

MOLECULAR BIOLOGY OF THE CBHII CELLULASE ENZYME

FINAL REPORT

TO

SOLAR ENERGY RESEARCH INSTITUTE

SUBCONTRACT NO. HK-7-07122-1

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I. Introduction

Enzymatic hydrolysis processes have the ability to convert cellulose to fermentable sugars for fermentation to ethanol at efficiencies approaching 100%. The preferred enzymes for this process are produced from mutants of the filamentous fungus, Trichoderma reesei. This microorganism secretes large amounts of an enzyme system which is capable of totally breaking down cellulose to fermentable sugars. However, further improvement is necessary in the cost and rate of production, as well as the efficacy of the enzyme in the hydrolysis process if such processes are to be commercialized. This contract employed genetic engineering/molecular biological techniques to carry out studies to better understand the action of one component of the cellulase enzyme system and attempt to improve its activity through modifications to the enzymes composition and structure.

Cellulases represent a multi-component enzyme system which act synergistically to hydrolyze crystalline cellulose to glucose. Three types of enzymatic activity participate in these reactions:

- Endoglucanases act on internal linkages in a relatively random manner to produce shorter chain fragments;
- Exo-cellobiohydrolases (exoglucanases) cleave cellobiosyl units from the non-reducing end of cellulose polymer chains; and
- β -glucosidases cleave glucosyl units from the non-reducing end of cellooligosaccharides.

The exoglucanase activity is provided by two separate enzymes, cellobiohydrolase (CBH) I and II. CBHII represents approximately 15% of the total cellulase enzyme produced by most strains of T. reesei. CBHII may be the best single enzyme for saccharifying cellulose. When supplemented with β -glucosidase, CBHII exhibits the highest rate and activity on amorphous forms of cellulose [1]. Native CBHII is relatively stable, attacks solid substrates, and requires no cofactors for activity.

The nucleotide sequence encoding CBHII (cbh2 gene) was published independently by Chen et al. and by Teeri et al. in 1987. The genomic clone is 1608 base pairs long and contains three introns (49, 56, and 90 base pairs, respectively). The native CBHII protein is 447 amino acids in length, giving it an apparent molecular weight of 46,000 daltons and is about 18-20% carbohydrate by weight.

Conventional programs for improving cellulase activity or the rate at which it is produced by fungi use mutation/selection techniques. In these, a parent strain is subjected to

ultraviolet light or chemical mutagens and the surviving mutants are subject to screening techniques to identify mutants with the desired properties. Using the techniques of genetic engineering/molecular biology it is now possible to make specific, desired changes in the structure of the enzyme to better understand its mode of operation and to improve its activity.

Genencor is a fully-integrated industrial enzyme company which has strong capabilities in protein biochemistry, microbial physiology, fermentation, molecular biology, and bacterial and fungal genetics. Successful expression in Aspergillus of heterologous genes encoding commercially interesting enzymes (proteases, lipases, cellulases and chymosin) has been achieved and further modification of native proteins using a molecular biology approach has led to recombinant enzymes with altered substrate specificity, improved stability and improved catalytic efficiency [2, 3, 4].

Genencor manufactures a low cost, liquid cellulase product, GC 123, at commercial scale. This product is derived from T. reesei and includes the major components of the cellulase system as well as other activities, such as β -glucanases and β -D-xylanases.

To support the development of cellulase and to further explore the potential for other carbohydrase products, we have generated useful materials for biochemical and genetic studies. For biochemical studies we have isolated cellulase components and corresponding antibodies and have developed analytical techniques, including specific isolated genes encoding CBHI and EGI and have expressed these genes in research and production strains of the fungus Aspergillus nidulans. Aspergillus awamori, also a filamentous fungus, has been developed at Genencor as a model heterologous expression system. Due to its relatively low level of cellulolytic enzymes and its well described transformation system similar to that of A. niger, this fungus has been a very desirable host for the study of various cellulases from T. reesei.

This report will summarize our work, supported in part by SERI subcontract No. HK-7-07122-1, toward identifying the amino acid residues in CBHII which are important to catalysis. Once identified, it is reasoned that we can direct changes in structure in which some would lead to improvement in function (hydrolytic efficiency). We began by testing the hypothesis put forth by Chen et al. [5] and Dr. Ross D. Brown, Jr. (personal communication), namely that the catalytically important amino acid residues are located at position 175 (173) and 184. We used the approach of synthetic oligonucleotide directed site-specific mutagenesis to impart these changes and used heterologous expression in Aspergillus to obtain altered forms of the enzyme. The test for enzymatic activity was performed after purification of rCBHII's from crude culture broths.

In addition, we examined a second type of modification to CBHII structure. This modification to CBHII structure generates a smaller molecule which contains the putative substrate binding site and catalytic site. The latter portion of CBHII (the carboxy terminal end) will be omitted by deleting the 3' end of the gene beginning at the start of the third intron.

II. Objectives

The objectives of the subcontract with SERI were to use the cbh2 gene to accomplish the following:

- ° Express "wildtype" cbh2 in Aspergillus awamori.
- ° Modify the amino acid residues (175 (173) and 184) that have been postulated to be important in catalysis and express in A. awamori.
- ° Delete the 3' end (carboxy terminal end) of the cbh2 gene and express in A. awamori.

Milligram quantities of the above (except 3 - see below) have been provided to Dr. Michael E. Himmel at SERI.

III. Approach

The CBHII structure-function studies were accomplished in the absence of any crystallographic information. Our approach is outlined in Figure 1.

IV. Molecular Biology

A. Recombinant CBHII

The nucleotide sequence and deduced amino acid sequence of the gene encoding CBHII is given in Figure 2. Both the genomic and intronless DNA sequences encoding CBHII and various plasmid vectors were used for expression of mature CBHII in Aspergillus awamori [5]. We constructed expression "cassettes" [6] containing the entire coding regions for both genomic and intronless forms of the cbh2 gene by introducing a unique BglII site 24 base pairs upstream of the start codon and a unique NheI site 21 base pairs downstream of the stop codon (Figure 3). These cbh2 cassettes were inserted into the Aspergillus awamori expression vector pGPT-pyrG1 which employs the glucoamylase promoter and terminator regions and a selectable pyrG gene from A. nidulans (Figure 4).

The cbh2 plasmid was used to transform a pyrG auxotroph of A. awamori. After growing transformants for 4-5 days using maltose for induction of the glucoamylase promoter, cultures were harvested by filtration. Secretion of recombinant CBHII was examined by western blotting analysis (see Figure 5). The western blot results indicated that

1. the cbh2 coding region was successfully transcribed and translated by A. awamori, and
2. A. awamori can recognize and process T. reesei CBHII signal sequence, resulting in efficient secretion of rCBHII.
3. A. awamori can correctly splice intron sequences in T. reesei genes.
4. A. awamori apparently glycosylates the CBHII enzyme in a different manner than T. reesei, giving the impression that it is hyperglycosylated.

Evidence for enzyme activity was obtained by incubating 100ul of culture supernatant overnight in a 1% solution of phosphoric acid swollen cellulase (PSC). Reactions were stopped by boiling for 10 minutes. Residual PSC was removed by centrifugation and 100ul of supernatant was analyzed for production of total reducing sugar using the method of Nelson-Somogyi [7, 8] and for production of cellobiose by HPLC. An example of the reducing sugar analysis is shown in Figures 6 and 7. Cultures were harvested between 4 and 5 days since the level of secreted protein and activity were found to be maximal. HPLC analysis was used to confirm that the product of reaction was cellobiose (Figure 8). Visualization of a single cellobiose peak on the HPLC trace shows that the rCBHII enzyme secreted by A. awamori is active and its specificity has not been affected by hyperglycosylation.

The screening of transformants for rCBHII was accomplished using both PSC activity and an ELISA assay. Throughout the study, we found similar results (ranking of transformants) with both methods. However, we had more confidence in the ELISA data (Table 1) because we found variable "low level" cellulase activity in control strains of Aspergillus. Aspergillus produces β -glucosidases and endoglucanases (not cellobiohydrolases) under specific culture conditions. The PSC activity screen was designed to yield data which would allow us to rank transformants. The early and overnight incubation times were chosen for convenience and the data does not reflect reaction kinetics.

B. Modified rCBHII's

The region between aspartic acid residue 175 (D175) and glutamic acid residue 184 (E184) of CBHII is hypothesized to be in the active site and may be involved in the initial proton donation and stabilization of the resulting carbonium ion intermediate, respectively [5, 9, 10] (Figure 9). To confirm that these residues are, indeed, important in catalysis and to further examine the relative activities of rCBHII's (modified in these positions) will lead to a better understanding of cellulases and, moreover, may result in an improved cellulase.

We prepared gene constructions (Figure 10) to impart the following modified rCBHII molecules:

1. Glu-184 to Gln-184 (note: Gln-184 is denoted either as Q184 or E184Q), and
2. Asp-173 and Asp-175 to Asn-173 and Asn-175 (note: Asn-173, 175 is denoted either as N173, N175 or D173N, D175N).

Support for the hypothesis that these residues are catalytically important will result if any or all of the above listed modified forms of CBHII are inactive.

The important catalytic and cellulose binding domains for CBHII are presumed to be located at one end of CBHII (N-terminal end) [5, 11]. Therefore, the carboxy-terminal end of CBHII appears to lack function, beyond contributing to tertiary structure. We explored this idea by deleting the latter half of CBHII beyond amino acid residue 232 (this point marks the beginning of the third intron in the cbh2 gene). The modified form was approximately one-half the size of the native CBHII and, if active, may have a higher specific activity.

Screens for transformants containing single or double amino acid modifications were performed as described above except that ELISA data served as the basis for ranking transformants (Table 2). The top three transformants were used to generate protein for purification and characterization studies.

The truncated cbh2 gene (deletion form) was inserted into appropriate plasmid vectors to transform Aspergillus. Northern blots showed that abundant quantities of mRNA were produced (Figure 11) and ELISA data (Table 3) confirmed the presence of immunological reactive material against CBHII in the culture broth.

However, repeated attempts to visualize a smaller protein on SDS and western gels failed. Furthermore, with the exception of the first test for activity using PSC, which gave mixed results, all subsequent tests for activity yielded negative results.

A logical reason for these observations is proteolysis. The significant reduction in the size of CBHII will, of course, affect the tertiary structure and disulphide bonding pattern. The net result is likely a molecule which is more sensitive to proteolysis. We will continue work on this potentially very interesting form of CBHII.

V. Protein Purification and Characterization

A. Purification of wildtype recombinant CBHII by FPLC

Concentrated broth containing rCBHII at approximately 8mg/ml was equilibrated with 5mM sodium phosphate, pH 7.8, and then loaded onto a monoQ column (1 ml loaded at a flow rate of 0.5ml/min). Bound protein was eluted off the column using a step gradient. The elution buffer used was 50mM sodium phosphate pH 7.5 + 0.5M NaCl.

The CBHII protein appeared in two fractions: the flow through and the first elution peak. The first peak eluted at 8% elution buffer (Figure 12).

SDS and western gel analysis of column fractions (Figure 13) confirmed that rCBHII binds weakly, if at all, to monoQ under these conditions. It should be noted that native CBHII behaves similarly and that we did not find conditions of buffer, pH and ionic strength that allowed binding of CBHII to the ion exchange resin.

Gel analysis showed that recombinant CBHII was of lower mobility than native CBHII (Figure 14). The recombinant CBHII gave a broad heterogeneous band due to hyperglycosylation. Indeed, some of additional glycosylation is N-linked glycosylation as evidenced by experiments using endo glycosidase H (endo H). Endo H cleaves glycosyl moieties from N-linked glycoproteins. Treatment with endo H caused an increase in mobility of rCBHII almost equal to native CBHII (Figure 14).

B. Purification of Recombinant D173N/D175N and E184Q CBHII's

The above procedure was repeated using concentrated D173N/D175N and E184Q broths. The elution profile for the rE184Q broth was similar to that of the wildtype rCBHII broth (see Figure 15).

The elution profile for the rD173N/D175N broth showed significantly less protein in the flow through peak (see Figure 16). SDS and Western analysis of this flow through peak showed little to no detectable protein. Additional cultures using positive D173N/D175N transformants were generated and these contained higher levels of immunologically reactive protein. The above procedure was repeated and pure D173N/D173N rCBHII was generated for characterization work.

C. Strain 12 Control (untransformed)

The same procedure used in the purification of the above rCBH's was repeated using A. awamori strain 12 (untransformed control) broth. The elution profile showed only a slight protein peak in the flow through. The first peak (corresponding to the 8% concentration of elution buffer) was smaller than that seen with elution of wildtype and E184Q rCBHII broths (Figure 17).

D. Specific Activity of Native and Recombinant CBHII's on PSC

One microgram each of the CBHII forms were incubated in 1% PSC in 50mM sodium acetate buffer, pH 4.8, for various times. Total reducing sugar was determined in the liquid fraction according to the method of Nelson and Somogyi [7, 8]. The results are shown in Figure 18 and Table 3.

Native CBHII, rCBHII and the E184Q rCBHII show similar activities on PSC after 30 minutes of incubation (Table 3). In contrast, the D173N, D175N rCBHII is completely inactive and thus, one or both of these residues are important in catalysis. In the next phase of work, we propose to determine which of these residues is responsible for catalytic function.

VI. Summary Statement and Future Work

Completion dates for work activities within this subcontract are given in Table 4. Evaluation of transformants (screening) and characterization studies required the most time.

This is the first report to our knowledge of the identification of the catalytically important residue(s) in any cellulase. Knowledge of this will allow us to focus on a limited region within CBHII for further study. We propose the following future work activities.

1. Determine if D173N and/or D175N or both are important in catalysis.

2. Modify the cysteine at position 172 (C172) to an alanine (A172). Compare gel patterns under reducing and non-reducing conditions (Rationale is discussed in separate proposal).
3. Modify the arginine at position 174 (R174) to an alanine (A174). Determine effect on activity and pH profile (Rationale is discussed in separate proposal).

In addition, we propose to continue work on the deletion form of CBHII.

VII. Materials and Methods

1. Fungal Strains

Aspergillus awamori strain GC12 (sometimes referred to as simply "strain 12") was derived from strain UVK143f (a glucoamylase hyper-producing mutant of strain NRRL 3112) by parasexual crossing of the following auxotrophic mutants: A. awamori GC5 (pyrG5), a uridine-requiring auxotroph isolated by selection on 5-flouoro-orotic acid [12] following mutagenesis of UVK143f with ultraviolet (this mutant is deficient in the enzyme orotidine-5'-monophosphate decarboxylase); A. awamori GC3 (argB3) which is an arginine-requiring auxotroph isolated by filtration enrichment [13] following nitrosoguanidine mutagenesis of UVK143f (this mutant is specifically deficient in the enzyme ornithine carbamoyl transferase).

2. Bacterial Strains, Cloning Vectors and Plasmids

Escherichia coli JM101 [14] was used for propagation of all plasmids and as a host for bacteriophage M13mp18 and M13mp19 [15].

The cloning vectors pUC218 and pUC219 are chimeric DNA phage-plasmid molecules derived from pUC18 and pUC19, respectively [15]. These vectors contain the intergenic region of the bacteriophage M13 [16] and when used in conjunction with the helper phage M13K07 generate single stranded DNA for use as template for site-directed mutagenesis [17].

3. Construction of the Vector pGPT-pyrG

The A. awamori transformation and expression vector, pGPT-pyrG (glucoamylase promoter terminator-pyrG), was constructed as follows: The vector (Figure 20) contains the A. awamori glucoamylase promoter region [18] and the A. niger glucoamylase terminator [19]. This vector also contains an origin of replication and ampicillin resistance gene from pBR322 [20] and the pyrG gene from

A. nidulans for selection of fungal transformants [21]. First, a 2.9 kb XhoI-BglII restriction fragment containing the A. awamori glucoamylase promoter region and a portion of the coding sequence was subcloned into XhoI and BglII digested pUC219. Secondly, a BglII site was introduced by oligonucleotide-directed mutagenesis at a site 11 nucleotides upstream of the start codon for glucoamylase. As a result, the 18 nucleotides including the start codon were 5' CATTAGATCTCAGCAATG 3'. The mutagenesis was verified by DNA sequence analysis. Next, the A. niger glucoamylase terminator region was obtained as a 1.7 Kb XbaI-HindIII fragment from plasmid pBR-GRG1 [22]. This fragment was subcloned into pUC100, a pUC18 derivative which contains the following polylinker sites: EcoRI, SacI, KpnI, SmaI/XmaI, BamHI, XhoI, BglII, ClaI, XbaI, SalI/AccI/HincII, PstI, SphI, and HindIII (C. Barnett, unpublished). The resulting plasmid, pUC-GT, was digested with XhoI and BglII, then ligated with a 1.9 Kb XhoI-BglII fragment containing the A. awamori glucoamylase promoter (described above). The plasmid derived from this ligation, designated pUC-GPT, was cleaved with XhoI and HindIII, and the 3.5 kb insert was cloned into pBR-XClink [22] which had been digested with the same enzymes. The vector which was produced, pBR-GPT, was then digested with PvuII and NruI and dephosphorylated. Into this vector fragment we inserted a 1.8 Kb ScaI-NaeI restriction fragment encoding the A. nidulans pyrG gene [21] to derive the expression vector pGPT-pyrG1.

4. Transformation Procedure for Aspergillus awamori

A. awamori strain GC12 was protoplasted as described [22] and transformed by an electroporation technique developed by Ward, et al. [23]. Approximately 4 transformants per microgram of DNA were obtained.

5. Culture Conditions

A. awamori CBHII transformants were grown in glucoamylase induction medium containing 1 g/l Bacto Peptone, 20 g/l Malt extract, 1 g/l Yeast extract (Difco, all), 6 g/l NaNO₃, 0.52 g KCl, 34 g/l KH₂PO₄, 1.0 g/l MgSO₄·7H₂O, 50 g/l maltose, 1g/l trace elements(1.0 g/l, FeSO₄·7H₂O, 8.8 g/l ZnSO₄·7H₂O, 0.4 g/l CuSO₄·5H₂O, 0.15 g/l MnSO₄·4H₂O, 0.10 g/l Na₂B₄O₇·10H₂O, 50 mg/l [NH₄]₆Mo₇O₂₄·4H₂O), 10 ml/l met-bio solution (50 g/l L-methionine, 200 mg/l d-Biotin), 0.1% tween 80, 50 mg/l streptomycin. All cultures were inoculated with conidia to a final concentration of 1X10⁶/ml and grown for 4 days at 37° on a rotary shaker (New Brunswick Scientific Company, Inc. at 200 rpm). Cultures were filtered through glass wool (Corning Glass Works) and filtrates collected for enzyme purification.

6. Isolation and Analysis of Nucleic Acids

A. awamori DNA and RNA were isolated as described previously [24]. Total genomic DNA samples from selected A. awamori CBHII transformants were digested with an appropriate restriction enzyme, fractionated on 0.5% agarose gels, blotted to Nytran (Schleicher & Schuell, Keene, NH), and analyzed for the presence of CBHII specific fragments by the method of Southern [25]. Total RNA from A. awamori transformants was electrophoretically fractionated on 1% formaldehyde-agarose gels, blotted to Nytran membranes and analyzed for CBHII specific mRNA by the Northern blot method [26]. Probes used for hybridization were radioactively labeled by nick-translation [27].

7. Site Specific Mutagenesis

Oligonucleotide-directed site specific mutagenesis was performed using the method described by Carter [17].

8. Enzyme Purification

One liter cultures of CBHII expressing transformants were concentrated twenty-fold and buffer exchanged with 5mM sodium phosphate buffer, pH 7.8. Recombinant CBHII protein was purified by FPLC using a Pharmacia MonoQ cation exchange column equilibrated in 5mM sodium phosphate buffer at pH 7.8. A 1 ml sample was loaded onto the MonoQ column at a flow rate of 0.5 ml/mm.

Bound protein was eluted from the column using a step gradient. The elution buffer used was 50mM sodium phosphate pH 7.5 + 0.5M NaCl.

9. Protein Determination

See Pierce product 23220/23225, BCA Protein Assay Reagent.

10. Reducing Sugar Determination

The spectrophotometric measurement of the release of reducing sugar residues has been employed extensively in following the action of polysaccharide hydrolases. This method has been described by Nelson and Somogyi [7, 8]. The Somogyi reagent was filtered before use and the absorbance at 510 nm was measured within a half-hour after addition of the chromagen.

11. HPLC

Reaction supernatants were analyzed by HPLC on a Bio-Rad HPX65A column at 80°C. Glucose and cellobiose (Sigma) were used as standards.

12. Antibody analysis

Antibodies specific for T. reesei CBHII were isolated by ion-exchange chromatography. Production of anti-sera to CBHII has been described previously [28, 29].

Immunoblotting (Western blotting) techniques were performed as described previously [30]

Enzyme-linked-immunosorbent assays have been described previously [22].

13. Endo H Treatment

Samples were buffer exchanged with 0.5M sodium citrate, pH 5.0, then denatured at 95°C for five minutes. After cooling to room temperature, samples were incubated with purified endo-H (final concentration of endo-H was 0.2mg/ml in the reaction mixture). Reaction time was 24 hours at room temperature. Normal SDS PAGE and Western blot methods were used for protein migration analysis.

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TABLE 1

ELISA SCREEN OF rCBHII TRANSFORMANTS:
 CBHII IMMUNOLOGICAL REACTIVE MATERIAL IN SHAKE FLASK CULTURES
 (4.5 DAYS)

| <u>Transformant #</u> | <u>rCBHII (ug/ml)</u> | <u>Transformant #</u> | <u>rCBHII (ug/ml)</u> |
|-----------------------|-----------------------|-----------------------|-----------------------|
| Genomic 1a | <0.05 | Intronless 1a | 28 |
| 1b | <0.05 | 1b | 30 |
| 2a | <0.05 | 2a | 12 |
| 2b | <0.05 | 2b | NA |
| 3a | 31 | 3a | 11 |
| 3b | 19 | 3b | 12 |
| 4a | 10 | 4a | 36 |
| 4b | 13 | 4b | 35 |
| 5a | 2 | 5a | 16 |
| 5b | 3 | 5b | 8 |
| 6a | 11 | 6a | 47 |
| 6b | 10 | 6b | 38 |
| 7a | 11 | 7a | <0.05 |
| 7b | 12 | 7b | <0.05 |
| 8a | <0.05 | 8a | 14 |
| 8b | <0.05 | 8b | 19 |
| 9a | 12 | 9a | <0.05 |
| 9b | 9 | 9b | <0.05 |
| 10a | 15 | 10a | 51 |
| 10b | 13 | 10b | 53 |
| Control | 0 | Control | 0 |

TABLE 2

ELISA SCREEN OF MODIFIED rCBHII TRANSFORMANTS:
 CBHII IMMUNOLOGICAL REACTIVE MATERIAL IN SHAKE FLASK BROTH CULTURES
 (4.5 DAYS)

| <u>Transformant #</u> | <u>rCBHII (ug/ml)</u> | <u>Transformant #</u> | <u>rCBHII (ug/ml)</u> |
|-----------------------|-----------------------|-----------------------|-----------------------|
| E184Q | | E184Q | |
| Genomic | | Intronless | |
| 1a | 7 | 1a | 4 |
| 1b | 7 | 1b | 2 |
| 2a | 16+ | 2a | 3 |
| 2b | 17+ | 2b | 4 |
| 3a | 20+ | 3a | 3 |
| 3b | 14+ | 3b | 5 |
| 4a | 13+ | 4a | <0.05 |
| 4b | 13+ | 4b | <0.05 |
| 5a | 14 | 5a | - |
| 5b | 14 | 5b | - |
| 6a | 8 | 6a | - |
| 6b | 9 | 6b | - |
| 7a | <0.05 | 7a | - |
| 7b | <0.05 | 7b | - |
| 8a | <0.05 | 8a | - |
| 8b | <0.05 | 8b | - |
| 9a | 15 | 9a | 6 |
| 9b | >20 | 9b | 8 |
| 10a | 3 | 10a | 5 |
| 10b | 3 | 10b | 8 |
| Control | 0 | Control | 0 |
| D173N, D175N | | D173N, D175N | |
| Genomic | | Intronless | |
| 1 | 22 | 1 | 30 |
| 2 | <0.05 | 2 | <0.05 |
| 3 | <0.05 | 3 | 3 |
| 4 | <0.05 | 4 | 15 |
| 5 | <0.05 | 5 | 18 |
| 6 | <0.05 | 6 | <0.05 |
| 7 | 5 | 7 | 5 |
| 8 | <0.05 | 8 | <0.05 |
| 9 | <0.05 | 9 | <0.05 |
| 10 | <0.05 | 10 | <0.05 |
| Control | 0 | Control | 0 |

TABLE 3

ELISA SCREEN OF DELETION FORM OF rCBHII TRANSFORMANTS:
CBHII IMMUNOLOGICAL REACTIVE MATERIAL IN SHAKE FLASK BROTH CULTURES
(4.5 DAYS)

| <u>Transformant #</u> | <u>rCBHII (ug/ml)</u> |
|-----------------------|-----------------------|
| Deletion | |
| Genomic 1 | 1 |
| 2 | 0.5 |
| 3 | 26 |
| 4 | 14 |
| 5 | <0.05 |
| 6 | 9 |
| 7 | <0.05 |
| 8 | <0.05 |
| 9 | 1 |
| 10 | <0.05 |
| Control | 0 |

TABLE 4

SPECIFIC ACTIVITY OF NATIVE AND RECOMBINANT
FORMS OF CBHII ON CELLULOSE (PSC)

| CBHII | SPECIFIC ACTIVITY ($\mu\text{mol}/\text{min}/\text{mg}$) |
|--------------------------|---|
| Native | 9.2 |
| Recombinant | 8.7 |
| Recombinant E184Q | 9.0 |
| Recombinant D173N, D175N | <0.01 |

TABLE 5
WORK ACTIVITY TIMELINE

| ACTIVITY | COMPLETION DATE | | | |
|--|-----------------|--------|--------------|---------------|
| | WT | E184Q | D173N, D175N | Δ COOH |
| Gene constructions | 7/87 | 7/87 | 9/87 | 11/87 |
| + transformants | 8/87 | 8/87 | 10/87 | 12/87 |
| E I A | 8/87 | 8/87 | 10/87 | 12/87 |
| PSC (RS, HPLC) | 8/87 | 8/87 | 10/87 | 12/87 |
| Western blots (+/-endo H) | 8/87 1/88 | - - | - - | - - |
| Northern blots | 12/87 | - | - | 1/88 |
| Southern blots | 10/87 | 11/87 | 12/87 | 1/88 |
| Culture broth (\geq 500 ml) | 8/87 | 8/87 | 10/87 | 2/88 |
| Purification | 2/88 | 3/88 | 6/88 | - |
| Characterization (gels, pI, activity) | 4/88 | 5/88 | 6/88 | - |

FIGURE 1

CBHII STRUCTURE-FUNCTION STUDIES

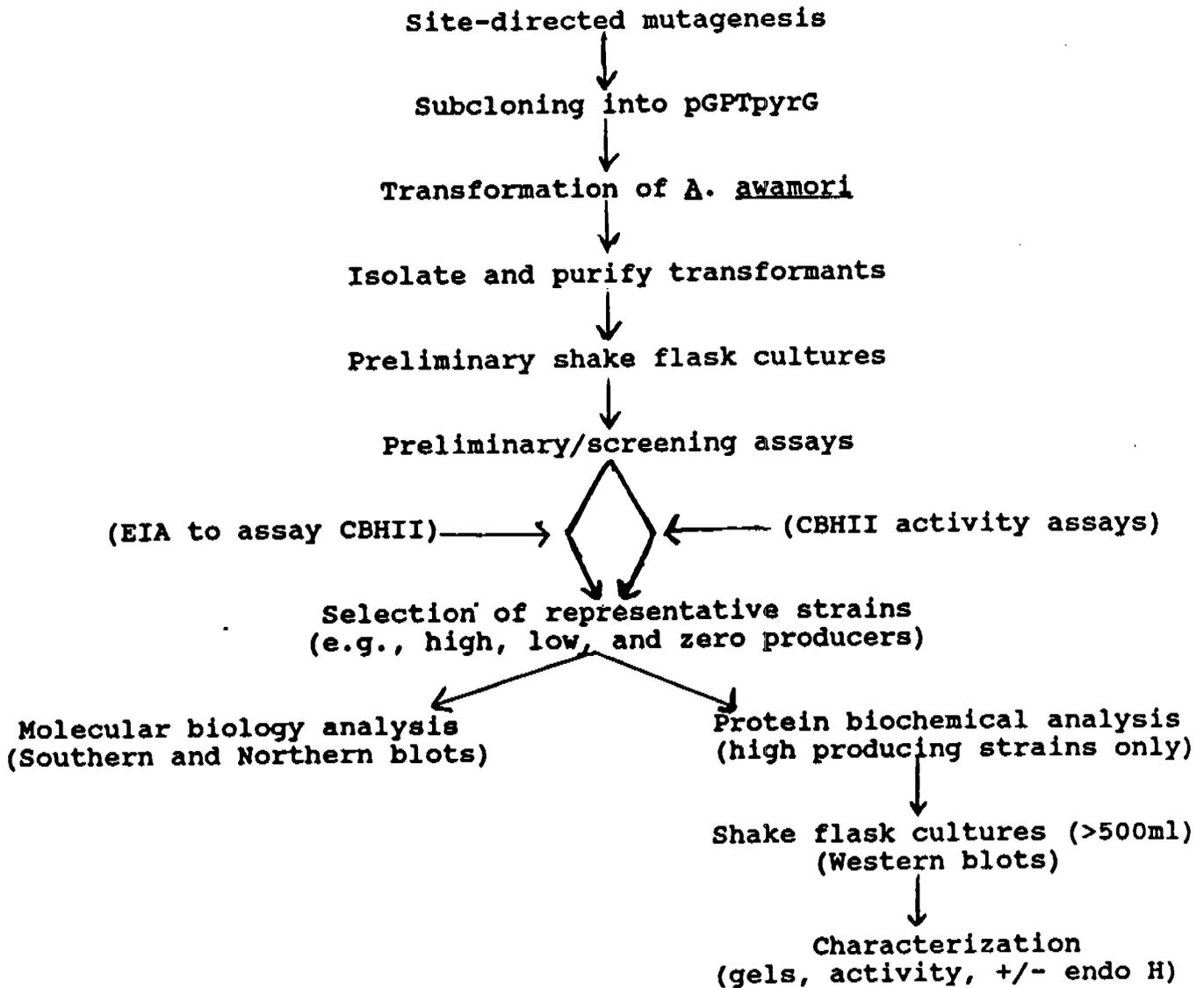


FIGURE 2

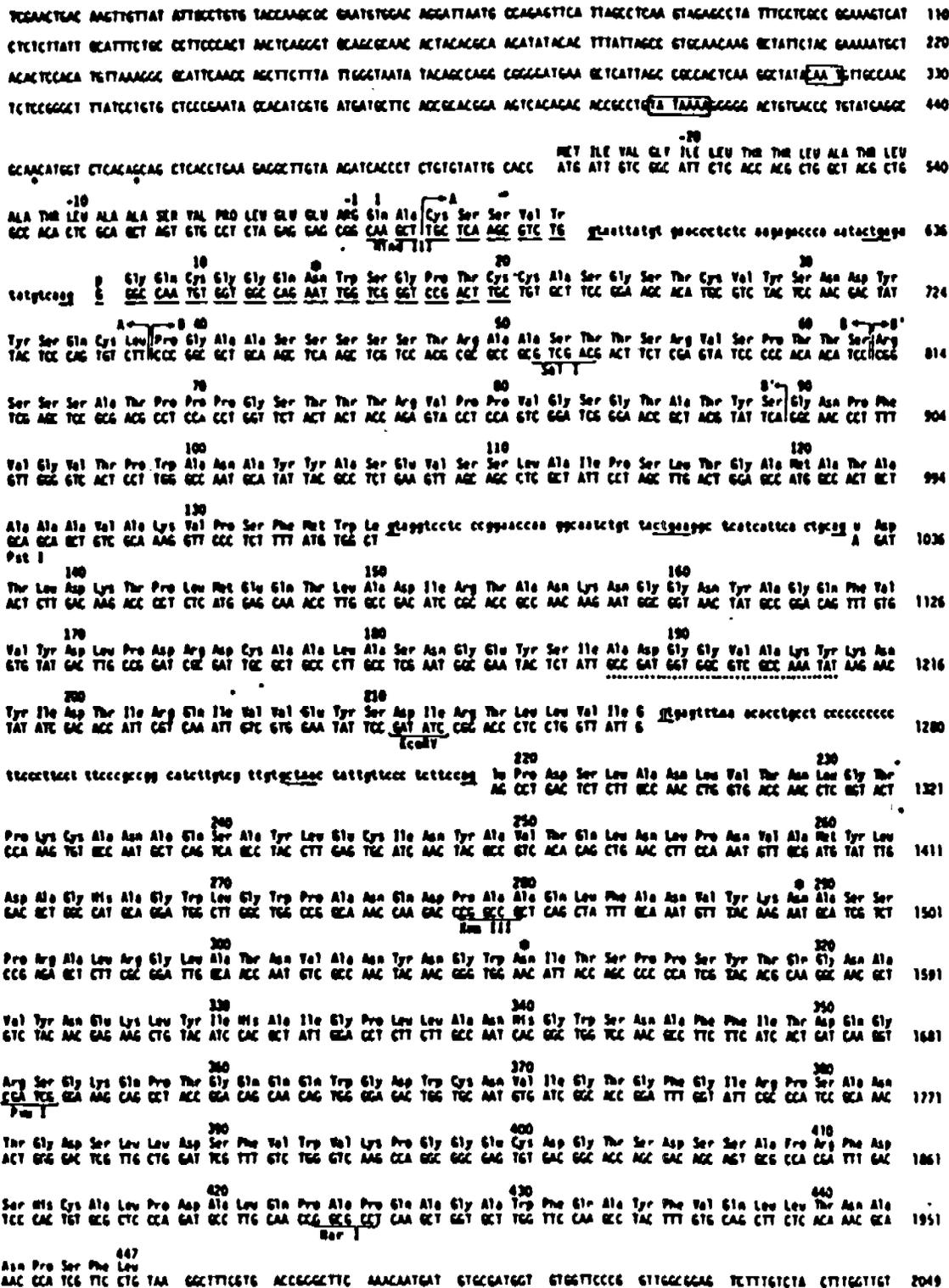
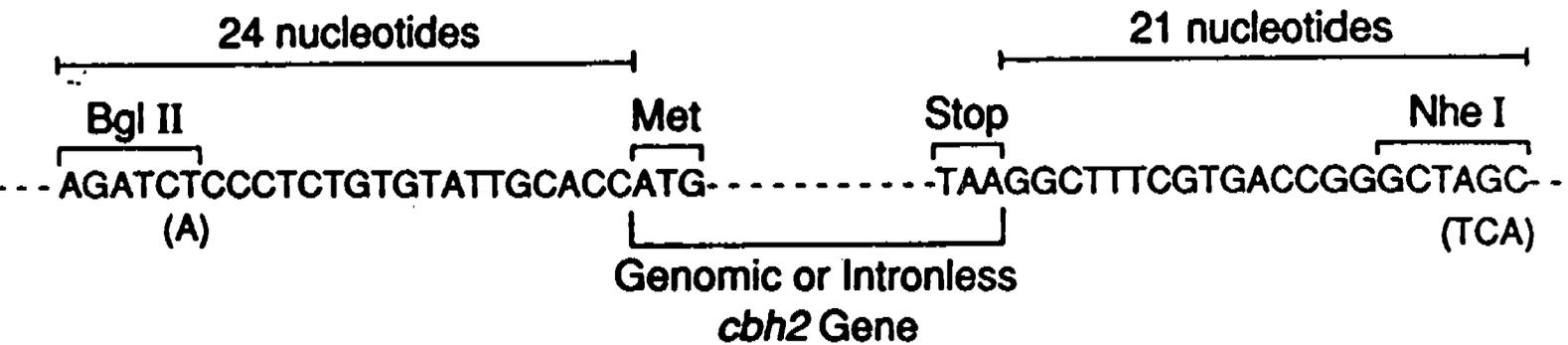


Fig. 1. Nucleotide sequence and the derived aa sequence of the *cbh2* gene. Residues from aa position -24 to position -1 are not found in the mature protein. The N-terminal aa sequence of 20 aa previously determined for the CBH II protein (Fägerstam and Pettersson, 1980) is denoted by lines below the codons. The highly homologous sequences common to all *T. reesei* cellulases are indicated in the sequence by A, B and B' and the putative N-glycosylation sites are shown by asterisks. In addition to the sequence blocks A, B and B', only an octapeptide at aa 188-195 (dotted line under the sequence) is found homologous to the CBH I mature protein at aa 149-157. The significance of this homology is not clear. Introns are indicated by small letters and the putative splicing signals are underlined. The putative TATA-box and the CAAT-sequence in the 5'-flanking region are boxed. The two major transcription start points are shown by dots (under the sequence).

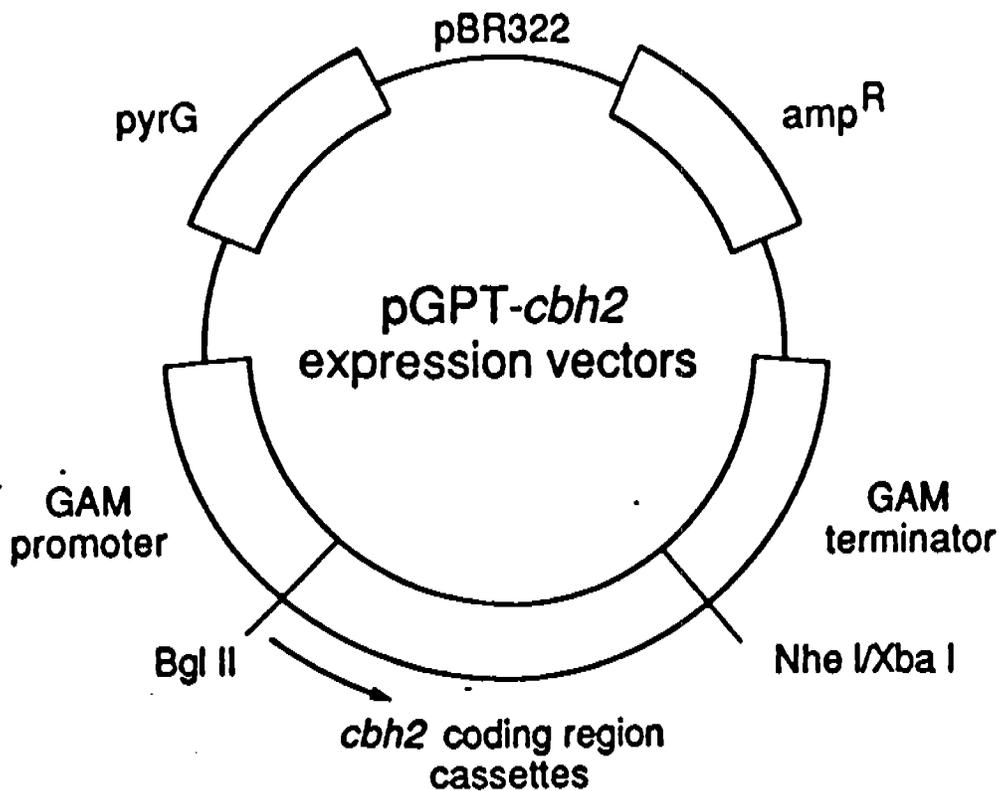
FIGURE 3

Nucleotide Sequence of 5' and 3' *cbh2* Regions



The 5' end of the CBHII gene was mutagenized to insert a Bgl II restriction site and the 3' end was mutagenized to insert an Nhe I restriction site to facilitate fusion to vector promoter and terminator elements. All mutageneses were performed using primer extension mutagenesis.

FIGURE 4



The Aspergillus expression vector, pGPT-pyrG1 has the glucoamylase (GAM) promoter and the GAM terminator with Bgl II and Xba I sites to facilitate insertion of CBHII. Insertion of the CBHII gene into pGPT-pyrG1 for expression in A. awamori.

LEFT GEL: Coomassie blue stained SDS polyacrylamide gel of selected *A. AWAMOXI* CBHII transformants.
RIGHT GEL: Duplicate SDS polyacrylamide gel transferred to nitrocellulose, incubated with rabbit polyclonal alpha-CBHII, and developed with I-125 conjugated *Staphylococcus aureus* protein A (western botted).
BOTH PANELS: Lane A: Purified genomic rCBHII from *A. AWAMOXI*.
Lane B: Purified *T. reesei* CBHII (5 3/4 g).
Lane C: Partially purified rCBHII from *A. AWAMOXI* culture broth including genomic rCBHII and a 68Kd unrelated impurity.
Lane D: Purified intronless rCBHII from *A. AWAMOXI*.

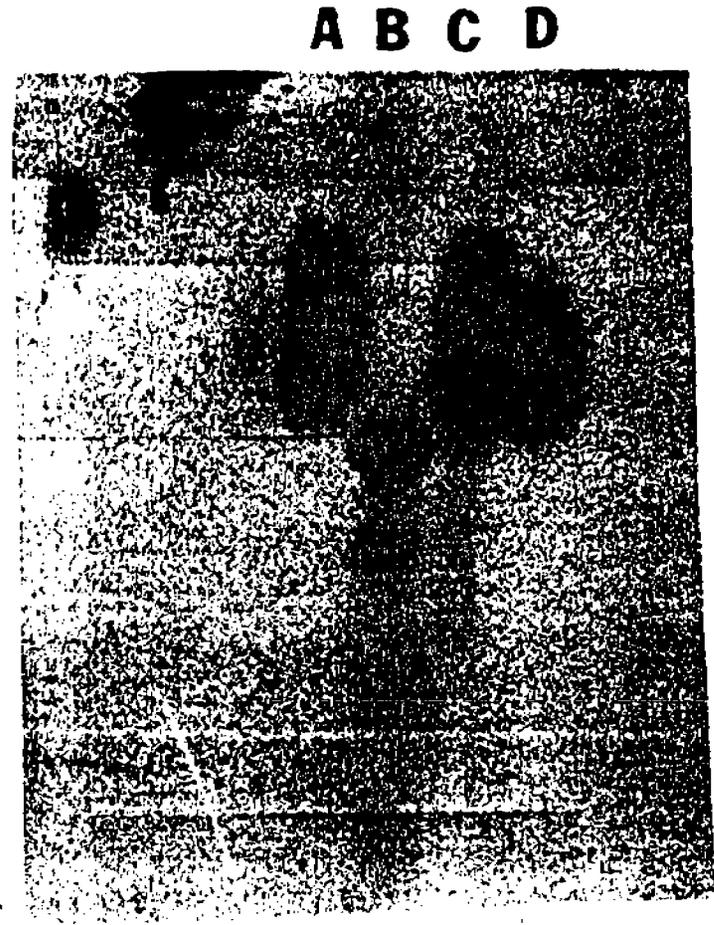
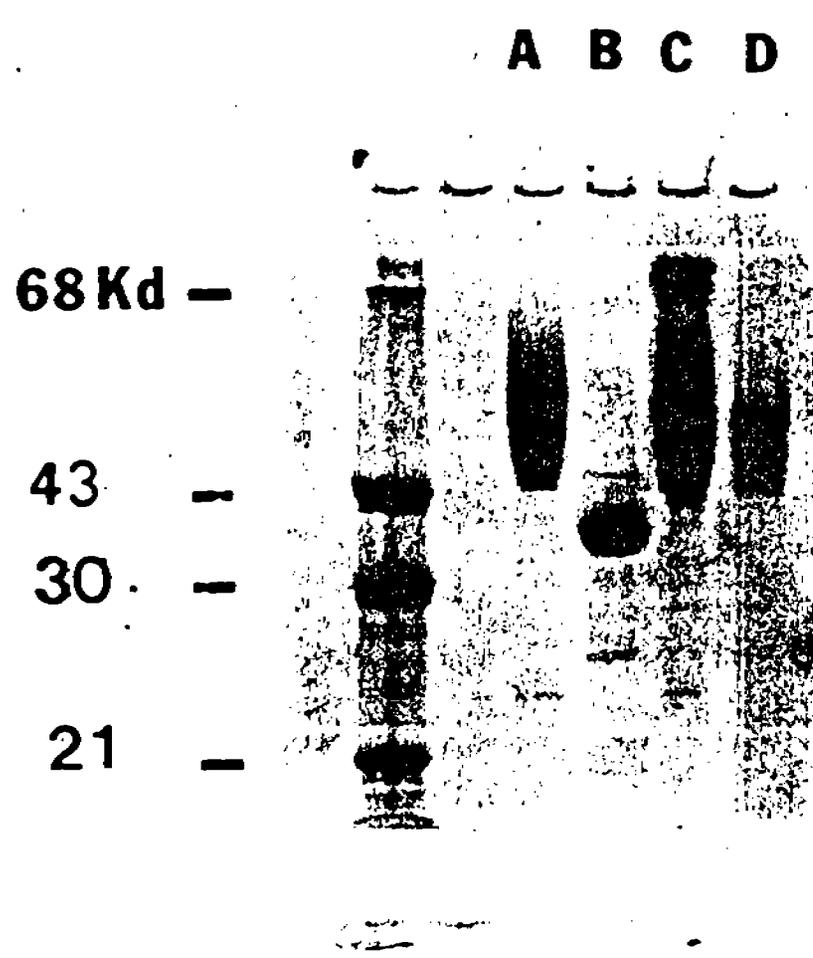


FIGURE 6

Activity Screen of CBHII Transformants in *Aspergillus*

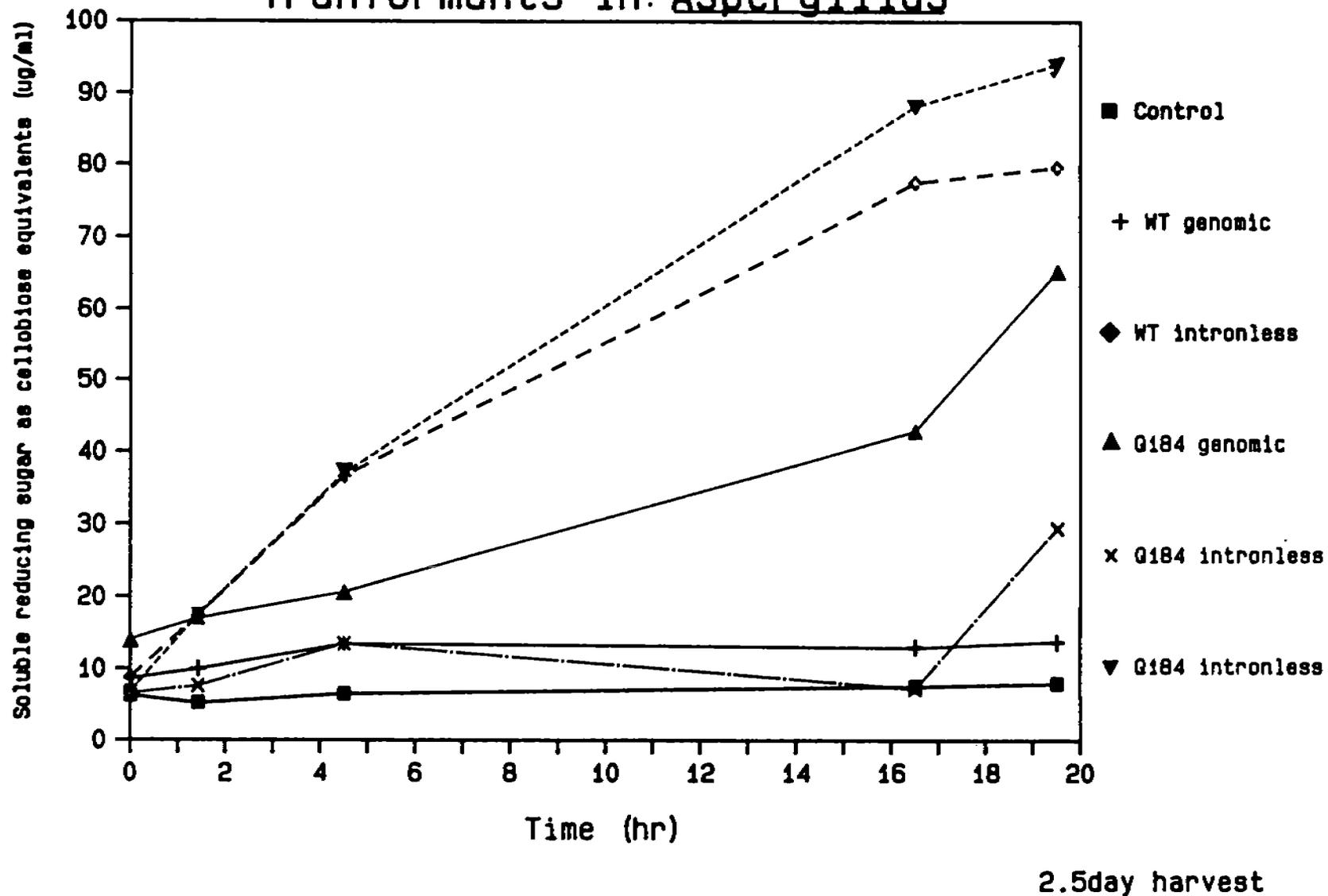


FIGURE 7

Activity Screen of CBHII Transformants in Aspergillus

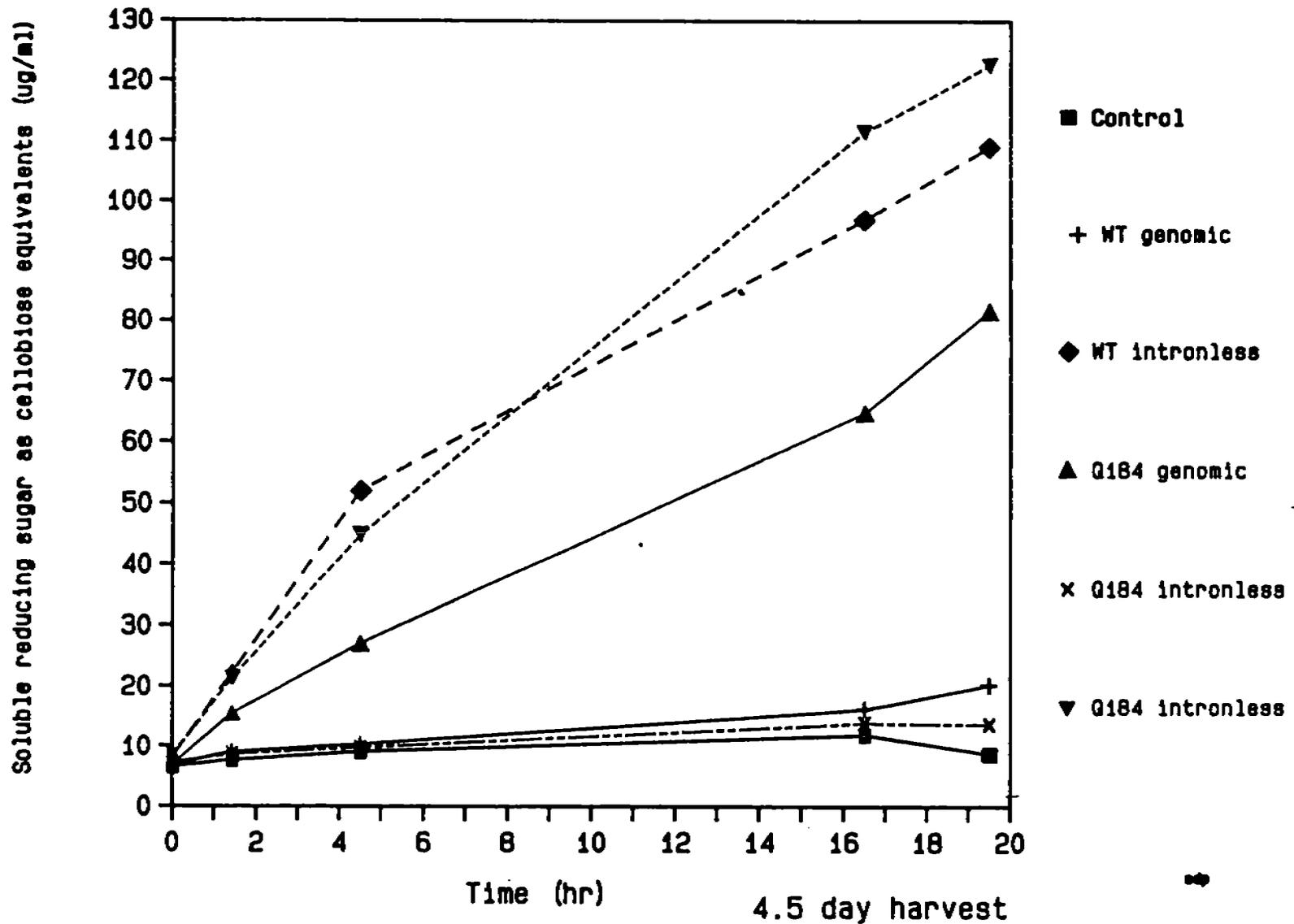
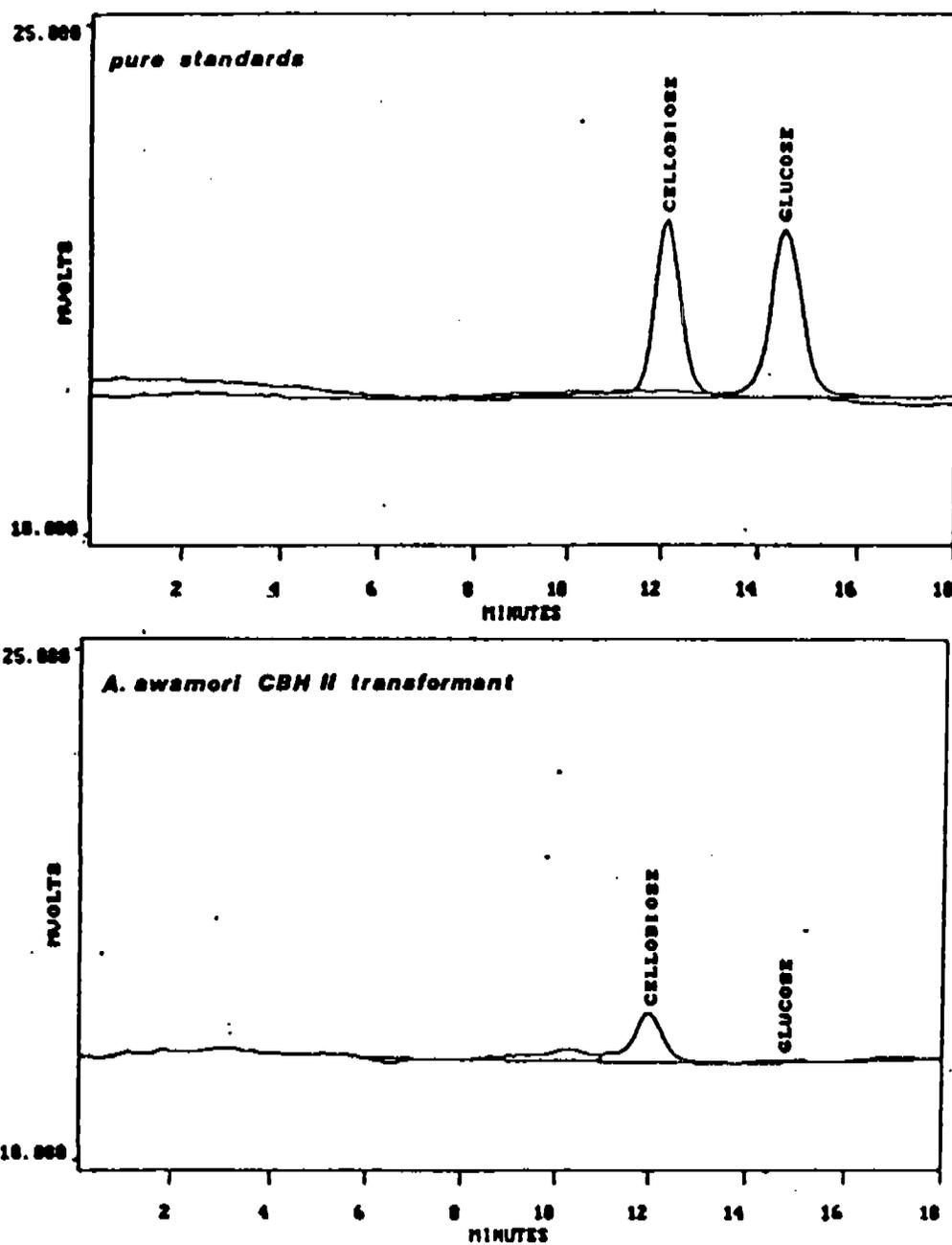


FIGURE 8



Reaction products of recombinant CBHII (rCBHII) analyzed by HPLC on a Bio-Rad HPX65A column using 85°C water for elution. Top figure shows glucose and cellobiose at 1 mg/ml as standards. Bottom figure shows reaction products (cellobiose) from an overnight incubation of 100 3/4l of recombinant CBHII culture filtrate with phosphoric acid swollen cellulose (PSC).

FIGURE 9

HYPOTHETICAL MECHANISM FOR GLUCAN HYDROLYSIS
 CATALYZED BY B-D-GLUCANASES

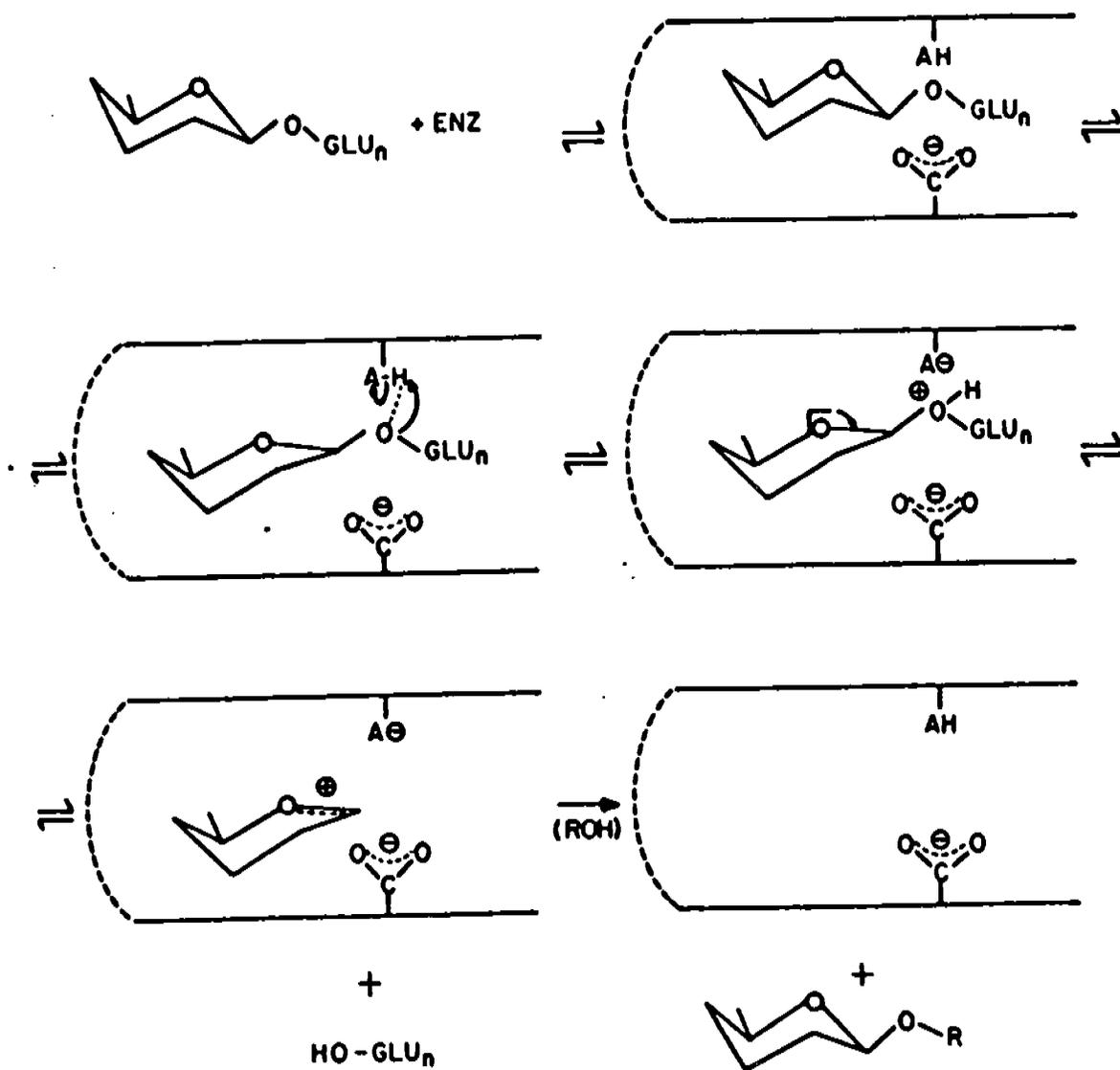


FIGURE 10

cbh2 Coding Region Cassettes

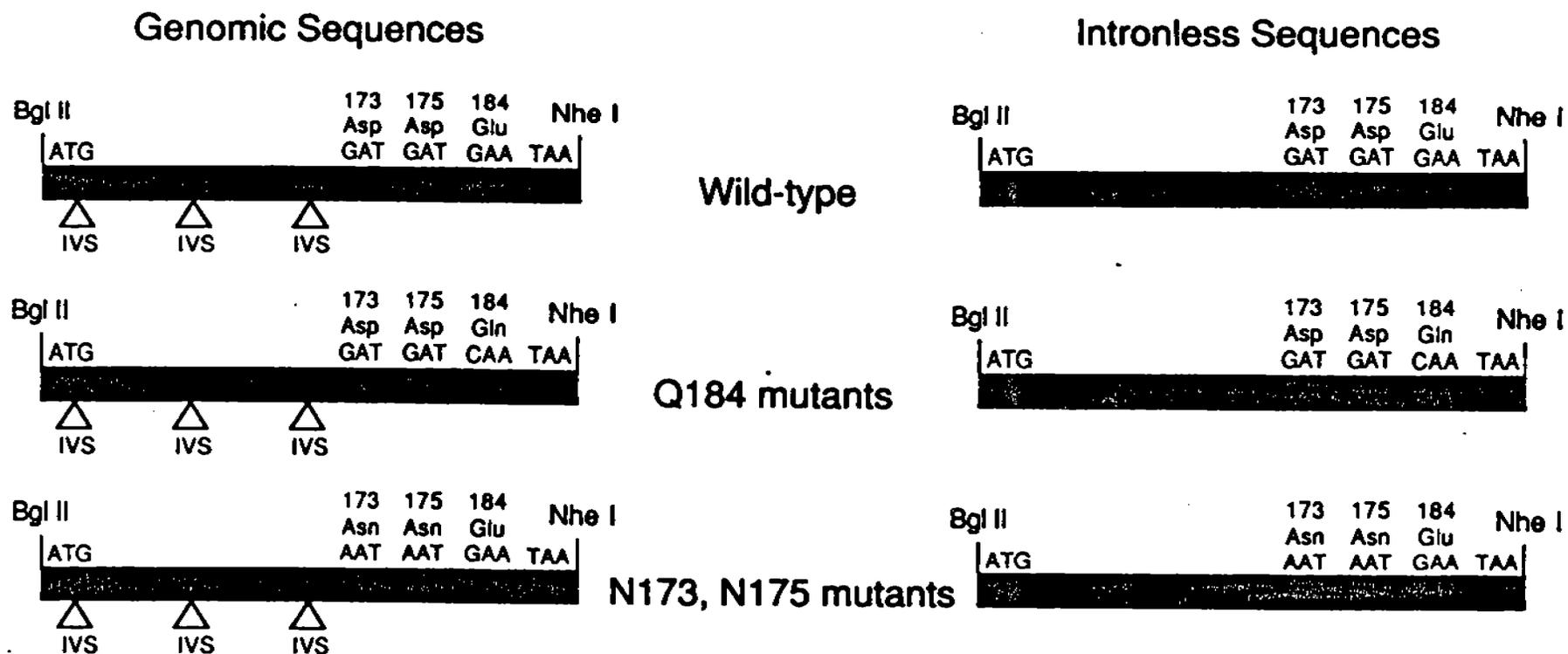


FIGURE 11

NORTHERN BLOT OF COOH-rcBHII TRANSFORMANTS

Lanes A-D are RNA from transformants 2-5. Lanes E and F are negative and positive controls, respectively.

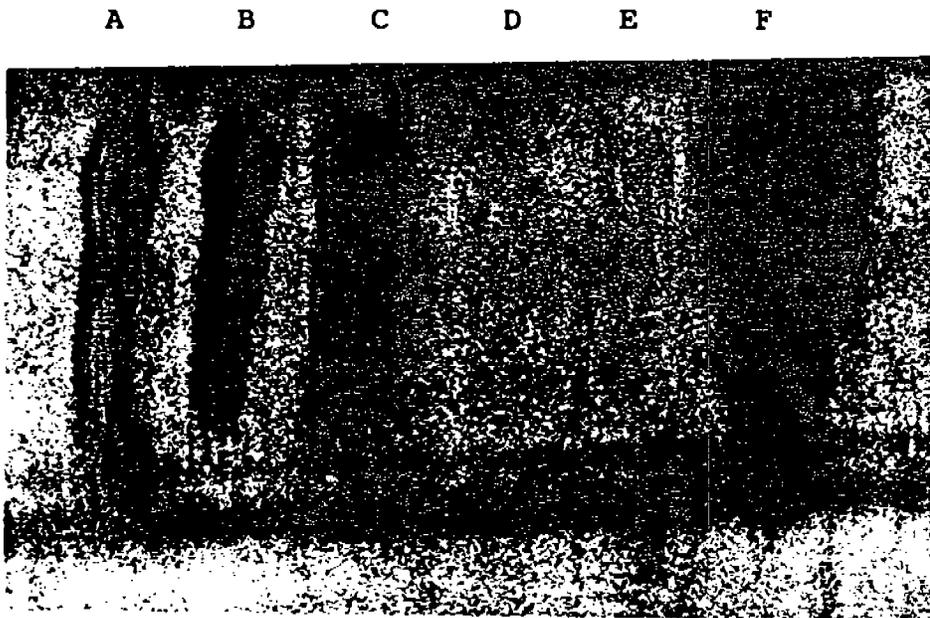


FIGURE 12

FPLC TRACE: PURIFICATION OF WILDTYPE CBHII ENZYME

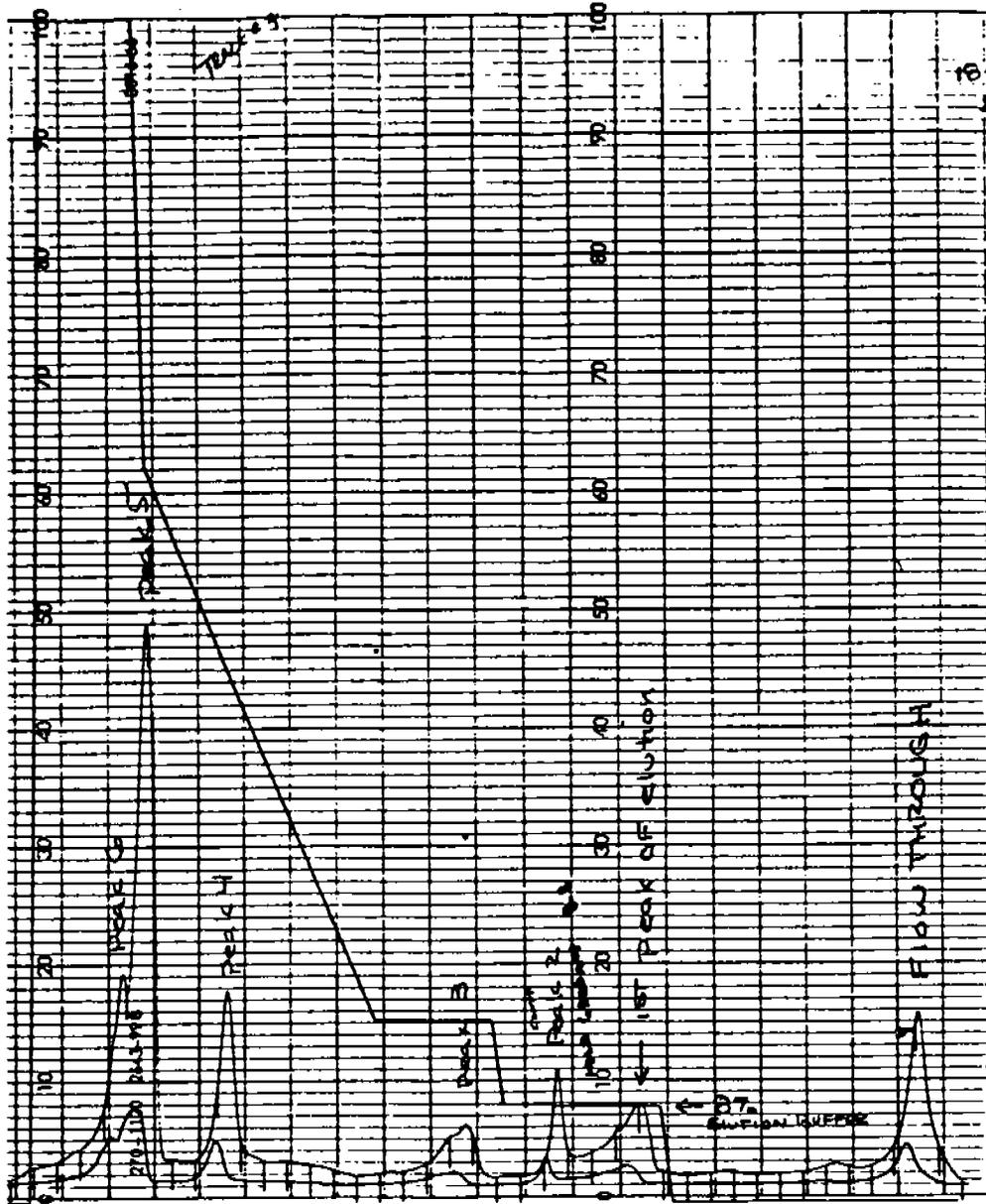
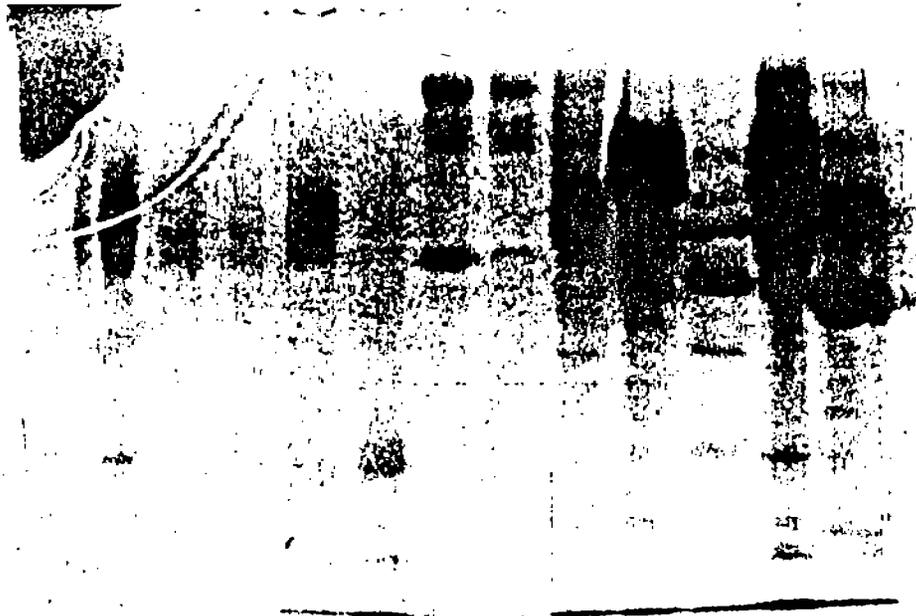


FIGURE 13

SDS AND WESTERN GEL ANALYSIS OF PROTEINS ELUTED OFF
MONO Q COLUMN
WILD TYPE rCBHII PURIFICATION

Lanes A-J are column fractions. Lanes A-C are flow through; D-E
are peak1; F-J are peaks 2-6; K is column starting material; L is
native CBHII (note: impure).



A B C D E F G H I J K L

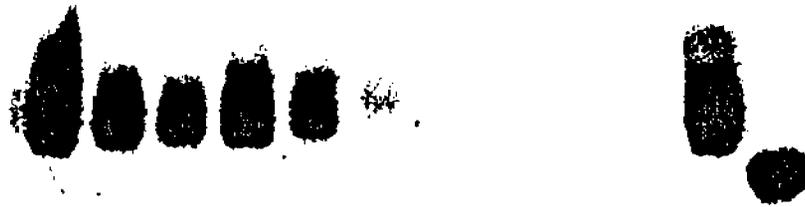


FIGURE 14

SDS AND WESTERN GELS ANALYSIS OF +/- ENDO H TREATED
RECOMBINANT AND NATIVE CBHII.

Lane A is standards; lane B is endo H; lanes C and D are rCBHII
without and with endo H; and lanes E and F are native CBHII (top
band) with and without endo H.

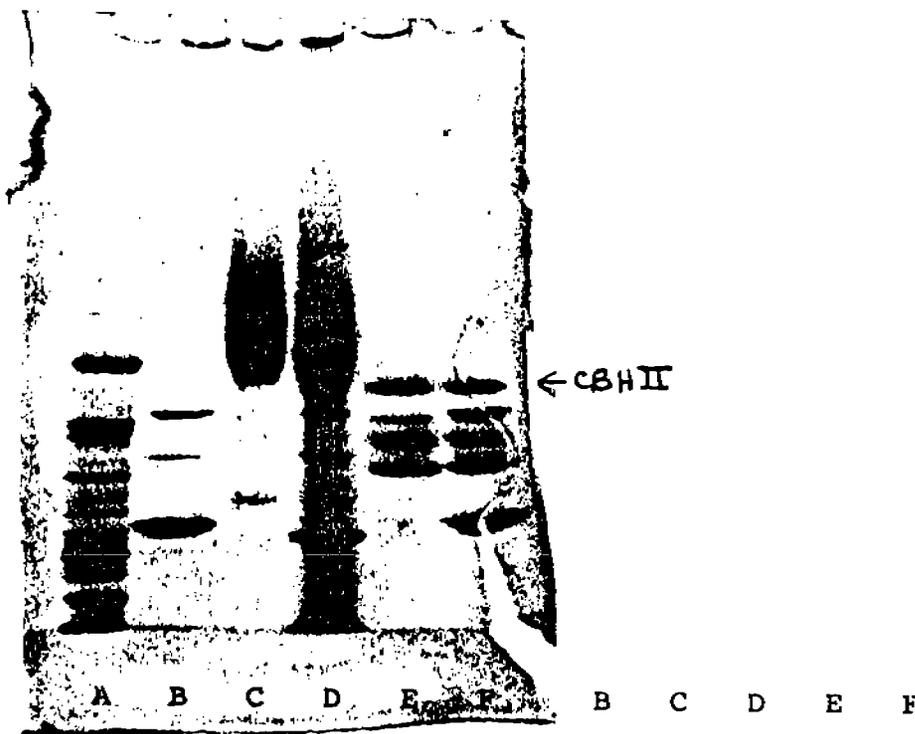


FIGURE 15

FPLC TRACE: PURIFICATION OF Q184 MUTANT CBHII ENZYME

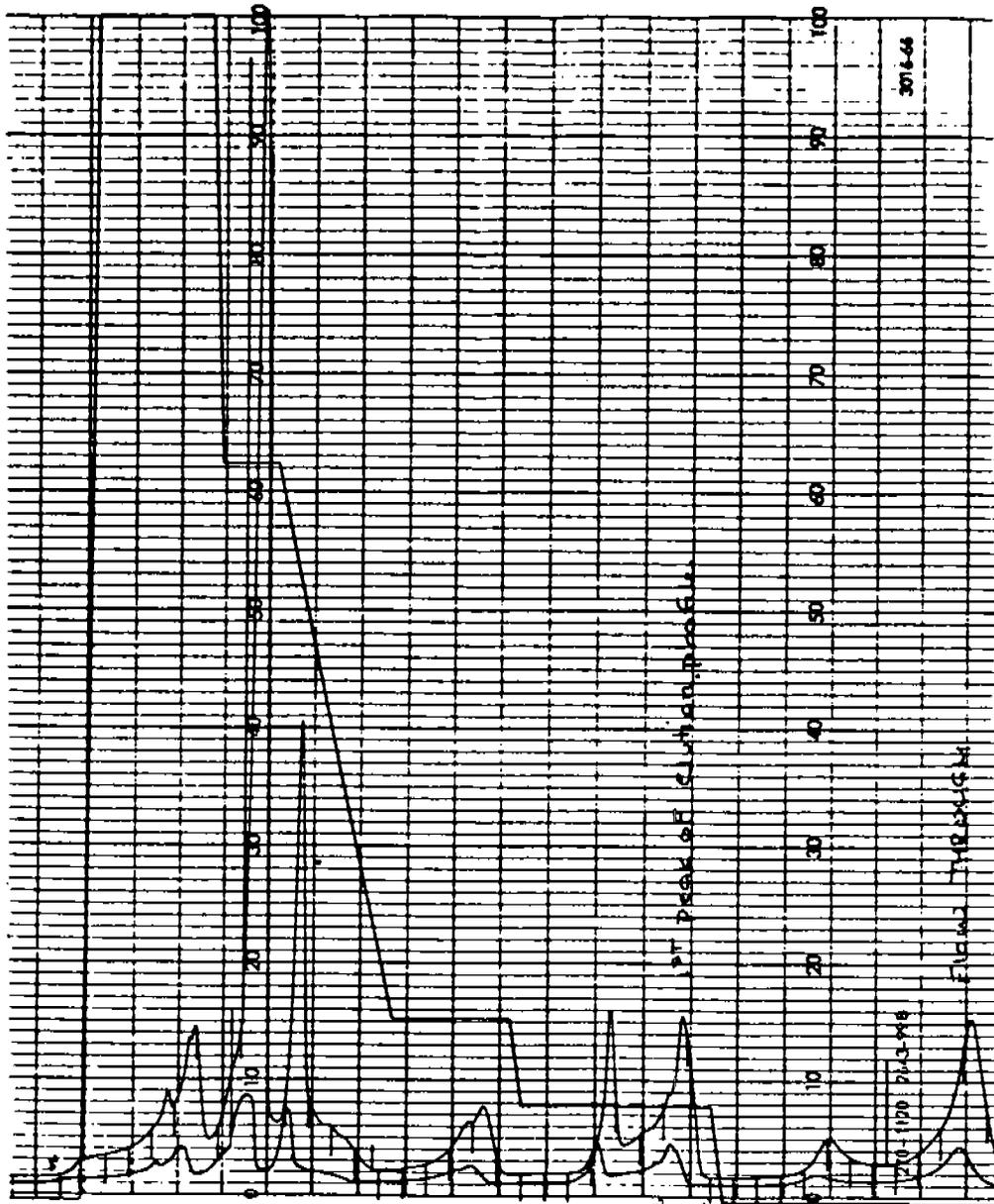


FIGURE 16

FPLC TRACE: PURIFICATION OF D173N/D173N MUTANT CBHII ENZYME

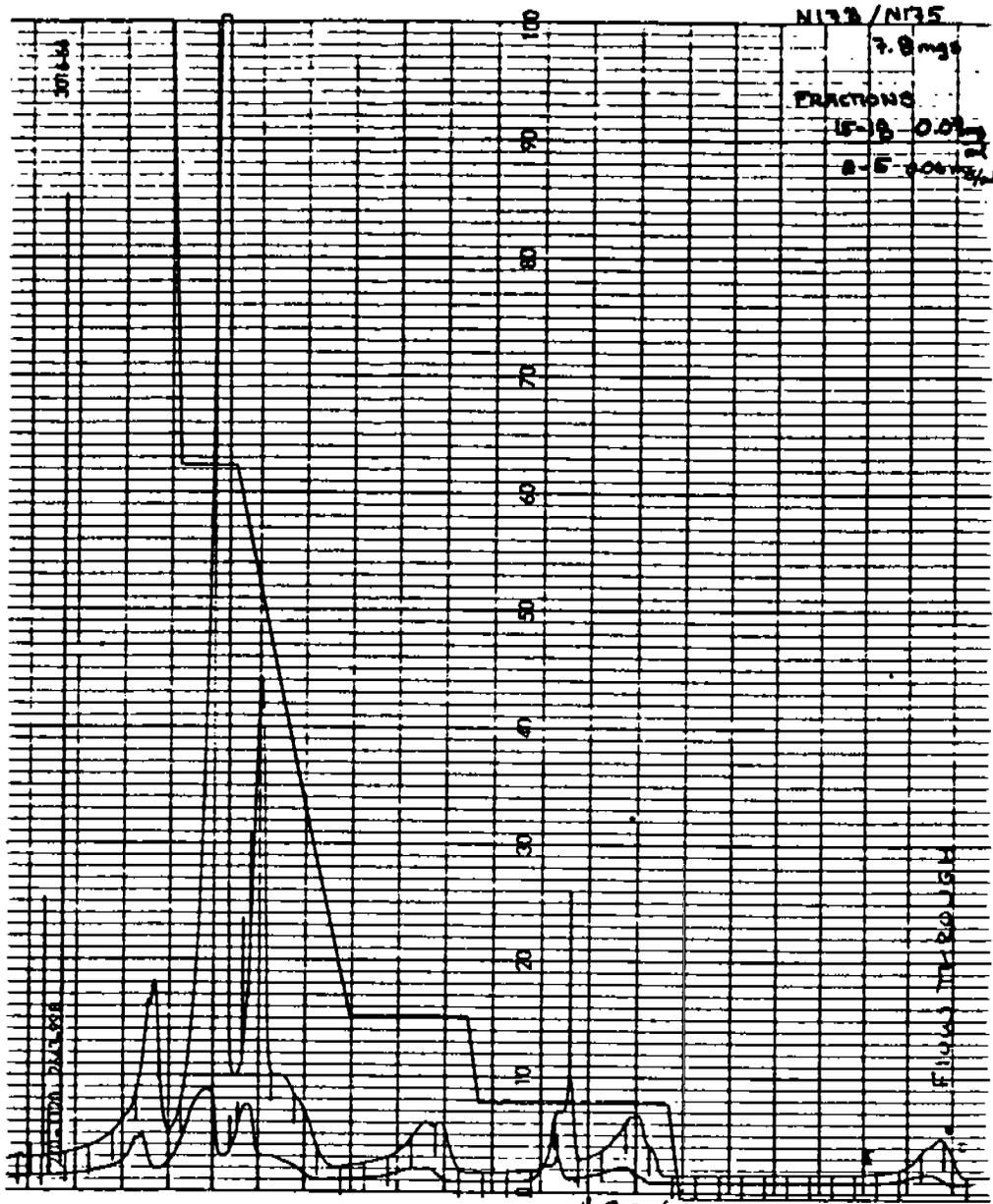


FIGURE 17

FPLC TRACE: STRAIN 12 CONTROL BROTH

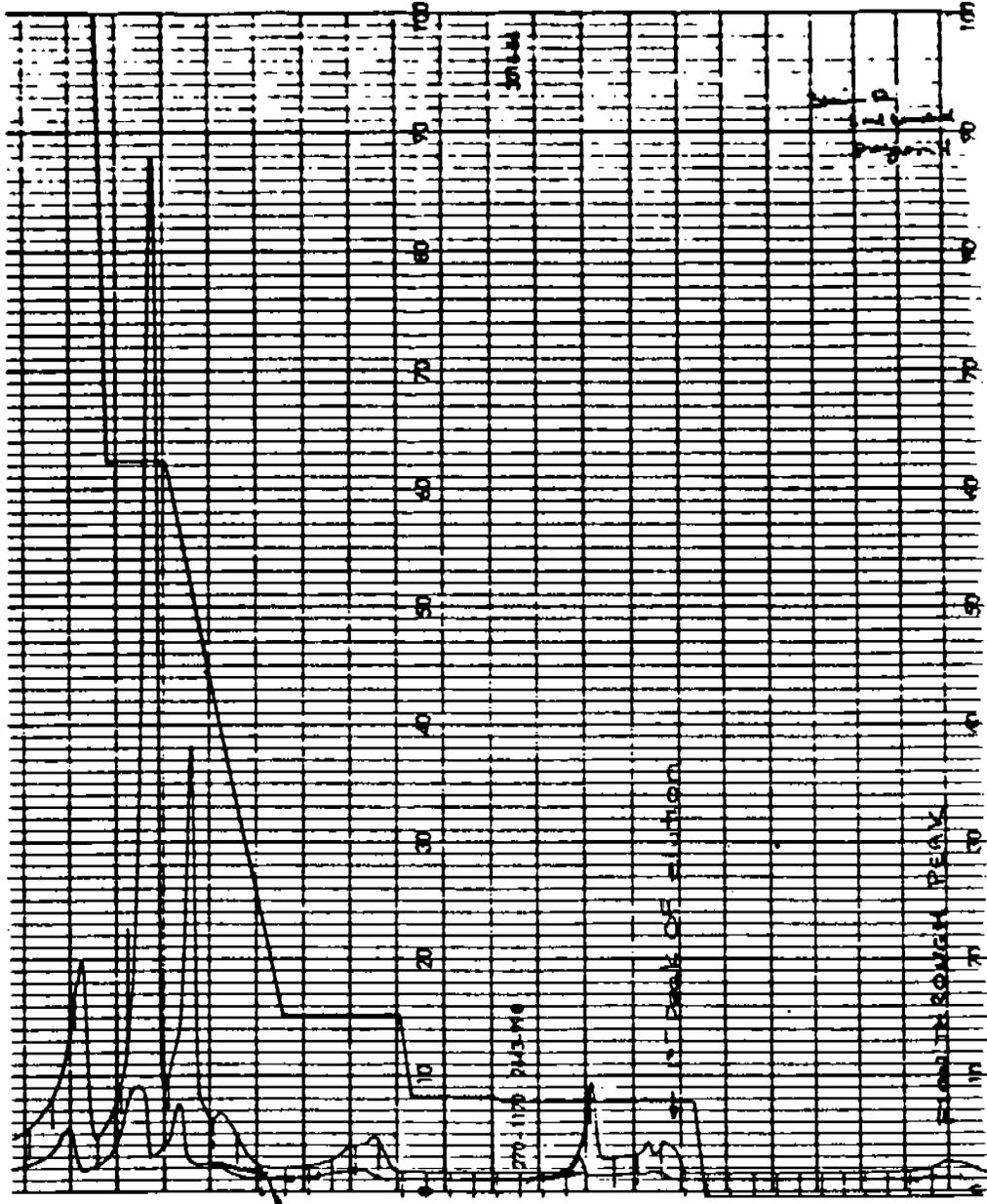


FIGURE 18

