mM ß-cyclodextrin. Samples (0.1 $\mu$g) were electrophoresed on a 0.1% sodium dodecylsulfate-10% polyacrylamide gel (b) or on a 0.1% sodium dodecylsulfate-15% polyacrylamide (c). After electrophoresis, the proteins were electroblotted. The transferred proteins were immunostained with anti-maltose-binding protein or anti-glucoamylase I and gold conjugated Protein A

cross-linked amylose, the later procedure was simpler and faster. Furthermore, purification using cross-linked amylose affinity chromatography was preferred because only the functional starch-binding domain or MBP-SBD fusion proteins were adsorbed and then specifically eluted from the affinity matrix.

We found that only cytoplasmic fusion proteins contained nonfunctional starch-binding domain fragments as indicated by the anti-glucoamylase I immunoblot in Figure 5b. The immunoblot analysis of periplasmic MBP-SBD fusion proteins revealed that no fusion protein was eluted with 10 mM maltose (Figure 5c, peak 1). The nonfunctional fraction constituted between 40 and 50% of the total fusion protein concentration (Figure 5b). Nonfunctionality of the starch-binding domain may be the result of improper folding of the fragments in the reducing environment of the E. coli cytoplasm (Fahey, 1977). Although possibly improperly folded, these nonfunctional starch-binding fragments were not degraded proteolytically.

Production of MBP-SBD106 using either cytoplasmic or periplasmic expression systems was lower compared to the expression of MBP-SBD122 and MBP-SBD136 (Table 1) probably due to proteolytic degradation of the fusion protein. Both SBD136 and SBD122 possess one disulfide bond formed between Cys-509 and Cys-604 (Svensson, personal communication). SBD106 which has only one cysteine residue, cannot form the disulfide bond as formed in SBD136 or SBD122. Lack of this disulfide bond

Table I. Amount of purified MBP-SBD fusion proteins from one liter of culture

| Fusion protein | Cytoplasmic system (mg) | Periplasmic system (mg) |
|---|---|---|
| MBP-SBD106 | 7.1 ± 6.4[b] | 1.7 ± 0.2[a] |
| MBP-SBD122 | 21.2 ± 1.1[b] | 4.2 ± 0.5[c] |
| MBP-SBD136 | 13.5 ± 2.8[c] | 4.2 ± 0[a] |

[a]Average of two purifications

[b]Average of three purifications

[c]Average of four purifications

may contribute to exposing one or more protease recognition sites on the protein. Proper folding of a heterologous protein in E. coli has been shown to be important in protecting the protein from host proteolytic system. The yield of MBP-SBD122 was higher than that of MBP-SBD136 (Table 1) although both proteins posses the same disulfide bond. This discrepancy is not understood, but we speculate that the additional 14 amino acid long peptide in MBP-SBD136 exposed certain protease recognition sites of the molecule which made the protein more susceptible to proteolysis.

The amount of purified functional MBP-SBD fusion proteins obtained with the cytoplasmic expression system were generally higher than those obtained with the periplasmic expression system (Table I). Because the yield of MBP-SBD fusion proteins produced by using the periplasmic system was lower

than that produced by using cytoplasmic system, the efficiency of exporting the fusion protein to periplasmic space was checked. From 500 ml of culture, 2.5 mg of MBP-SBD122 was purified from the cold osmotic shock fluid. After removal of the periplasmic proteins, only 0.1 mg of MBP-SBD122 was purified from the cell-free extract. Because only approximately 5% of the periplasmic fusion protein remained in the cytoplasm, the lower yield was due to a lower expression level and not due to an inefficiency of targeting the protein to the periplasmic space. The cytoplasmic expression system was subsequently used for isolation and purification of the three starch-binding fragments.

The purity (>95%) of the isolated fusion proteins was determined by sodium dodecylsulfate-polyacrylamide gel electrophoresis (Figure 6a), and the identity of the fusion proteins was confirmed by hybridization with polyclonal antibody against glucoamylase I (Figure 6b). The isolated fusion proteins showed a stepwise increase in molecular weight as expected.

### Isolation of starch-binding fragments from MBP-SBD fusion proteins

Cytoplasmic starch-binding fragments were separated from the maltose-binding protein by digestion of the fusion proteins with factor $X_a$ under nondenaturing conditions suggesting that the tetrapeptide recognition site is

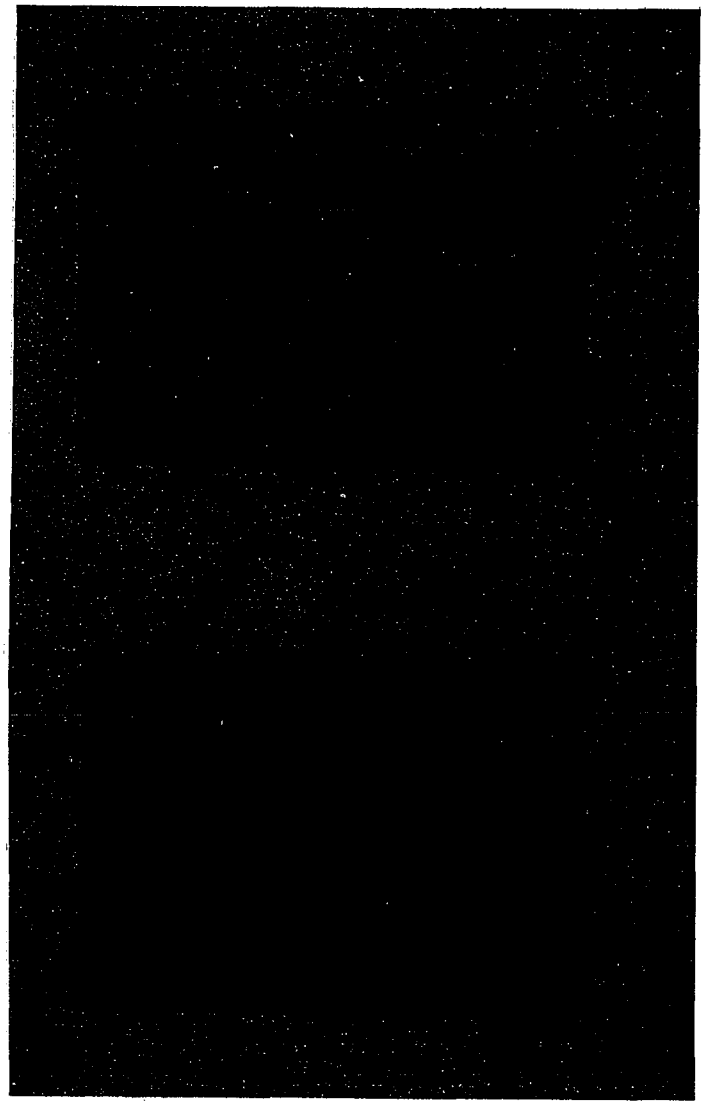


Figure 6. (a) Sodium dodecylsulfate-polyacrylamide gel electrophoresis analysis of purified MBP-SBD fusion proteins. 0.1 $\mu$g of cytoplasmic or periplasmic MBP-SBD fusion proteins were electrophoresed on a 0.1% sodium dodecylsulfate-10% polyacrylamide gel and were stained with silver stain. (b) Immunoblots of purified MBP-SBD fusion proteins. The electroblotted proteins were immunostained with anti-GAI and gold conjugated Protein A. Lanes: 1, MBP-SBD106; 2, MBP-SBD122; 3, MBP-SBD136

accessible to the protease. In some cases, such as MBP-ß-galactosidase fusion protein, the proteolysis had to be performed under denaturing conditions in order to obtain acomplete digestion (Maina et al., 1988). For the purpose of this work, digestion under nondenaturing conditions is preferable because a functional starch-binding domain is required. Digestion under denaturing conditions will require unfolding of the fusion protein before digestion and refolding afterwards. It has been shown that some proteins could not fully and properly refold after unfolding. The digestion of the fusion proteins was completed after approximately 12 hours, as judged from the disappearance of MBP-SBD band on sodium dodecylsulfate-polyacrylamide gel electrophoresis (Figure 7).

The starch-binding fragments resulting from factor $X_a$ digestion of MBP-SBD fusion proteins were separated from maltose-binding protein on a cross-linked amylose column as described for the MBP-SBD fusion protein. Maltose-binding protein was eluted with 10 mM maltose, whereas each starch-binding domain was efficiently eluted with 500 mM maltose or 10 mM ß-cyclodextrin. This result is in agreement with previous observations (Savel'ev, 1990) that ß-cyclodextrin binds strongly ($K_d$ = 56 $\mu$M) to starch-binding domain whereas maltose is a poor ligand with $K_d$ of 8800 $\mu$M (approximately 100 times weaker). Hence, binding of the starch-binding domain to cross-linked amylose and its elution with 500 mM maltose or 10

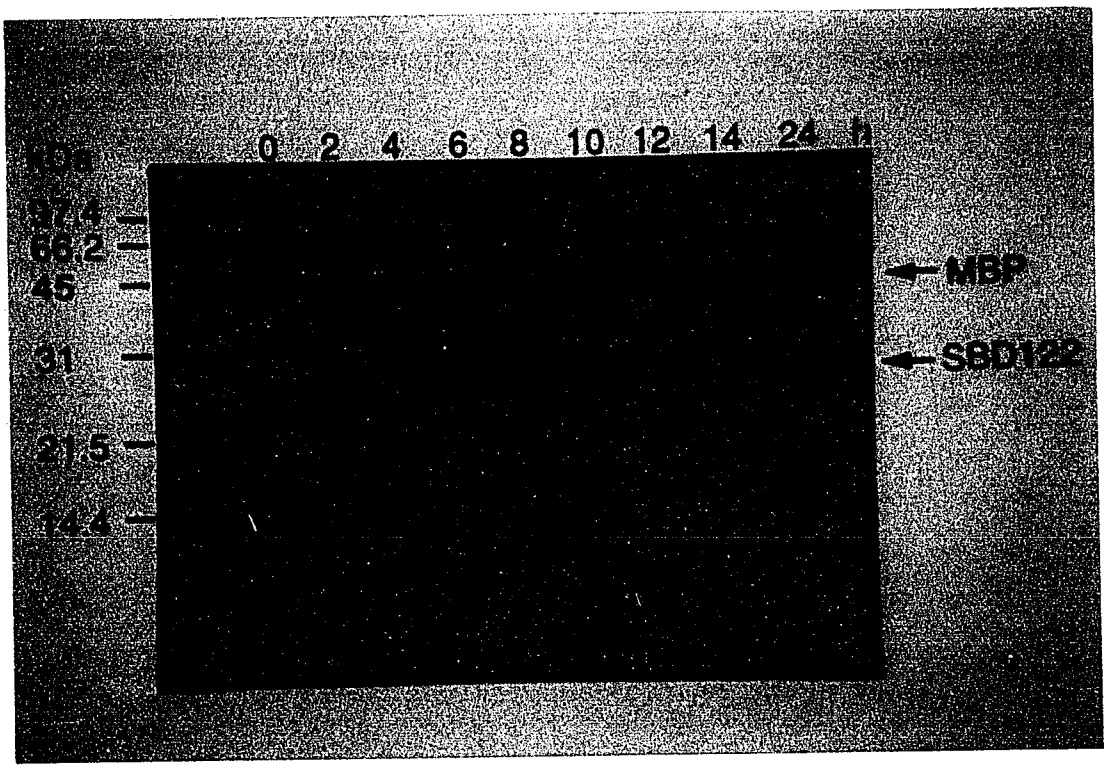



Figure 7. Sodium dodecylsulfate-polyacrylamide gel electrophoresis (0.1% sodium dodecylsulfate, 15% polyacrylamide) analysis of the factor $X_a$ digest of MBP-SBD122. The fusion protein was digested with factor $X_a$ (1 unit/50 $\mu$g) in 20 mM Tris.HCl (pH 8.0), 100 mM NaCl, 2 mM $CaCl_2$ and 1 mM $NaN_3$, and samples were taken at different time intervals. The presence of protein bands were visualized using silver staining

mM ß-cyclodextrin can be used to measure the functionality of starch-binding domain in binding to granular starch. Figure 8 shows an example of the fractions obtained from the purification of SBD122. There was no SBD122 in the fraction eluted with 10 mM maltose (Lane 4 in Figure 8). The fraction eluted from the cross-linked amylose column with 500 mM maltose appeared as a single protein band on sodium dodecylsulfate-polyacrylamide gel electrophoresis. This band hybridized with anti-glucoamylase I but not with anti-maltose-binding protein (Lane 5 in Figure 8). The same results were obtained for SBD106 and SBD136.

We have purified 74, 54, and 55 μmole of SBD136, SBD122, and SBD106, respectively, from 100 μmole of the corresponding MBP-SBD fusion proteins. The reduction in yield is probably due to nonspecific degradation of starch-binding domain by factor $X_a$. The adsorption and specific elution of starch-binding domain fragments from cross-linked amylose indicates the retention of functionality of the starch-binding domain fragments upon cleavage of the respective fusion proteins by factor $X_a$. The ability to isolate functional starch-binding fragments ranging in size from 106 to 136 amino acid suggests that all of these peptides are properly folded and can act as independent domains. Our results are in agreement with the recent report by Williamson et al. (1992a,b), who found that proteolytically produced fragments of glucoamylase I corresponding to residues 499-616 and 509-616 acted as

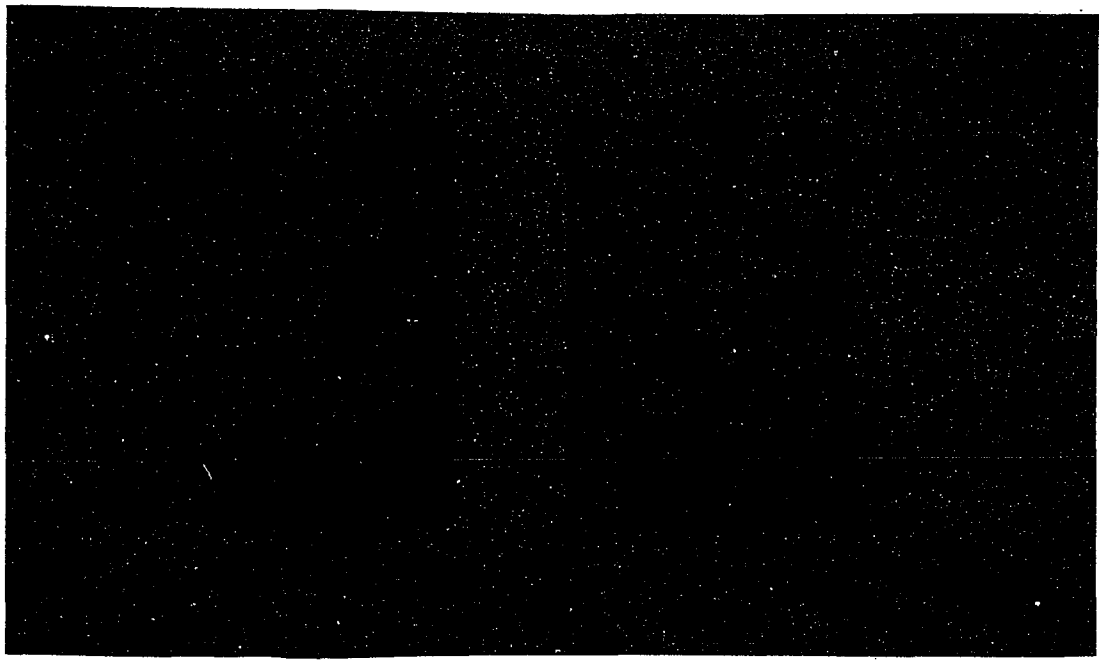


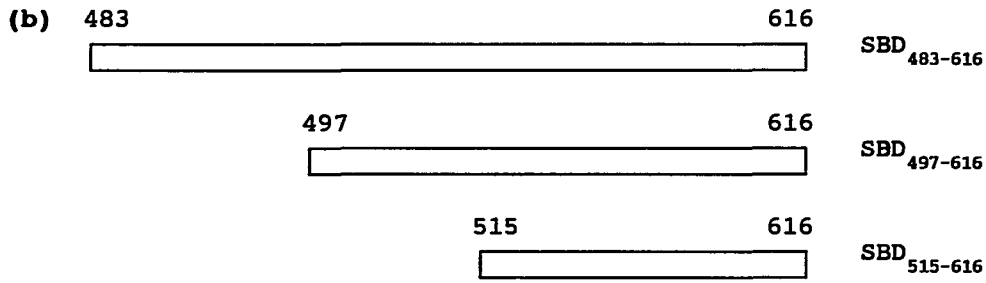
Figure 8. Immunoblots of fractions obtained from the purification of SBD122 on cross-linked amylose. Fractions taken during purification were electrophoresed on a 0.1% sodium dodecylsulfate-15% polyacrylamide gel and then electroblotted. Lanes: 1, MBP-SBD122; 2, factor $X_a$ digest of MBP-SBD122; 3, unbound fraction; 4, fraction eluted with 10 mM maltose; 5, fraction eluted with 500 mM maltose

independent starch binding domains. However, we do not imply that the ability of each of the starch-binding domain fragments to bind to starch is the same.

**Production of starch-binding fragments in E. coli**

Because we have shown that starch-binding domain fragments separated from MBP-SBD fusion proteins by factor $X_a$ digestion are functional in binding to starch, we wanted to find out whether these different starch-binding fragments could be produced independently in E. coli. We used the E. coli expression vector pKK233-2 to construct and express three different plasmids ($pSBD_{515-616}$, $pSBD_{497-616}$, and $pSBD_{483-616}$) that encode starch-binding fragments of 102, 120, and 134 amino acids long (Figure 9). The three starch-binding fragments were produced at very low levels, which made their isolation and purification impossible. An immunoblot of cell-free extracts from isopropyl-ß-D-thiogalactoside-induced cultures (Figure 10) indicates the presence of a protein band that hybridizes with polyclonal antibody against glucoamylase I. $SBD_{483-616}$ was produced at a higher level than either $SBD_{515-616}$ or $SBD_{483-616}$. This finding suggests that $SBD_{483-616}$ is less susceptible to the intracellular E. coli proteolytic system than $SBD_{515-616}$ and $SBD_{483-616}$, which were almost undetectable on the immunoblot (Figure 10). This unsuccessful expression of starch-binding domain in E. coli may have been due to improper folding of intracellularly produced starch-binding domain,

which made the peptide susceptible to E. coli proteases. SBD102 is the most unstable, whereas SBD120 seems to be the most stable of the three peptides. This observation is consistent with our results presented above. The starch-binding fragment consisting of about 120 amino acid seems to be the optimal peptide size for a given expression system based on the yield, stability, and starch-binding ability.




(c) $pSBD_{483-616}$: ...ccatg gGA TCC GGC AGC GTG ACC TCG ACC AGC AAG ACC ACC GCG ACT GCT AGC
AAG ACC AGC ACC AGT ACG TCA TCA ACC TCC TGT ACC ACT CCC ACC GCC GTG
GCT GTG ACT...GCcatgg...

$pSBD_{497-616}$: ...ccatg gCT AGC AAG ACC AGC ACC AGT ACG TCA TCA ACC TCC TGT ACC ACT CCC
ACC GCC GTG GCT GTG ACT ...GCcatgg...

$pSBD_{515-616}$: ...ccatg gTG GCT GTG ACT ...GCcatgg...

Figure 9. Cloning of starch-binding fragments in pKK233-2. (a) C-terminal 146 amino acid of A. awamori glucoamylase I (amino acid 471-616) in one-letter code (Nunberg et al., 1984). (b) Three different sizes of cloned starch-binding fragments. Numbers indicate amino acid from glucoamylase I. (c) Cloning strategy. Lower case letters indicate sequences from the vector, and bold lower case letters indicate adaptor used

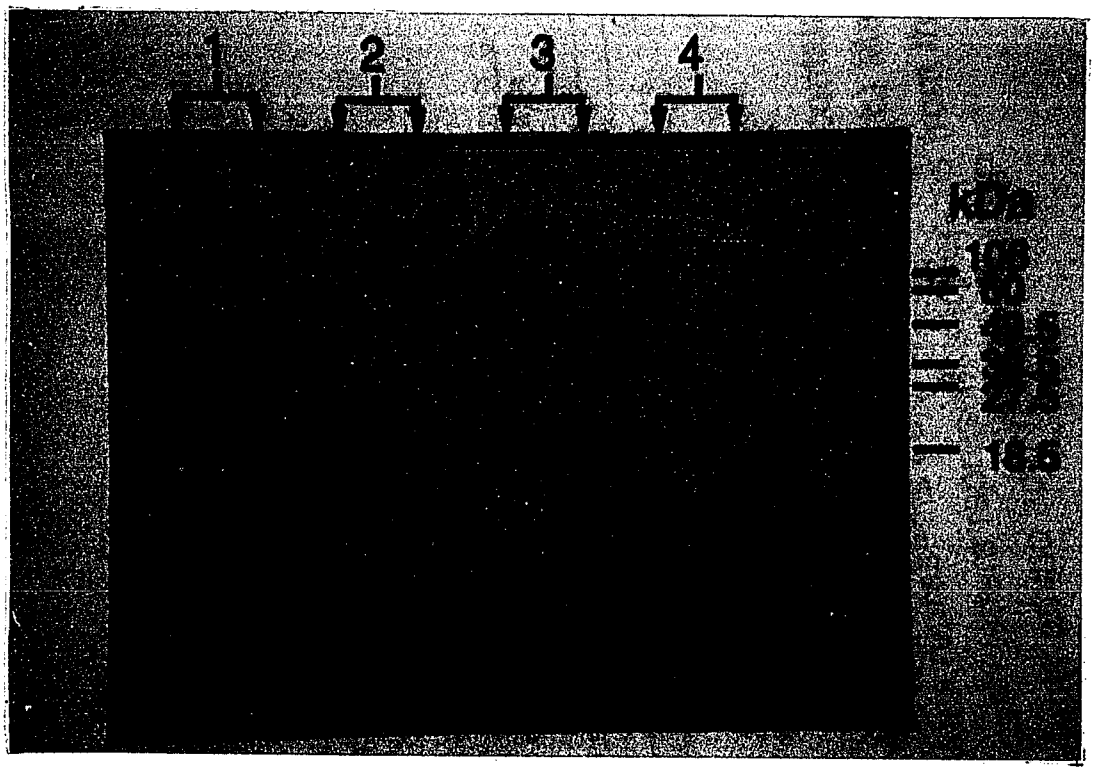



Figure 10. Immunoblot analysis of starch-binding fragments produced in E. coli. Lanes: 1, negative control; 2, $SBD_{515-616}$; 3, $SBD_{497-616}$; 4, $SBD_{483-616}$. After centrifugation, 3 $\mu$g of the resulting cell free extract was separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis (0.1% sodium dodecylsulfate-15% polyacrylamide) and then immunoblotted. Protein bands were immunostained with first anti-glucoamylase I and then gold conjugated Protein-A and enhanced using silver enhancement

# CONCLUSIONS

SBD120 can be produced independently in E. coli by direct expression. The expression levels were very low because of proteolysis in E. coli, which makes this expression system impractical for production of the starch-binding domain. Fusing starch-binding domain to maltose-binding protein significantly protected the starch-binding domain from proteolysis, although factor $X_a$ has reduced the yield of the starch-binding domain due to nonspecific degradation of starch-binding domain.

MBP-SBD fusion proteins adsorbed to cross-linked amylose and were specifically eluted with 10 mM ß-cyclodextrin or 500 mM maltose indicating that the starch-binding domain behaved as a separate domain that folded independently and properly to retain its starch-binding function. After cleavage from MBP-SBD fusion proteins, the starch-binding fragments retained completely their starch-binding activity.

We have demonstrated that a functional starch-binding domain can be produced in E. coli, and that O-glycosylation is not required for binding to starch. Our data support previous indications that the starch-binding domain is a separate domain which does not require the catalytic domain or O-glycosylated linker of glucoamylase I for proper folding and functioning (Chen et al., 1992; Libby et al., 1991). Also, our experience with different fusions indicates that a 119-122

amino acid long starch-binding domain is optimal in size for proper folding and functioning.

Using gene fusion approach, rather than extremely tedious purification of the proteolytic digest of glucoamylase I (Williamson et al., 1992a), we can produce and purify different lengths of starch-binding fragments in a three-step process.

This work is another example, besides ß-galactosidase fusions, which demonstrates the use of the starch-binding domain of Aspergillus glucoamylase I for protein purification.

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# PAPER 3. CHARACTERIZATION OF THE STARCH-BINDING DOMAIN OF *ASPERGILLUS* GLUCOAMYLASE I

## ABSTRACT

In this paper we report the characterization of three peptide fragments (SBD106, SBD122, and SBD136) containing the starch-binding domain of Aspergillus sp. glucoamylase I. These starch-binding fragments were produced by proteolysis of maltose-binding protein fusion proteins possessing different lengths of starch-binding fragments produced in Escherichia coli (Kusnadi et al., submitted). SBD106, SBD122, and SBD136 have molecular weight values of 9.7, 13.7, and 16.4 kDa respectively. Their amino acid compositions were similar to those deduced from their amino acid sequences. The first 23 amino acids sequence at the N-terminal of SBD122 correspond to amino acids 495-517 of Aspergillus glucoamylase I. The C-terminus sequencing of maltose-binding protein possessing SBD122 showed that Arg was the C-terminal amino acid.

SBD106, SBD122, and SBD136 bound specifically to ß-cyclodextrin with dissociation constant values of 34.3, 43.2, and 51.4 $\mu$M, respectively. SBD106 and SBD122 also interacted with maltoheptaose with dissociation constant values of 540 and 570 $\mu$M, respectively. The binding of these two ligands to all three starch-binding fragments affected one or more Trp and Tyr residue(s). Amino acids 511-616 of Aspergillus glucoamylase I contains the starch-binding domain as has been suggested by Svensson et al. (1989), and Chen (1992). The produced starch-binding fragments were not glycosylated, but

interacted with both ß-cyclodextrin and maltoheptaose, suggesting that glycosylation was not crucial for the functioning of the starch-binding domain. However, the isolated starch-binding domain fragment might have a somewhat different conformation than that of the native starch-binding domain.

# INTRODUCTION

Glucoamylase from Aspergillus exists in two major molecular forms; glucoamylase I and glucoamylase II (Lineback et al., 1969, Pazur et al., 1971; Svensson et al., 1982). The primary structure of glucoamylase II is identical to that of glucoamylase I (Svensson et al., 1983a,b) from amino acid 1 to 512 (Svensson et al., 1986). Glucoamylase I has a large catalytic domain, a highly O-glycosylated domain, and a starch-binding domain (Svensson et al., 1983b, Gunnarsson et al., 1984; Evans et al., 1990). The catalytic domain comprises residues 1-440, and is composed of seven consecutive binding subsites for glucosyl residues (Hiromi et al., 1983). The highly O-glycosylated domain consists of residues 441-512, and is rich in Ser and Thr. The role of the O-glycosylated domain is to maintain the catalytic domain and the starch-binding domain apart at a fixed distance (Aleshin et al., 1992; Williamson et al., 1992a). It also stabilizes the catalytic and the starch-binding domain (Williamson et al., 1992b). The starch-binding domain comprises residues 513-616 which allows glucoamylase I to adsorb to and hydrolyze granular starch (Svensson et al., 1982). Glucoamylase II, which lacks the 104 C-terminal amino acids, has about 50 times less adsorbability to granular starch (Dalmia and Nikolov, 1991).

Even though the three dimensional structure of

glucoamylase I is not yet available, there are indications that the starch-binding domain is an independent domain from the catalytic domain, and that the two domains are separated by a highly glycosylated region of about 10 nm long (Williamson et al., 1992a; Kusnadi et al. submitted). The starch-binding domain of glucoamylase I interacts with various carbohydrates, such as acarbose (Dalmia, 1990; Svensson and Sierks, 1992), α-cyclodextrin, ß-cyclodextrin, and γ-cyclodextrin (Savel'ev et al., 1990; Svensson and Sierks, 1992; Dalmia and Nikolov, submitted), maltooligosaccharides (Savel'ev et al, 1990; Dalmia, 1990), amylose (Savel'ev et al., 1990). The strongest interactions were achieved with longer maltooligosaccharides and cyclodextrins, with dissociation constant values ranging from 6 to 19 μM for ß-cyclodextrin (Savel'ev et al., 1990; Svensson and Sierks, 1992) and 10 μM for amylose with degree of polymerization of 40 (Savel'ev et al., 1990).

To determine the location of the starch-binding domain, ß-galactosidase fusion proteins possessing various sizes of starch-binding domain of Aspergillus glucoamylase I were constructed. All ß-galactosidase fusion proteins adsorbed to granular starch because of the presence of starch-binding domain (Chen et al., 1991a, b). The ß-galactosidase fusion protein containing amino acid 488-616 of glucoamylase I (BSB119) had the strongest adsorption to granular starch. Its granular starch adsorption was inhibited by maltose,

isomaltose, and cellobiose with decreasing efficiency suggesting that the starch-binding domain had a better specificity for α-1,4 glucosidic linkage than α-1,6 or ß-1,4 glucosidic linkages (Dalmia and Nikolov, submitted). BSB119 also interacted with α-, ß-, and γ-cyclodextrin, maltooligosaccharides and acarbose (Dalmia and Nikolov, submitted), and the strongest interaction was achieved with maltodextrin with degree of polymerization of 10 and ß-cyclodextrin, as has been shown for glucoamylase I. The strength of the interaction of the starch-binding domain in any of the ß-galactosidase fusion proteins with starch and starch-like molecules could not be determined because of proteolytic degradation of the produced ß-galactosidase fusion proteins. Between 50 and 70% of the produced ß-galactosidase fusion proteins were degraded resulting in a mixture of fusion proteins containing one, two, three and four starch-binding domains (Chen et al., 1991a,b; Dalmia and Nikolov, submitted).

Glycosylated starch-binding fragments (residues 509-616, 499-616, and 471-616) were isolated from protease digest of native glucoamylase I using a time consuming purification procedure of 45 times repeated ion exchange chromatography (Williamson et al., 1992a). The three glycosylated starch-binding fragments showed strong interactions with ß-cyclodextrin and granular starch (Belshaw and Williamson, 1991; Williamson et al., 1992a). The O-glycosylated region in glucoamylase I did not play a significant role in binding to

soluble or insoluble substrates (Williamson et al., 1992a; Hayashida et al., 1992), but the O-glycosylated decapeptide of glucoamylase I (498-KTSTSTSSTS-508) stabilized the conformation of the starch-binding domain (Williamson et al., 1992b).

To overcome the problems associated with the ß-galactosidase fusion protein system, we fused the starch-binding domain to the C-terminal of a monomeric protein, maltose-binding protein, flanked by a factor $X_a$ cleavage site (Kusnadi et al., submitted). Functional starch-binding fragments were isolated by cleaving them from the respective maltose-binding protein fusion protein using factor $X_a$ (Kusnadi et al., submitted).

This work addresses interactions of the isolated starch-binding fragments produced in E. coli with ß-cyclodextrin and maltoheptaose. The strength of the interactions were determined by estimating the dissociation constants ($K_d$) for the ligands using a difference spectroscopy method. By comparing the estimated $K_d$ values with those reported for native glucoamylase I and starch-binding fragments isolated from the native glucoamylase I, we will discuss the role of glycosylation and folding on the functionality of the starch-binding domain, as well as the applicability of the E. coli expression system for the production and purification of proteins using starch as an adsorbent.

# MATERIALS AND METHODS

## Production of starch-binding domain

In this study, three different sizes of starch-binding fragments were used, SBD106, SBD122, and SBD136, which respectively consist of residues 511-616, 495-616, and 481-616 of glucoamylase I. The three starch-binding fragments were prepared following the procedure described in Chapter 2. The concentration of the purified starch-binding fragments were determined using $A_{280}$, with molar extinction coefficient of 30600 $M^{-1}$ $cm^{-1}$ (Gill and von Hippel, 1989)

## Molecular weight determination

The molecular weight was determined using high performance size exclusion chromatography (HPSEC) on 9.4 mm ID x 25 cm Zorbax GF-250 column (Dupont, Wilmington, DE). Between 20 and 50 $\mu$g of purified fusion protein, starch-binding fragments, and starch-binding fragments mixed with carbonic anhydrase and cytochrome C were injected in a total volume of 20 $\mu$l. The mobile phase containing 0.1 M ammonium phosphate (pH 7.0) and 0.02% $NaN_3$ was set at 0.5 ml/min. A molecular weight calibration curve was obtained by plotting the retention time of each molecular weight standard against its log molecular weight. Molecular weight standards (bovine serum albumin, ovalbumin, carbonic anhydrase, and cytochrome C) were purchased from Sigma Co., St. Louis, MO. Retention

times of the proteins in the mixture were compared and converted into molecular weight values using the standard curve.

### Amino acid composition

About one nmol of the purified starch-binding domain was electrophoresed on a 13.5% polyacrylamide gel (Laemmli, 1970) as described in Chapter 2, and then electroblotted to a piece of polyvynilidene fluoride membrane (Bio-Rad, Richmond, CA). The electroblotting procedure was the same as that in Chapter 2, except that 1x 3-[cyclohexylamino]-1-propanesulfonic acid (CAPS) buffer was used. The 1x CAPS buffer was made by mixing 100 ml of 10x CAPS buffer (100 mM CAPS, pH 11), 100 ml of methanol and 800 ml of deionized $H_2O$ (Matsudaira, 1987). After electroblotting, the membrane was washed extensively with $H_2O$ and stained for 1-2 minutes with Coomassie blue (0.1% Coomassie Blue R-250 in 40% methanol/1% acetic acid), and then destained with 50% methanol/10% acetic acid for 15 minutes with 3-5 changes of destaining solution. The destained membrane was rinsed with water and air-dried. Amino acid analysis of the protein bound on the dried membrane was performed by the Iowa State University Protein Facility (Ames, IA). The on-membrane amino acid analysis was performed using norleucine as an internal standard. The protein band was cut-off and placed in a clean hydrolysis vial containing 500 $\mu$l of 6 N HCl. The vial was purged with argon gas and placed in a

pre-heated oven at 165°C for 45 minutes. The tube was then lyophilized in a Speed Vac. The membrane was removed and placed on a Model 420H derivatizer (Apllied Biosystems, Inc.) sample slide, and the automated derivatization and on-line phenylthiocarbamyl amino acid analysis was started. The free amino acids were derivatized under basic condition with phenylisothiocyanate to produced phenylthiocarbamyl-amino acids, which were separated on a narrow-bore HPLC system using a reverse-phase C-18 silica column. The phenylthiocarbamyl chromophore was detected at 254 nm.

**N- and C-terminus sequencing**

The N-terminus sequence of SBD122 was determined on the same sample using the Edman degradation method (Iowa State University Protein Facility). An Applied Biosystems model 477A protein sequencer and a 120A PTH amino acid analyzer were used.

The C-terminus sequence was performed using MBP-SBD122 fusion protein. Before performing C-terminus sequencing, MBP-SBD122 was first denatured and carboxymethylated as follows (Applied Biosystems User Buletin, 1988). The purified MBP-SBD122 was dialyzed extensively with $H_2O$ and lyophilized in an aliquot of 100 $\mu$g. Each 100 $\mu$g aliquot was resuspended with 500 $\mu$l of 6 M Guanidine-HCl buffered with 0.25 M Tris-HCl, 1 mM ethylenediamine tetraacetic acid pH 8.5. The mixture was flushed with $N_2$ and incubated in the dark at room temperature.

After denaturation, the protein was carboxymethylated by adding iodoacetamide (Sigma, Co., St. Louis, MO). Iodoacetamide was added at a 1.5 fold molar excess of the total sulfhydryl groups in the fusion protein (the iodoacetamide stock was made by dissolving 100 mg iodacetamide per ml of ethanol). The reaction mixture was then incubated for another 30 minutes at room temperature. Carboxymethylation was stopped by adding 100 times molar excess of 1-mercaptoethanol, and then the protein was dialyzed extensively with $H_2O$ to remove all salts. The protein concentration was determined from the amino acid composition.

The carboxymethylated MBP-SBD122 was digested with carboxypeptidase P (Boehringer Mannheim, Indianapolis, IN) at pH 3.8. In a 1.5 ml microfuge tube, 200 µl of MBP-SBD122 (25 pmol/µl) was mixed with 560 µl of 0.03 M NaOAc pH 3.8 (Applied Biosystems Inc.), 80 µl norleucine (50 pmol/µl), and 8 µl carboxy peptidase P (1.74 mg/100 µl $H_2O$). The reaction mixture was incubated at room temperature and at different time intervals 40 µl samples were transferred into a tube containing diisopropyl ethylamine (Applied Biosystems Inc.) to stop the reaction. A control reaction (reaction mixture without MBP-SBD122) at the same pH was also performed. Samples from the control reaction mixture were taken at the same intervals as in the digestion reaction mixture. Twenty µl aliquots of each sample were subjected to amino acid analysis to determine the amount of amino acids released (Iowa

State Protein Facility).

## Difference spectroscopy

Ligand-induced perturbation of absorption spectra was determined at ambient temperature (20°C±2°C), using double-chamber cuvettes with light paths of 4.375 mm. Absorption spectra were recorded on a Cary Model 1501 spectrophotometer with the On-line Instruments modification that permits direct recording and analyzing of spectra by a computer. The sample and reference cells containing starch-binding fragment (30-70 μM, in 5 mM sodium acetate, pH 4.5) and the buffer, in either chamber were scanned five times at 315-245 nm. The ligand (0-3 mM) was added to the protein sample and the buffer reference in aliquots ranging from one to 15 μl. The same volume of buffer, which matched that of the ligand aliquots, was added to the buffer sample and protein reference, and the mixture was scanned. For each ligand concentration five spectra were recorded from 315 to 245 nm. Each spectra consisted of 180 data points, and each point was the average of nine readings. The spectra were averaged, corrected for the dilution factor and baseline drift, and then smoothed using the 17-point smoothing procedure. The difference in absorbance (ΔA) between the complex and the free starch-binding fragment was calculated using Equation 1 (Belshaw and Williamson, 1991).

$$\Delta A_{286} = [(A_{286}-A_{280}) + (A_{286}-A_{290})]/2 \qquad (1)$$

where $A_{280}$, $A_{286}$, and $A_{290}$ are the absorbance at 280, 286, and 290 nm, respectively.

The molar absorptivity ($\epsilon$) was calculated using Equation 2 (Bashford, 1987).

$$\epsilon = A/c.l \qquad (2)$$

where A is the absorbance, $\epsilon$ is molar absorptivity [$M^{-1}cm^{-1}$], c is the protein concentration [M] and l is light path [cm].

The dissociation constants of SBD106, SBD122, and SBD136 for ß-cyclodextrin and maltoheptaose were calculated using equation given below:

$$b = e_0(f)/(K_d + f) \qquad (3)$$

where b, f and $e_0$ are the concentrations of bound ligand, free ligand, and total binding sites, respectively.

The uniformity of the binding sites was determined using the Scatchard equation (Bagshaw and Harris, 1987).

$$b/f = -1/K_d(b) + e_0 \qquad (4)$$

Assuming that one mole of ligand binds to one mole of starch-binding domain fragment, the concentration of the bound ligand was calculated as:

$$b = (\Delta A/\Delta A_{max})e \qquad (5)$$

where $\Delta A$ and $\Delta A_{max}$ are the difference and maximum difference in the absorbance between the ligand and the starch-binding domain fragment complex and free starch-binding domain, and e is the concentration of the starch-binding domain fragment used. The $\Delta A_{max}$ was determined by using a non-linear curve fitting procedure of Sigma-Plot software (Sigma-Plot).

$$\Delta A = \Delta A_{max} * L/(K_{app}+L) \qquad (6)$$

where L is the added ligand concentration, and $K_{app}$ is the apparent dissociation constant.

# RESULTS AND DISCUSSIONS

## Molecular weight determination

From sodium dodecylsulfate-polyacrylamide gel electrophoresis analysis, the molecular weight of all three different sizes of starch-binding domain were estimated to be above 20 kDa. Based on the amino acid sequence of the three starch-binding fragments molecular weights of 11.6, 13.1, and 14.4 kDa were expected for SBD106, SBD122, and SBD136, respectively. To resolve this molecular weight discrepancy, size exclusion chromatography was performed. Table 1 summarizes the molecular weights of starch-binding domain and MBP-SBD fusion proteins obtained from high-performance size exclusion chromatography. From the difference between the molecular weights of MBP-SBD fusion proteins and the maltose-binding protein, estimated by size exclusion chromatography, molecular weights of 9.7, 13.7, and 16.4 kDa were calculated for SBD106, SBD122, and SBD136, respectively.

When purified SBD122 sample was spiked with carbonic anhydrase and cytochrome C standards, and analyzed by size exclusion chromatography, two distinct peaks were observed (Figure 1). The retention time of the first peak (18.01 minutes) corresponded to that of the carbonic anhydrase. The second peak (20.64 minutes) had a shoulder (21.78 minutes) suggesting that there were two components that were not separated completely. Since the retention time of 21.78

Table 1. Average molecular weight of MBP-SBD fusion proteins and SBD fragments

| Protein | Expected MW[a] (kDa) | Estimated MW[b] (kDa) | Calculated MW[c] (kDa) |
|---|---|---|---|
| MBP-SBD106 | 53.4 | 57.2 | |
| SBD106 | 11.6 | ND | 9.7 |
| MBP-SBD122 | 54.9 | 61.2 | |
| SBD122 | 13.1 | 16.6 | 13.7 |
| MBP-SBD136 | 56.2 | 63.9 | |
| SBD136 | 14.4 | 16.9 | 16.4 |
| MBP | 41.7 | 47.5[d] | |

MW: molecular weight

ND: not determined

[a]Determined based on amino acid sequence

[b]Determined based on molecular weight standard curve, and averaged from two observations.

[c]Determined by subtracting the estimated molecular weight of maltose-binding protein from that of the fusion proteins.

[d]Average of five measurements.

ND: not determined.

minutes corresponded to the retention time of the cytochrome C, the second peak was attributed to SBD122 with an estimated molecular weight of 16.6 kDa. A similar elution profile was obtained using SBD136 and the molecular weight of SBD136 was estimated as 16.9 kDa. This molecular weight value was higher than the calculated value obtained from the difference of

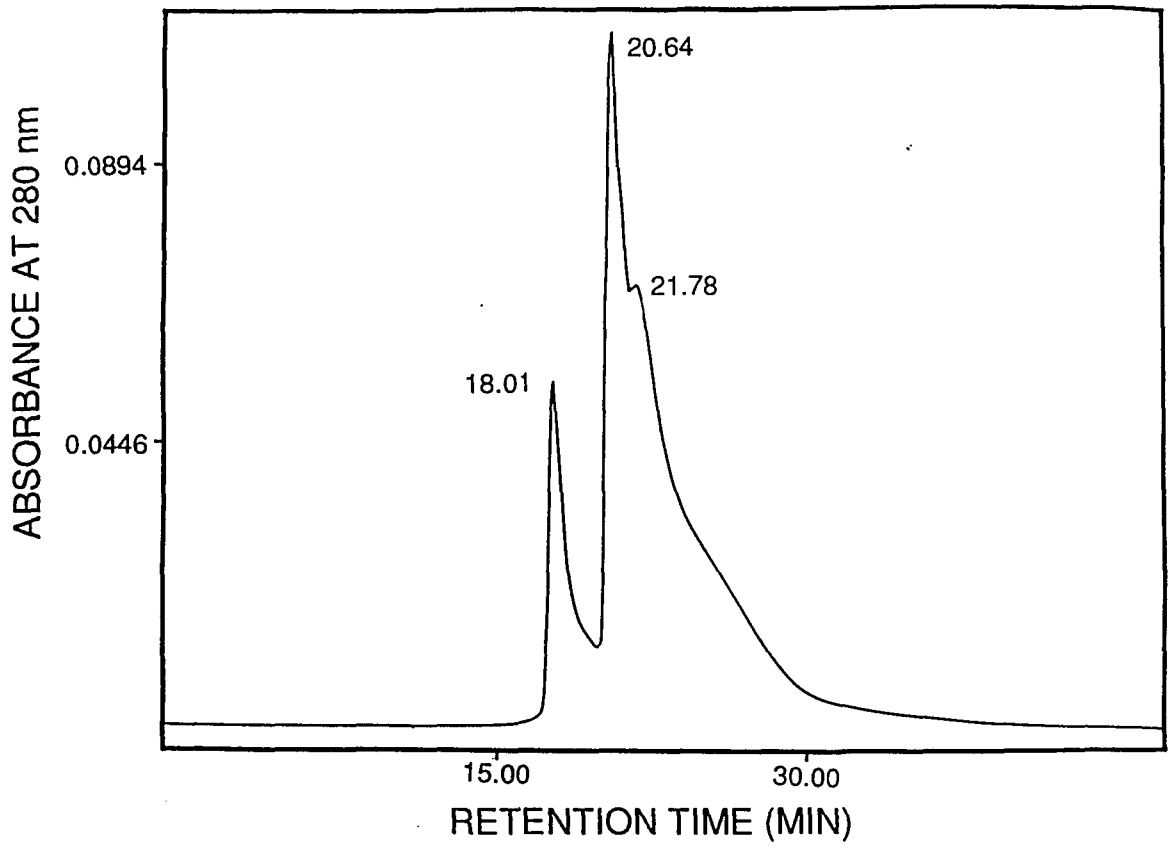


Figure 1. Size exclusion high performance chromatogram of pure SBD122 mixed with cytochrome C and carbonic anhydrase.

molecular weight between maltose-binding protein and MBP-SBD122 (Table 1). Because the difference between the expected and the estimated molecular weight of maltose-binding protein was 12%, we believe that the 17 kDa molecular weight of SBD122 and SBD136 is acceptable, and within the experimental error. The size exclusion chromatography results thus indicated that the isolated starch-binding fragments had comparable molecular weights with those expected from their amino acid sequences. However, we cannot explain the reason for the retardation of the mobility of the starch-binding fragments on the polyacrylamide gel.

**Amino acid analysis**

To further verify their identity, amino acid analysis of SBD106, SBD122 and SBD136 was performed. The amino acid compositions of the isolated starch-binding fragments were in good agreement with those of glucoamylase I peptides 511-616, 495-616, and 481-616 (Table 2). The number of Ser and Thr residues were lower than expected in all three starch-binding fragments due to the instability of these two amino acids during acid hydrolysis. The exact amounts of Ser and Thr can be determined by performing three separate hydrolysis at varying times and extrapolating the line to time zero after plotting molar concentration versus time. The number of Gly residues was higher than expected as well, perhaps due to a contamination during sample preparation for amino acid

Table 2. Amino acid compositions of SBD106, SBD122 and SBD136

| Amino | SBD106 | | SBD122 | | SBD136 | |
|---|---|---|---|---|---|---|
| Acids | Expected | Calc. | Expected | Calc. | Expected | Calc. |
| Asx | 11 | 8 | 11 | 10 | 11 | 10 |
| Glx | 10 | 8 | 10 | 10 | 10 | 10 |
| Ser | 11 | 10 | 17 | 14 | 21 | 18 |
| Gly | 6 | 8 | 6 | 7 | 8 | 13 |
| Arg | 3 | 3 | 3 | 3 | 3 | 4 |
| Thr | 17 | 12 | 23 | 19 | 28 | 22 |
| Ala | 8 | 8 | 10 | 10 | 10 | 10 |
| Pro | 5 | 4 | 5 | 4 | 6 | 5 |
| Tyr | 6 | 5 | 6 | 6 | 6 | 5 |
| Val | 8 | 8 | 8 | 8 | 9 | 9 |
| Ile | 5 | 5 | 5 | 5 | 5 | 6 |
| Leu | 6 | 6 | 6 | 6 | 6 | 7 |
| Phe | 3 | 3 | 3 | 3 | 3 | 3 |
| Lys | 2 | 2 | 3 | 3 | 4 | 4 |
| Cys | 1 | ND | 2 | ND | 2 | ND |
| Trp | 4 | ND | 4 | ND | 4 | ND |

ND: not determined.

Calc.: calculated.

analysis. Gln and Asn could not be detected because both amino acids were converted to the corresponding carboxylic acids. Thus, the resulted Asp and Glu were actually the sum of Asp and Asn and Glu and Gln, respectively. The number of Val and Ile residues calculated were very similar to the expected values even though both amino acids are difficult to break. The high recovery of Val and Ile might explain the

lower recoveries of Ser and Thr. The number of Trp and Cys residues could not be determined by this method because they were destroyed during acid hydrolysis. The calculated amino acid compositions for the three starch-binding fragments were within the experimental error. Thus, the amino acid analysis results confirmed that we have isolated the correct size of starch-binding fragments. To further identify the identity of these starch-binding fragments, N- and C-terminus sequencing were performed on SBD122 and MBP-SBD122, respectively.

### N- and C-terminus sequencing

The results of N-terminus sequencing are tabulated in Table 3. The fourth and fourteenth cycles in the first run were not detected because the PTH-amino acids were lost due to the blockage of the tubings in the instrument, which could be the result of contaminants such as sodium dodecylsulfate or salts in the sample. No PTH-amino acids could be identified in cycles number 15, 17, 19, and 23 in the first run, and cycles number 15, 16, and 22 in the second run because of low amount. However, by overlapping the sequence determined from the first and second runs, 22 amino acids were able to be aligned, except amino acid in cycle number 15 in both runs. By checking the amino acid sequence of SBD122, this amino acid was assigned as Cys. This assignment is supported by the fact that unmodified cysteine is destroyed during Edman degradation and gives a blank cycle. Thus, from the N-terminus sequencing

Table 3. Amino acid recovered during 23 cycles of Edman degradation

| Cycle | Amino Acid | |
|---|---|---|
| | Run 1 | Run 2 |
| 1 | Ala | Ala |
| 2 | Thr | Thr |
| 3 | Ala | Ala |
| 4 | - | Ser |
| 5 | Lys | Lys |
| 6 | Thr | Thr |
| 7 | Ser | Ser |
| 8 | Thr | Thr |
| 9 | Ser | Ser |
| 10 | Thr | Thr |
| 11 | Ser | Ser |
| 12 | Ser | Ser |
| 13 | Thr | Thr |
| 14 | - | Ser |
| 15 | - | - |
| 16 | Thr | - |
| 17 | - | Thr |
| 18 | Pro | Pro |
| 19 | - | Thr |
| 20 | Ala | Ala |
| 21 | Val | Val |
| 22 | Ala | - |
| 23 | - | Val |

of SBD122 we concluded that the first 23 amino acid of SBD122 are the same as residues 495-517 of glucoamylase I (495-ATASLTSTSTSSTSCTTPTAVAV-517) indicating that no vector derived sequence remained attached to the starch-binding domain after factor $X_a$ digestion. Although we only performed N-terminus sequencing on SBD122, we believe that factor $X_a$ also cleaved SBD106 and SBD136 from the maltose-binding protein at the junction, leaving no vector-derived sequence on both starch-binding fragments.

Because all starch-binding fragments and MBP-SBD fusion proteins should have the same C-terminal amino acids (Arg), MBP-SBD122 was used to identify the C-terminal amino acid. Figure 2 shows the amino acid recovery after carboxy peptidase P digestion of MBP-SBD122. The last four expected amino acids at the C-terminus of MBP-SBD122 are Asp-Thr-Trp-Arg. After three minutes of carboxy peptidase P digestion 28 pmol Ala, which was not expected at all, was recovered. At this time point Ala recovery was higher compared to Arg, Gly and Thr. Also, Ala recovery was almost the same during the 125-minute of digestion. This suggests that Ala appeared in the analysis as a result of sample contamination. The observed Gly was also attributed to a contamination. The recovery of Arg increased from 18 pmol after three minutes to 43 pmol after 125 minutes of digestion, suggesting the release of Arg as the first amino acid at the C-terminal. Trp was expected as a second amino acid from the C-terminal, but could not be

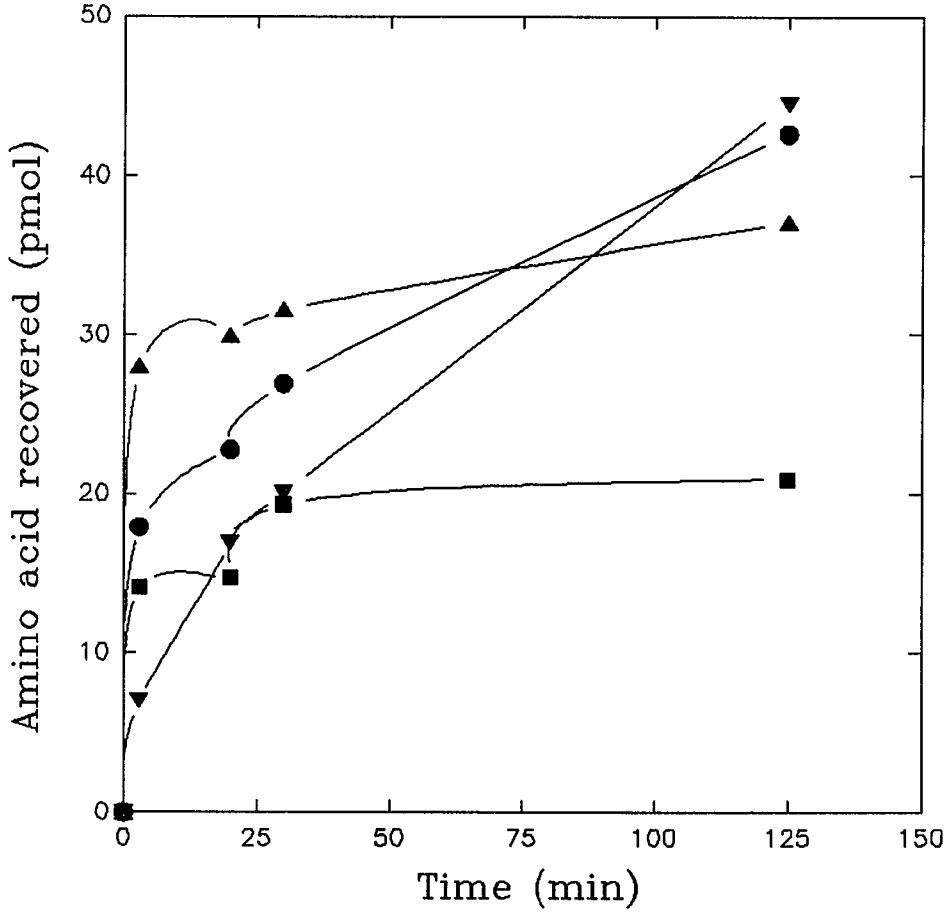


Figure 2. Kinetic analysis of amino acids released from carboxy peptidase P digestion of MBP-SBD122 fusion protein. Sample at each time point was taken subjected to analysis after phenylisothiocyanate derivatization. (•) Arg, (▲) Ala, (▼) Gly, and (▪) Thr

detected. Overall, the C-terminus sequencing was not very successful, and further investigation will be needed to find out why Ala was detected, and to modify the procedure so that Trp can be detected.

## Interaction of starch-binding fragments with ß-cyclodextrin

The ultraviolet difference spectra of SBD106, SBD122, and SBD136 induced by titration with ß-cyclodextrin (Figure 3) show four characteristic peaks, with absorbance maxima at 278 nm, 286-287 nm, 294-295nm, and 303-304 nm. The peak observed around 278 nm suggests that one or more Tyr residues were affected (Svensson and Sierks, 1992) upon binding of ß-cyclodextrin to starch-binding fragments. The ligand-induced perturbation changes in the region of 280 to 295 nm are characteristics of the red-shifted absorbance bands of indole chromophore in one or more Trp residues due to their transfer to a less polar environment upon binding (Ananthanarayanan and Bigelow, 1969). The ligand-induced perturbation changes around 300 nm are also characteristic of the indole chromophore due to changes in the electrostatic environment of the chromophore (Anathanarayanan and Bigelow, 1969). Since ß-cyclodextrin is not a charged molecule, it could be speculated that the conformational change of the starch-binding domain that occurred upon binding of ß-cyclodextrin to the starch-binding domain, changed the electrostatic environment of the

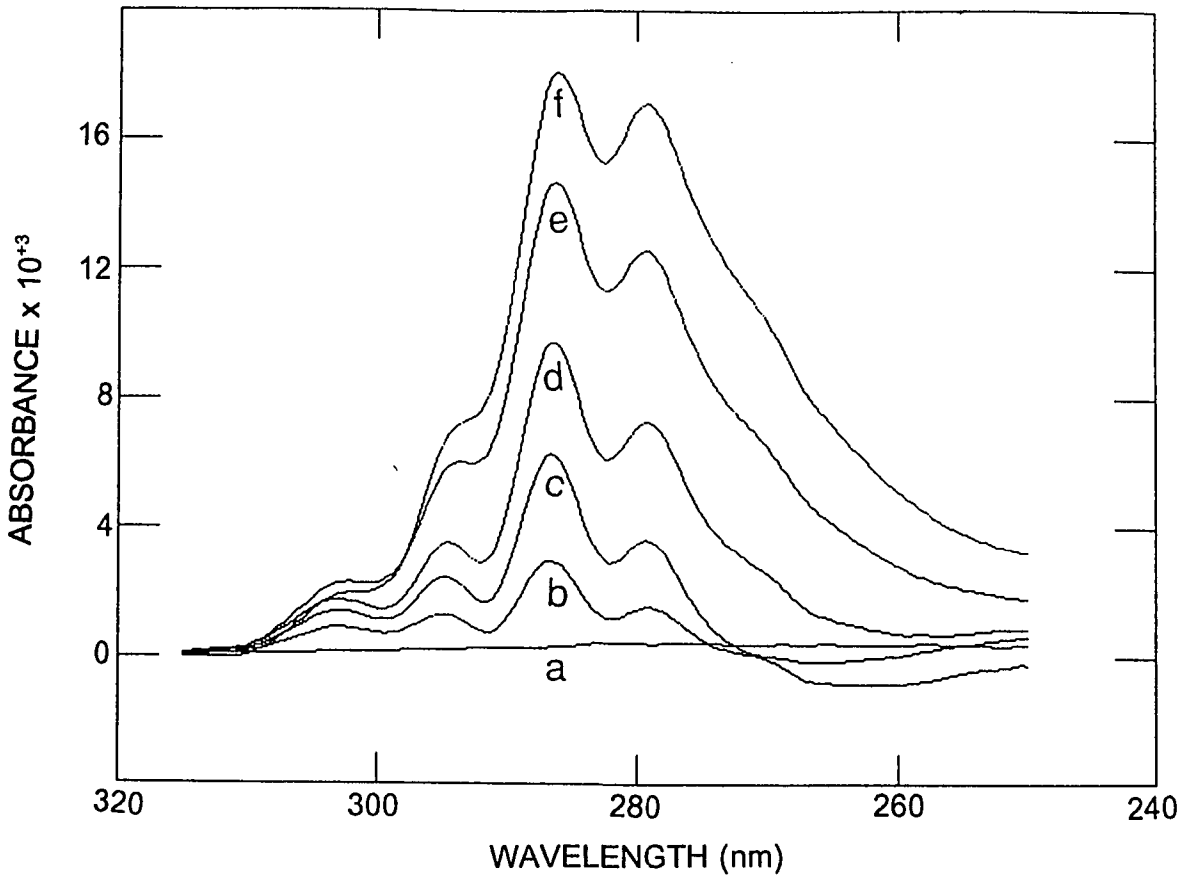


Figure 3. Ultraviolet difference spectra of SBD122 induced by ß-cyclodextrin. (a) 0, (b) 39.8, (c) 79.4, (d) 183, (e) 297, and (f) 554 μM ß-cyclodextrin

Trp residue(s) in the starch-binding domain (Ohnishi et al., 1975).

The involvement of Trp residues in the interaction of a protein to carbohydrate residues through a hydrophobic or hydrogen bonding has been recently demonstrated (Quicho, 1986; Rouvinen et al., 1990; Vyas et al., 1991). Ultraviolet difference spectra, similar to that shown in Figure 3, have also been observed when Aspergillus glucoamylase I (Svensson and Sierks, 1992) or glycosylated fragment of glucoamylase I (amino acids 471-616) of Aspergillus (Belshaw and Williamson, 1991) was perturbed by ß-cyclodextrin. The chemical modification of Trp residues of A. niger suggested that the Trp residues, Trp-590 and Trp-615, participated in granular starch adsorption (Svensson et al., 1986). Furthermore, the deletion of the eight C-terminal amino acid of glucoamylase I diminished the enzyme's granular starch adsorptivity (Chen, 1992), supporting the importance of Trp-615. The three starch-binding fragments investigated here contain these two Trp residues, and hence the obtained ultraviolet difference spectra of starch-binding domain-ß-cyclodextrin complex are due to indole chromophore perturbation.

The maximum change of $\Delta A$ was observed at 286 nm (Figure 3), and the dissociation constant ($K_d$) values for the starch-binding fragments were calculated based on $\Delta A_{286}$. The titration curve of $\Delta A_{286}$ of SBD106, SBD122, and SBD136 against ß-cyclodextrin concentrations is shown in Figure 4. $\Delta A_{286}$

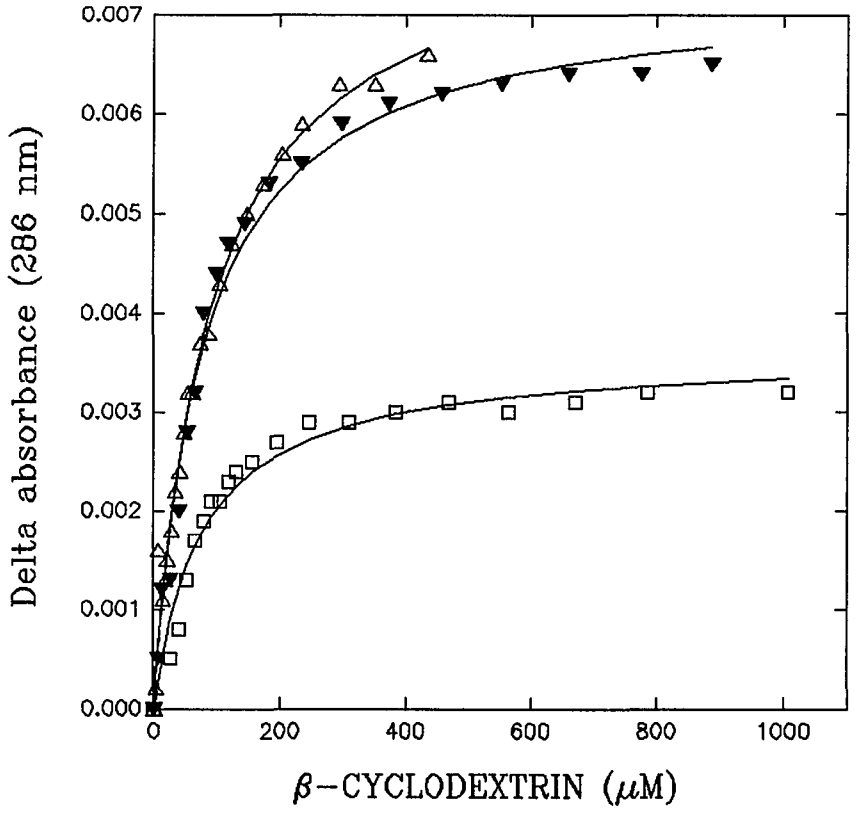


Figure 4. Titration of starch-binding fragments with ß-cyclodextrin. Dependence of ΔA on the ß-cyclodextrin concentrations. Titrations were performed in 5 mM sodium acetate pH 4.5 at 20°C. (△) 74.3 μM SBD106, (▼) 77.4 μM SBD122, and (□) 39.9 μM SBD136

increased hyperbolically with the ß-cyclodextrin concentrations, and it followed the Langmuir adsorption isotherm. The maximum change in molar absorptivity values at 286 nm ($\Delta\varepsilon_{286}$) for SBD106, SBD122, and SBD136 calculated by using Equation (2) were 246, 217, and 200 $M^{-1}cm^{-1}$, respectively (Table 4). Our observed $\Delta\varepsilon_{286}$ values were between five to six times lower than those reported for the perturbation of glucoamylase I with ß-cyclodextrin (1600 $M^{-1}cm^{-1}$) or α-cyclodextrin (1200 $M^{-1}cm^{-1}$) (Svensson and Sierks, 1992). The higher reported $\Delta\varepsilon_{286}$ for the perturbation of glucoamylase I with ß-cyclodextrin than for our isolated starch-binding fragments is most likely the result of the presence of the catalytic and the O-glycosylated domains in glucoamylase I. Glucoamylase II, which lacks the 104 C-terminal amino acids also interacted with ß-cyclodextrin with $\Delta\varepsilon_{286}$ of about 600 $M^{-1}cm^{-1}$ (Svensson and Sierks, 1992). The $\Delta\varepsilon_{286}$ for the SBD122 was approximately two times lower than that reported for the similar size of glycosylated fragment (residues 471-616) of glucoamylase I (500 $M^{-1}cm^{-1}$) (Belshaw and Williamson, 1991).

The Scatchard plots shown in Figure 5 are linear suggesting the presence of homogeneous binding sites. The $K_d$ values calculated by using Equation (3) were tabulated in Table 4. SBD106, SBD122, SBD136 have $K_d$ values of 35, 39, and 64 $\mu$M, respectively. The $K_d$ value increases as the length of the starch-binding fragment increases. We have observed that maltose, which was used to elute the starch binding domain

Table 4. $\Delta\varepsilon_{max}$ and $K_d$ values for ß-cyclodextrin binding to starch-binding domain

| Protein | $\Delta\varepsilon_{286max}$ ($M^{-1}cm^{-1}$) | $K_d$ ($\mu M$) |
|---|---|---|
| SBD106 | 246 ± 6 | 35.4 ± 2.1 |
| SBD122 | 221 ± 8 | 33.8 ± 2.5 |
| | 213 ± 9 | 43.0 ± 6.2 |
| SBD136 | 190 ± 39 | 57.1 ± 6.1 |
| | 209 ± 6 | 70.0 ± 6.3 |

from the cross-linked amylose during purification, could not be completely removed during dialysis. The isolated starch-binding fragments contained between 0.2 to 0.4 mM maltose as estimated by using phenol-sulfuric acid method. This low amount of maltose did not have a significant effect on the ß-cyclodextrin interaction, because ß-cyclodextrin binds to the starch-binding domain of Aspergillus glucoamylase 1000 times more strongly than maltose (Savel'ev et al.,1990). SBD106 had the lowest $K_d$ value suggesting that the starch-binding domain is overlapped or located within amino acids 511-616 of glucoamylase.

Glycosylated starch-binding fragments of similar length isolated from a protease digest of glucoamylase I had $K_d$ values of 1.7, 3.0 and 3.3 $\mu M$ for amino acids 471-616, 499-616, and 509-616 fragments, respectively (Belshaw and Williamson, 1991; Williamson et al., 1992). The O-glycosylated residues 499-508 of Aspergillus glucoamylase I

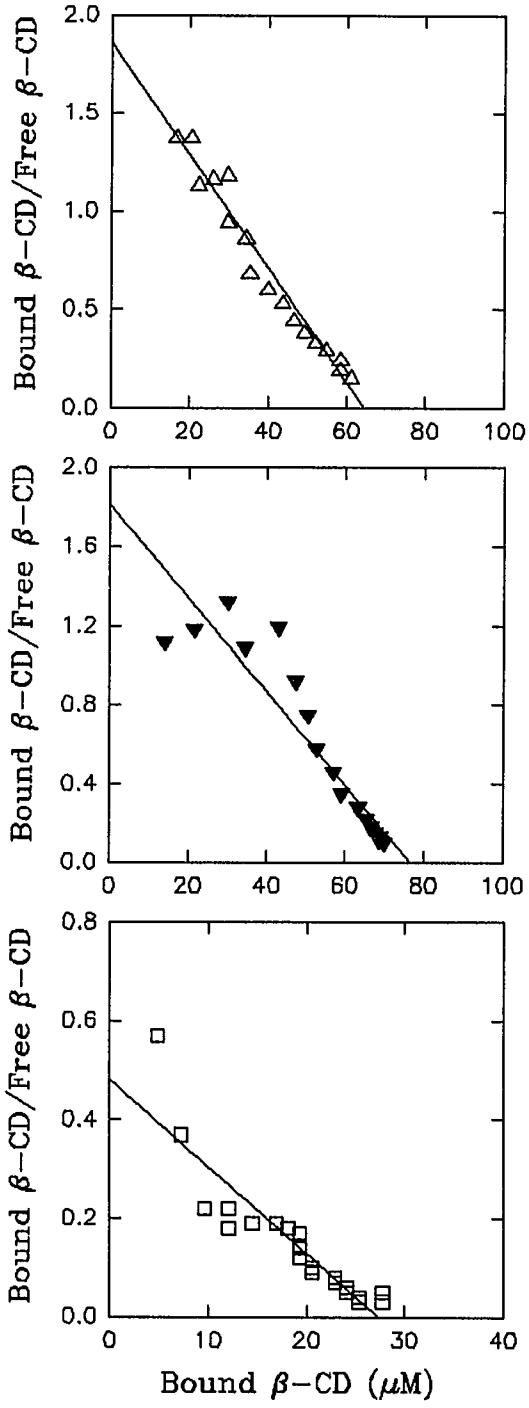


Figure 5. Scatchard plot of SBD106 (△), SBD122 (▼), and SBD136 (□) titration with ß-cyclodextrin

were important in stabilizing the starch-binding domain of the enzyme (Williamson et al., 1992b). Lack of this stabilizing glycopeptide in amino acids 499-616 and 509-616 fragments increased almost two folds their respective $K_d$ values. This observation suggests that this glycopeptide (amino acids 499-508) might have an impact on the folding and conformational stability of the starch-binding domain. Thus, the higher $K_d$ values for SBD106, SBD122, and SBD136 compared to those of the glycosylated starch-binding fragments isolated from native glucoamylase I probably reflect different conformations among them.

Svensson and Sierks (1992) reported that ß-cyclodextrin bound to the starch-binding domain of Aspergillus glucoamylase I with $K_d$ value of 19 $\mu$M, which was six to ten times higher than that reported for the glycosylated glucoamylase I fragments. The difference could be the result of difference in the stoichiometry of the interaction of ß-cyclodextrin with the starch-binding domain. Belshaw and Williamson (1991) reported that one mole of starch-binding domain bound two moles of ß-cyclodextrin, in contrast to one to one stoichiometry reported by Svensson and Sierks (1992), and Savel'ev et al. (1990). We estimated the $K_d$ values for the interaction of ß-cyclodextrin with the isolated starch-binding fragments by assuming a stoichiometry of one to one. Even though the $K_d$ value reported by Svensson and Sierks (1992) is lower, it is in better agreement with our reported values for

the three starch-binding fragments.

The studies with the glycosylated starch-binding fragments and native glucoamylase measured the interactions of the starch-binding domain in its native functional conformation. SBD106, SBD122, and SBD136 were isolated from the corresponding MBP-SBD fusion proteins. Therefore, different conformation or conformational stability could be an explanation for the different $K_d$ values measured with ß-cyclodextrin.

**Interaction with maltoheptaose**

In general, the maltoheptaose-induced ultraviolet difference spectra of SBD106, SBD122, and SBD136 were very similar to those induced by ß-cyclodextrin. The spectra show peaks at 284-285 nm and 293-294 nm, and a shoulder around 303 nm (Figure 6). The shoulder at 303 nm suggests that the electrostatic changes in the vicinity of one or more Trp residue(s) were not as strong as the changes brought about by ß-cyclodextrin.

The maximum $\Delta A$ was observed at 285 nm (Figure 6), and hence the $K_d$ values for starch-binding fragments were calculated based on $\Delta A_{285}$. The titration curves of $\Delta A_{285}$ of SBD106, SBD122, and SBD136 against maltoheptaose concentrations are shown in Figure 7. As with ß-cyclodextrin, $\Delta A_{285}$ increased hyperbolically with the maltoheptaose concentrations, and they followed the Langmuirian type

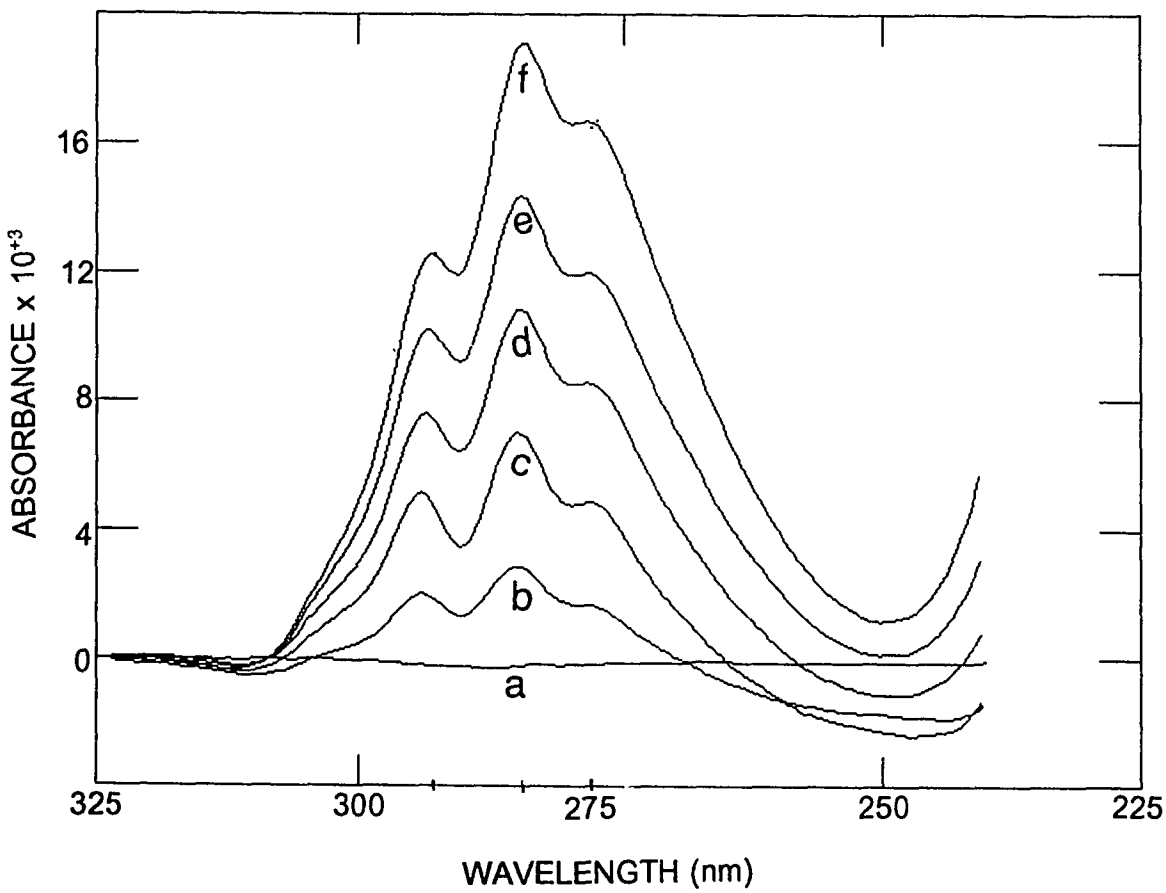


Figure 6. Ultraviolet difference spectra of SBD136 induced by maltoheptaose. (a) 0, (b) 133, (c) 462, (d) 658, (e) 980, and (f) 1675 $\mu$M maltoheptaose

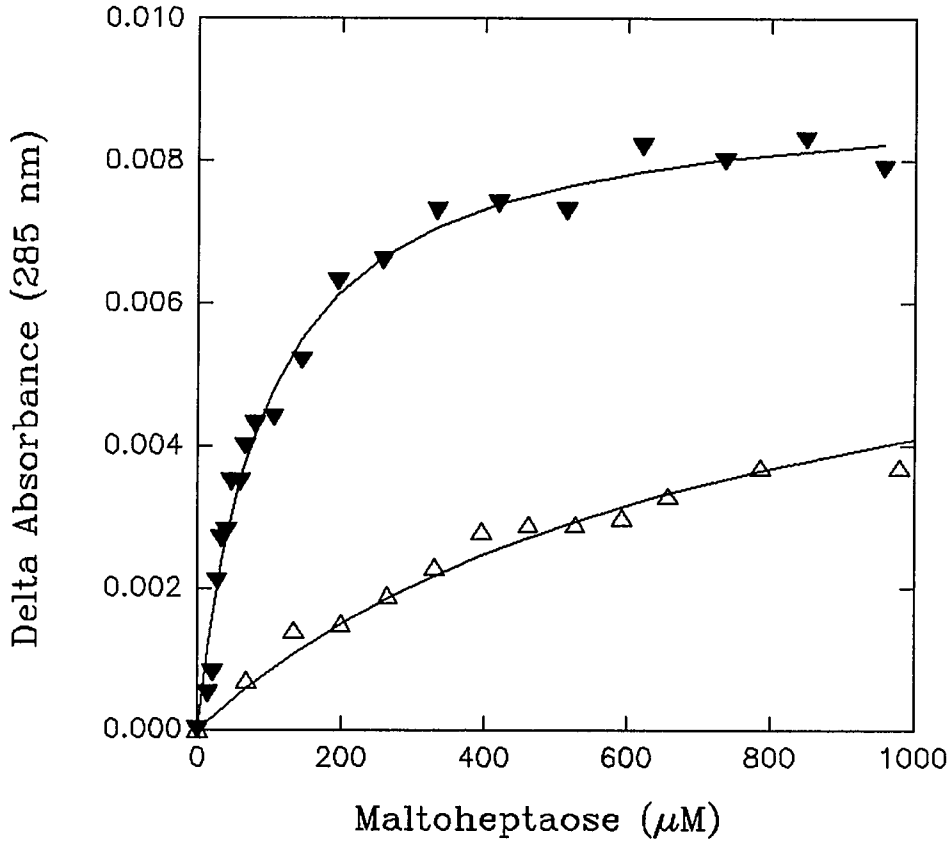


Figure 7. Titration of starch-binding fragments with maltoheptaose. Dependence of ΔA on the maltoheptaose concentration. Titrations were performed in 5 mM sodium acetate pH 4.5 at 20°C. (△) 64.2 μM SBD106, and (▼) 55.6 μM SBD122

isotherm. The maximum molar absorptivity values at 285 nm($\Delta\epsilon_{285}$) for SBD106 and SBD122 calculated from Equation 2 were, respectively, 256, and 152 $M^{-1}cm^{-1}$ (Table 5). Replicated observations need to be performed in order to ensure that the $\Delta\epsilon_{285}$ for SBD106 was in fact 40% higher than that of SBD122. We attributed the lower $\Delta\epsilon_{285}$ value for SBD122 than for SBD106 to the variation in sample preparation.

Table 5. $\Delta\epsilon_{max}$ and $K_d$ values for maltoheptaose binding to starch-binding domain

| Protein | $\Delta\epsilon_{285max}$ [a] ($M^{-1}cm^{-1}$) | $K_d$ [a] (mM) |
|---|---|---|
| SBD106 | 256 ± 7 | 711 ± 66 |
| SBD122 | 152 ± 12 | 497 ± 113 |

The Scatchard plots of the binding of maltoheptaose to SBD106 and SBD122 given in Figure 8 are linear suggesting that there is only one type of specific binding. SBD122 have lower $K_d$ value for maltoheptaose than SBD106 (Table 5), which are one order of magnitude higher than those for ß-cyclodextrin. The $K_d$ value for the interaction of ß-cyclodextrin with the starch-binding domain of glucoamylase I was approximately one order of magnitude lower than that for maltoheptaose (Savel'ev et al., unpublished data), which is in agreement our value.

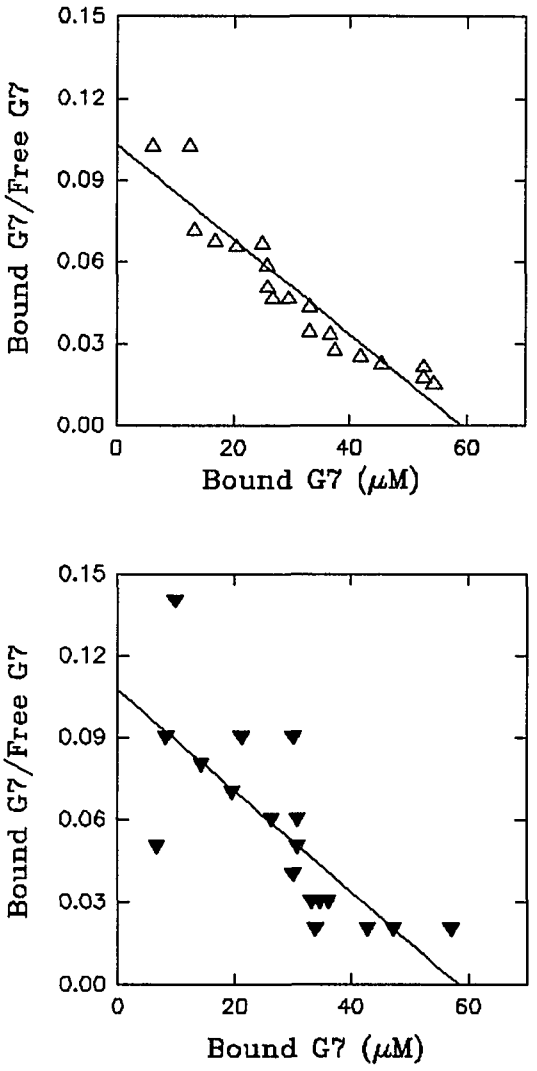


Figure 8. Scatchard plots of SBD106 (△) and SBD122 (▼) titration with maltoheptaose (G7)

The fact that the starch-binding fragments have the strongest affinity for ß-cyclodextrin, a cyclic heptamer of glucosyl residues with a diameter of about 10 Å suggests that the binding site is located on the surface of the starch-binding domain. This hypothesis is supported by our difference spectroscopy data, which showed that the difference spectra observed were the result of transfer of one or more Trp residue(s) from a polar environment to a less polar environment indicating that upon binding the pertinent Trp residue(s) was shielded by the nonpolar nature of the ß-cyclodextrin as well as maltoheptaose.

The weaker interaction of maltoheptaose with the isolated starch-binding fragments than of ß-cyclodextrin, indicates the importance of the conformational structure of the ligands. Even though both ß-cyclodextrin and maltoheptaose are glucose heptamers, ß-cyclodextrin is a cyclic molecule, whereas maltoheptaose is a linear molecule. The ring structure of ß-cyclodextrin probably fits in the binding site of the starch-binding domain allowing more than one glucosyl residues to interact with the starch-binding domain. Both $\alpha$- and $\gamma$-cyclodextrin also showed a weaker interaction with the starch-binding domain of glucoamylase I than ß-cyclodextrin (Savel'ev, et al., 1990; Svensson et al., 1992; Dalmia and Nikolov, submitted) indicating the importance of the size of the cyclic glucosyl oligomer.

# CONCLUSIONS

The produced starch-binding fragments had the correct molecular weights and amino acid compositions. Arg was at the C-terminus of SBD122 indicating that the translation of the genetic codes stop at the correct codon. Its N-terminus amino acid sequence is similar to amino acids 495-517 of glucoamylase I.

SBD106 have the strongest interaction with ß-cyclodextrin. However, our observation with the MBP-SBD fusion protein suggested that SBD106 when fused to maltose-binding proteins and produced in E. coli was the least stable in terms of proteolytic degradation, and SBD122 was the most stable. Thus, for the purpose of purification of a protein of interest, it would be advisable to use SBD122. When a 103 amino acid long starch-binding domain was fused to ß-galactosidase, the resulting fusion protein had two times lower affinity for granular starch adsorption than the fusion protein possessing longer starch-binding domain (119 or 133 amino acid long), indicating that 119 amino acid long starch-binding domain was of optimal size (Chen et al., 1991a,b).

This study confirmed that starch-binding domain is an independent domain, and that the glycosylation was not necessary for its binding to carbohydrates.

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# GENERAL CONCLUSION

Amino acids 497-616 fragment of glucoamylase I could be produced independently in E. coli using a cytoplasmic expression system. The expression level was very low, which makes this expression system impractical for production of the starch-binding domain.

We have isolated and characterized three different sizes of starch-binding fragments (SBD106, SBD122, and SBD136) from a protease digest of MBP-SBD fusion proteins. The starch-binding fragments isolated from MBP-SBD fusion proteins adsorbed to cross-linked amylose, and interacted with ß-cyclodextrin and maltoheptaose, indicating that the starch-binding domain behaved as a separate domain that folded independently and properly to retain its starch-binding function. The O-glycosylation of the starch-binding domain is not required for binding to starch.

Our experience with different fusions indicates that a 119-122 amino acid starch-binding domain is optimal in size for proper folding and functioning. Using recombinant DNA techniques, the starch-binding domain can be fused to a protein of interest, which can be purified using the starch-binding property of the starch-binding domain. The starch-binding domain can be cleaved from the protein of interest after purification if a specific protease cleavage site is incorporated into the fusion protein.

## Suggestions for future work

We have shown that the isolated starch-binding domain is functional in interacting with starch-like molecules. However, we still do not know the mechanism of the interaction. To characterize the nature of the starch binding domain, and its interaction with starch and starch-like molecules, more studies need to be performed.

A three-dimensional structure is important in studying structure-function relationship of a protein. Unfortunately, no three-dimensional structure is available for glucoamylase I. To overcome the shortcoming in the three-dimensional structure, the MBP-SBD fusion protein can be crystallized. Since the three-dimensional structure of maltose-binding protein is known, then we will be able to assign the three-dimensional structure of the starch-binding domain fused to it. It will also give us insight on how the starch-binding domain affects the three-dimensional structure of the maltose-binding protein. In order to use the starch-binding domain as an affinity tag for purification or immobilization of a protein of interest, fusing the starch-binding domain to the protein should not significantly affect its three-dimensional structure. The three-dimensional structure of cyclodextrin glucanosyltranferase which has a strong homology with the starch-binding domain can give us insight on the structure of the starch-binding domain.

Our data indicate that the isolated starch-binding

fragments have different conformation than that of the glycosylated starch-binding domain in the native glucoamylase I. To answer the importance of the glycosylation on the proper folding (as in the native starch binding domain), the starch binding domain can be produced in yeast or Aspergillus that will facilitate glycosylation of the produced protein.

Site-directed mutagenesis will answer the important amino acid residue(s) in binding. Substitution of Trp-590 and/or Trp-615 with Gly will answer the importance of the Trp residue(s). Nuclear magnetic resonance can also be used to determine the amino acid(s) affected upon binding of ligands. Furthermore, nuclear magnetic resonance can be used to deduced the three dimensional structure of the starch-binding domain.

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