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Analysis of the Active Center of Branching Enzyme II from Maize Endosperm

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Analysis of the primary structure of mBEII, with those of other branching and amylolytic enzymes as reference, identifies four highly conserved regions which may be involved in substrate binding and in catalysis. When one of the amino acid residues corresponding to the putative catalytic sites of mBEII, i.e., Asp-386, Glu-441, and Asp-509, was replaced, activity disappeared. These putative catalytic residues are located in three different regions (regions 2–4) of the four highly conserved regions (regions 1–4) which exist in the primary structure of most starch hydrolases and related enzymes, including branching enzymes. Region 3, which contains Glu-441 as one of the putative catalytic residues, was located downstream of the carboxyl-terminal position previously reported. The importance of the carboxyl amino acid residues was also demonstrated by chemical modification of the branching enzyme protein using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide.

KEY WORDS: Branching enzyme; active center; site-directed mutagenesis; EDAC modification; α-amylase family.

1. INTRODUCTION

Starch branching enzyme (EC 2.4.1.18) plays a fundamental role in starch biosynthesis [for a review see Preiss (1991)] by cleaving α-1,4-glucosidic linkages and forming new α-1,6-glucosidic linkages. Multiple forms of branching enzyme have been identified in many plants, e.g., maize endosperm (Boyer and Preiss, 1978), pea seed (Smith, 1988), and rice endosperm (Mizuno *et al.*, 1993; Y. Nakamura *et al.*, 1992). The genes coding for the enzymes have been cloned from various sources, e.g., maize endosperm (Baba *et al.*, 1991; Fisher *et al.*, 1993), pea seed (Bhattacharyya *et al.*, 1990), potato tuber (Kossmann *et al.*, 1991; Poulsen and Kreiberg, 1993), and rice endosperm (Mizuno *et al.*, 1993; Y. Nakamura and Yamanouchi, 1992).

The cDNAs encoding the mature branching enzymes I and II of maize endosperm (mBEI and mBEII) have been expressed in *Escherichia coli* using the T7 promoter (Guan *et al.*, 1994*a,b*), establishing a basis for studying the relationship between structure and function of the mBEs using site-directed mutagenesis.

The homology observed in the primary structures of glycogen branching enzyme and amylolytic enzymes was first reported by Romeo *et al.* (1988). Later Baba *et al.* (1991) pointed out that branching enzymes contain the four highly conserved regions which are also present, and highly conserved, in α-amylases, pullulanase, isoamylase, and cyclodextrin glucanotransferases, and showed that the branching enzymes belong to the family of amylolytic enzymes. Structure–prediction and hydrophobic-cluster analysis of the enzymes mentioned above indicated that all the enzymes possess a catalytic (β/α)8-barrel (Jespersen *et al.*, 1991) as

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observed in the crystal structure of α-amylases (Matsuura *et al.*, 1984; Buisson *et al.*, 1987; Boel *et al.*, 1990) and cyclodextrin glucanotransferases (Klein and Schulz, 1991; Kubota *et al.*, 1991).

Neopullulanase catalyzes the hydrolysis of α-1,4-glucosidic linkages and α-1,6-glucosidic linkages as well as transglycosylation to form α-1,4-glucosidic linkages and α-1,6-glucosidic linkages (Kuriki *et al.*, 1988; Takata *et al.*, 1992). The replacements of several amino residues which constitute the active center of neopullulanase proved that one active center of the enzyme participated in all four reactions described above (Kuriki *et al.*, 1991). This work suggested that in addition to the structures of branching enzyme, α-amylase, pullulanase/isoamylase, and cyclodextrin glucanotransferase being similar, the catalytic mechanisms also may be similar (Takata *et al.*, 1992). The functions of these enzymes are as follows: branching enzyme catalyzes transglycosylation to form α-1,6-glucosidic linkages; α-amylase catalyzes hydrolysis of α-1,4-glucosidic linkages; pullulanase/isoamylase catalyzes hydrolysis of α-1,6-glucosidic linkages; and cyclodextrin glucanotransferase catalyzes transglycosylation to form α-1,4-glucosidic linkages. Based on these results, a definition of an enzyme family, the α-amylase family, was established that includes enzymes that catalyze hydrolysis and transglycosylation at α-1,4- and α-1,6-glucosidic linkages (Takata *et al.*, 1992; Kuriki, 1992; Svensson, 1994; Kuriki and Okada, 1995).

Here, we report that Asp-386, Glu-441, and Asp-509 of mBEII play critical roles in the enzymatic reaction as suggested by the conservation of these three residues in other enzymes of the α-amylase family. Preliminary chemical modification experiments showing the importance of carboxyl amino acid residues in mBEII are also described.

2. MATERIALS AND METHODS

2.1. Media

LB Medium (10 g of tryptone, 5 g of yeast extract, 10 g of NaCl in 1 L of deionized water, adjusted to *p*H 7.0 with NaOH) was used for culture of *Escherichia coli.* Broth 2 × YT (16 g of tryptone, 10 g of yeast extract, and 5 g of NaCl in 1 L of deionized water) was used for the preparation of phage DNA. Ampicillin was used at a final concentration of 100 μg/ml.

2.2. Bacterial Strains, Plasmids, and Phages

E. coli TG-1 [supE hsdΔ5 thi Δ(lac-proAB) F′(traD36 proAB+ lacIqlacZΔM15)] (Sambrook *et al.*, 1989) was used as a host for the site-directed mutagenesis and DNA manipulation. *E. coli* BL21 (DE3) [hsdS gal(cIts857 ind1 Sam7 nin5 lacUV5-T7 gene1] (Novagen, Madison, WI) was used to express the gene for wild-type and mutated mBEII under the control of the T7 promoter (Guan *et al.*, 1994*a,b*). Plasmid pET-23d-MBEII (*Apr* encoding the gene for mature mBEII) was described previously (Guan *et al.*, 1994*b*, 1995). M13mp19 was used for the preparation of single-stranded DNA (Messing, 1983).

2.3. Site-Directed Mutagenesis

Mutagenesis was done using the SculptorTM *in vitro* mutagenesis system (Amersham Corp., Amersham, England). Oligonucleotides (Fig. 1) were synthesized using an Applied Biosystems model 380A DNA synthesizer at the Macromolecular Facility, Department of Biochemistry, Michigan State University. The mutations were confirmed by DNA sequencing. DNA sequencing was done by the dideoxy chain-terminating method (Sanger *et al.*, 1977). The sequence reaction started from the M13 linker region with the universal primer or was primed by internally annealing 17-mer synthetic oligonucleotides.

2.4. Preparation and Purification of Wild-Type and Mutated BEII

E. coli BL21 (DE3) carrying a recombinant plasmid encoding wild-type or mutated mBEII gene was incubated overnight in LB medium containing 100 μg/ml ampicillin. This culture was then diluted 1:20 (v/v) in fresh LB medium containing 100 μg/ml ampicillin and the cells were grown at 37°C to mid-log phase (optical density at 600 nm = 0.6) before the expression of the mBEII cDNA was induced by addition of isopropyl-β-D-thiogalactopyranoside (final concentration 0.5 mM). Following

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Mutation            Template DNA   5'-GGT TTC CGT TTT GAT GGT GTG ACC-3'
                                      Gly Phe Arg Phe Asp Gly Val Thr
Asp386→   Asn   Oligonucleotide     3'-AAG GCA AAA TTA CCA CAC T-5'
      →   Glu                         3'-G GCA AAA CTT CCA CAC TGG-5'

                Template DNA   5'-GGG AAC TTC AAT GAG TAT TTT GGC-3'
                                      Gly Asn Phe Asn Glu Tyr Phe Gly
Glu408→   Gln   Oligonucleotide     3'-TTG AAG TTA GTC ATA AAA C-5'

                Template DNA   5'-GGA CTT TAT CCT GAG GCT GTA ACC-3'
                                      Gly Leu Tyr Pro Glu Ala Val Thr
Glu435→   Gln   Oligonucleotide     3'-GAA ATA GGA GTC CGA CAT T-5'

                Template DNA   5'-GTA ACC ATT GGT GAA GAT GTT AGT-3'
                                      Val Thr Ile Gly Glu Asp Val Ser
Glu441→   Gln   Oligonucleotide     3'-TGG TAA CCA GTT CTA CAA T-5'
      →   Asp                         3'-G TAA CCA CTA CTA CAA TCA-5'

                Template DNA   5'-GCT GAA AGT CAT GAT CAA GCA TTA-3'
                                      Ala Glu Ser His Asp Gln Ala Leu
Asp509→   Asn   Oligonucleotide     3'-CTT TCA GTA TTA GTT CGT A-5'
      →   Glu                         3'-T TCA GTA CTT GTT CGT AAT-5'
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Fig. 1. Synthetic oligonucleotide primers used for site-directed mutagenesis. Underlined nucleotides denote differences from the wild-type sequence.

growth at 25°C for 12 hr, cells were harvested by centrifugation (8000 × *g*, 5 min). The cell pellet was resuspended and lysed by sonication in 50 mM Tris acetate buffer (*p*H 7.5) containing 10 mM EDTA and 5 mM dithiothreitol (DTT). The lysed suspension was then centrifuged at 27,000 × *g* for 20 min and the supernatant (cell extract) was used as crude enzyme for preliminary assay of the mutated mBEIIs. Purification of wild-type or mutated mBEII from the cells was done as described previously (Guan *et al.*, 1994*b*). The mutated enzyme fractions which did not show detectable activity were analyzed by dot-blotting and antigen–antibody complex formation using antibody raised against mBEII (Guan *et al.*, 1994*b*).

2.5. Assay of BE Activity

BE activity was measured by three different assays as described previously (Guan and Preiss, 1993).

Assay

The phosphorylase a stimulation assay is based on the increased rate of synthesis of α-D-glucan (from α-D-glucose-1-phosphate) catalyzed by rabbit phosphorylase a (Hawker *et al.*, 1974) when branching enzyme is present. One unit of enzyme activity is defined as 1 μmol of glucose incorporated into α-D-glucan per min at 30°C.

Assay b

The branching linkage assay determines the number of branching linkages introduced into reduced amylose used as substrate (Takeda *et al.*, 1993). One unit of enzyme activity is defined as 1 μmol of branching linkage formed per min at 30°C.

Assay c

The iodine stain assay is based on monitoring the decrease in absorbance of the glucan–iodine complex (Boyer and Preiss, 1978). One unit of

enzyme activity is defined as the decrease in absorbance of 1.0 per min at 30°C.

2.6. Protein Assay

Protein concentration was measured with bicinchoninic acid protein assay reagent (Smith *et al.*, 1985) (Pierce Chemical Co., Rockford, IL), using bovine serum albmin as the standard.

2.7. Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS–PAGE) and Immunoblotting

SDS–PAGE was performed on 8% polyacrylamide gels according to the method of Laemmli (1970) and immunoblotting was carried out following the method of Burnette (1981). The primary rabbit antibodies, anti-mBEII and anti-*E. coli* BE (Holmes *et al.*, 1982), were diluted 1:2000 and 1:500, respectively, in phosphate-buffered saline containing 1.35% w/v teleostan gelatin (Sigma Co.). The antigen–antibody complex was detected using anti-rabbit immunoglobulin conjugated with alkaline phosphatase (USB, Cleveland, OH) (diluted 1:10,000) with 5-bromo-4-chloro-3-indolyl phosphate and nitrobluetetrazolium as substrates.

2.8. Chemical Modification of mBEII with 1-Ethyl-3-(3-Dimethylaminopropyl) Carbodiimide (EDAC)

EDAC modification was performed at 25°C by dissolving known quantities of EDAC in MES buffer (0.1 M, *p*H 6.5) containing phenethylamine (25 mM) as described by Plant *et al.* (1987). Reactions were initiated by adding enzyme and terminated by withdrawing aliquots and diluting them 20-fold with reaction mixture for branching enzyme assay. Residual BE activity was determined immediately after the chemical modification.

2.9. Other Procedures

Plasmid or M13 replicative form DNA were prepared by either the rapid alkaline extraction method (Sambrook *et al.*, 1989) or using the Qiagen Plasmid Maxi kit (Qiagen Inc., Chatsworth, CA). Treatment of DNA with restriction enzymes and ligation of DNA were done as recommended by the manufacturer. Transformation of *E. coli* with plasmid DNA and M13 single-stranded template DNA preparation were done as described elsewhere (Sambrook *et al.*, 1989).

3. RESULTS AND DISCUSSION

3.1. Identification of Amino Acid Residues that Participate in the Active Center of mBEII

Structure-prediction and hydrophobic-cluster analysis (Jespersen *et al.*, 1991) indicate that BE, α-amylase, pullulanase, isoamylase, cyclodextrin glucanotransferase, and neopullulanase possess a catalytic (β/α)8-barrel as seen in the crystal structures of α-amylases (Matsuura *et al.*, 1984; Buisson *et al.*, 1987; Boel *et al.*, 1990) and cyclodextrin glucanotransferases (Klein and Schulz, 1991; Kubota *et al.*, 1991). The existence of four highly conserved regions, putatively involved in substrate binding and catalysis, in these enzymes has also been described (Baba *et al.*, 1991; Takata *et al.*, 1992; Guan *et al.*, 1994*b*). Crystallographic analysis (Matsuura *et al.*, 1991) pointed to Asp-206 in region 2, Glu-230 in region 3, and Asp-297 in region 4 as catalytic sites of Taka-amylase A. In the case of neopullulanase, two Asp residues and one Glu residue, corresponding to those of Taka-amylase A, were proposed as the catalytic residues for neopullulanase (Kuriki *et al.*, 1991). Similarly, equivalent two Asp residues and one Glu residue were suggested for cyclodextrin glucanotransferases (Klein and Schulz, 1991; A. Nakamura *et al.*, 1992), cyclodextrinase (Podkovyrov *et al.*, 1993), and amylopullulanase (Mathupala *et al.*, 1993). Using random mutagenesis, Holm *et al.* (1990) found that these three residues are important for catalysis by α-amylase.

All the enzymes named above belong to the α-amylase family (Takata *et al.*, 1992; Kuriki, 1992; Svensson, 1994) and, since BE also belongs to the α-amylase family (Takata *et al.*, 1992; Kuriki, 1992; Svensson, 1994; Kuriki and Okada, 1995), two Asp and one Glu (corresponding to Asp-206 in region 2, Glu-230 in region 3, and Asp-297 in region 4 of Taka-amylase A) are possibly involved in catalysis by BE.

On this basis, we identified the amino acid

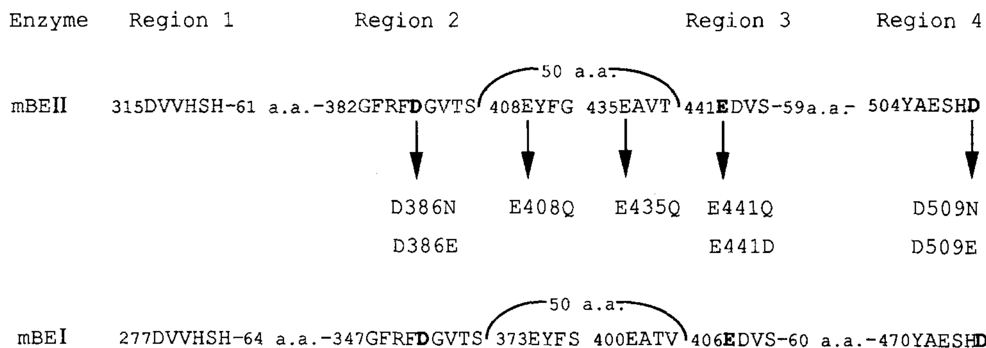

Fig. 2. Four highly conserved regions in mBEI and MBEII. Numbering starts from the first amino acid residue of the mature protein. Three carboxyl amino acid residues which are most likely to play an important role in catalysis are denoted in bold. Vertical arrows indicate the replacements of amino acid residues introduced by site-directed mutagenesis; a.a. denotes amino acid residues.

residues Asp-386, Glu-408, Glu-435, Glu-441, and Asp-509 as targets for substitutions in order to analyze the putative active center of mBEII (Fig. 2). Asp-386 and Asp-509 are located in regions 2 and 4, respectively (Fig. 2); Glu-408 was previously reported to be in region 3 (Baba *et al.*, 1991; Guan *et al.*, 1994*b*); Glu-435 is also conserved in mBEI and mBEII and rice BEI and BEIII (Guan *et al.*, 1994*b*) and Glu441 is completely conserved in all BEs sequence published so far (Guan *et al.*, 1994*b*).

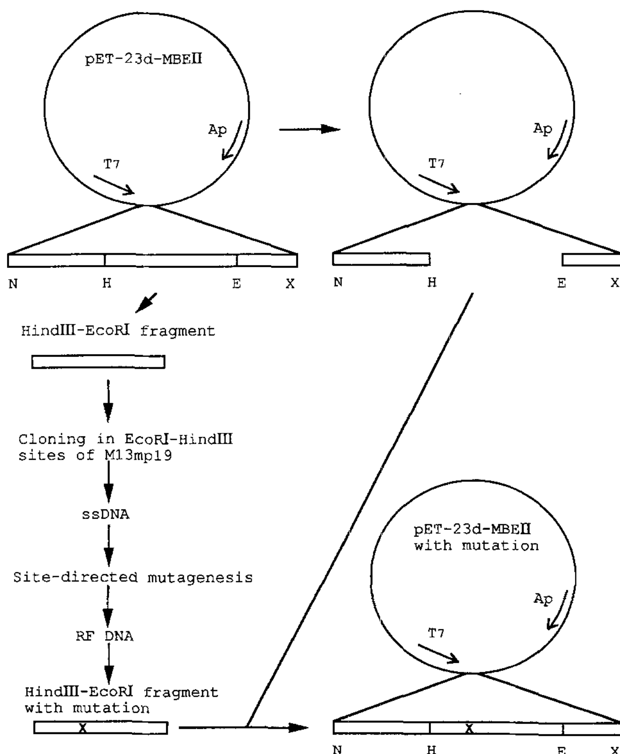

Fig. 3. Schemes of DNA construction and mutation in mBEII gene. X denotes mutation. *Nco*I, *Hind*III, *EcoR*I, and *Xho*I cleavage sites are indicated by N, H, E, and X, respectively. T7 and Ap are T7 promoter and ampicillin resistance gene, respectively.

3.2. Amino Acid Substitution by Site-Directed Mutagenesis and Preparation of Wild-Type and Mutated mBEIIs

The five target amino acid residues are within the piece of the mBE encoded by a 1073-bp *Hind*III-*EcoR*I fragment of plasmid pET-23d-MBEII (Fig. 3). The *Hind*III–*EcoR*I fragment was cloned into the *Hind*III–*EcoR*I sites of phage M13mp19 multiple-cloning sites. Single-stranded DNA was prepared from the phage and used as the template for site-directed mutagenesis (Fig. 3). Sequence analysis of the 1073-bp *Hind*III–*EcoR*I fragment verified that site-directed mutagenesis introduced the desired nucleotide changes and no second-site mutations. The whole mBEII gene is contained in a 2452-bp *Nco*I–*Xho*I fragment of pET-23d-MBEII (Fig. 3). Since both *Hind*III and *EcoR*I sites were unique in pET-23d-MBEII, the wild-type *Hind*III–*EcoR*I fragment was easily exchanged for the mutant fragment. The mBEII gene was located downstream from the T7 promoter in pET-23d-MBEII, and was expressed efficiently in *E. coli* BL21(DE3) by the addition of IPTG as an inducer.

E. coli BL21(DE3) carrying plasmid encoding wild-type (pET-23-dMBEII) or mutated mBEII gene (pET-23d-MBEII with mutation) was cultured, and wild-type or mutated enzyme, respectively, was prepared as indicated in Section 2.

Table I. BE Activity in Cell Extracts of *E. coli* Having Plasmid Encoding Wild-Type or Mutated mBEII Gene

Plasmid	Site and type of mutation	Activity (U/ml)	Protein (mg/ml)	Specific activity (U/mg)
pET-23d	NoMBEIIgene	3	6.4	0.5
pET-23d-MBEII	Wild-type	210	6.2	34
pET-23d-MBEII	D386N	4	6.1	0.7
pET-23d-MBEII	D368E	5	6.9	0.7
pET-23d-MBEII	E408Q	190	6.1	31
pET-23d-MBEII	E435Q	270	6.9	39
pET-23d-MBEII	E441Q	5	6.2	0.8
pET-23d-MBEII	E441D	6	6.7	0.9
pET-23d-MBEII	D509N	5	6.6	0.8
pET-23d-MBEII	D509E	7	7.6	0.9

3.3. Activity of Mutated mBEIIs

By using the crude enzyme prepared from *E. coli* cell extract, the activities of the eight mutated mBEIIs were estimated (Table I). When one of the target amino acid residues, Asp-386, Glu-441, or Asp-509, was replaced with its respective amide form or the alternate acid form, BE activity could be scarcely detected, as it was very similar to the background activity displayed by the control, i.e., *E. coli* BL21(DE3) carrying pET-23d (Table I).

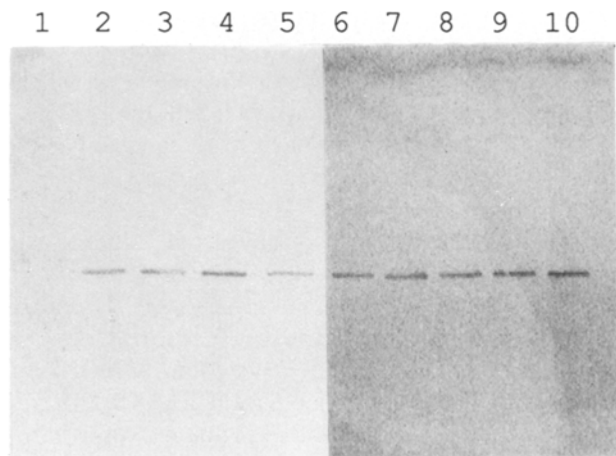

Fig. 4. Western immunoblot analysis of the cell extract of *E. coli* carrying wild-type and mutated mBEII genes. After SDS–PAGE of the samples (3 μg), the protein was transferred to a nitrocellulose membrane. The same samples indicated in Table I were loaded onto a gel. The cell extract were prepared from *E. coli* carrying pET-23d (lane 1), pET-23d-MBEII (lane 2), pET-23d-MBEII D386N (lane 3), pET-23d-MBEII D386E (lane 4), pET-23d-MBEII E408Q (lane 5), pET-23d-MBEII E435Q (lane 6), pET-23d-MBEII E441Q (lane 7), pET-23d-MBEII E441D (lane 8), pET-23d-MBEII D509N (lane 9), and pET-23d-MBEII D509E (lane 10).

The background activity was most likely that of the endogenous glycogen branching enzyme of the host cell, *E. coli* BL21(DE3). Glu-408 is located, as previously reported, in region 3, *a region highly conserved* (Baba *et al.,* 1991; Guan *et al.,* 1994*b*); and Glu-435 is also conserved in mBEI and mBEII and rice BEI and BEIII (Guan *et al.,* 1994*b*) (Fig. 2), and conservation suggested that the two residues might be essential. However, when Glu-408 or Glu-435 was replaced with Gln, the activities of the mutated enzymes sample were comparable to that of wild-type mBEII (Table I).

The expression of wild-type and mutated mBEII genes was analyzed by SDS–PAGE followed by Western blotting (Fig. 4). The results revealed the presence of a protein with a molecular weight identical to that of the wild-type enzyme. The Western blotting indicated no significant difference in the expression level of the wild-type and mutated mBEII gene products (Fig. 4).

3.4. Purification and Further Analysis of Mutated mBEIIs

Among the mutated mBEIIs described in Table I, D386N, E441Q, and D509E enzymes were purified to homogeneity from *E. coli* cell extract as described previously (Guan *et al.,* 1994*b*) (Fig. 5). Since these mutated mBEIIs did not show detectable activity during the purification process, the enzyme fractions were followed by dot-blotting and antigen–antibody formation with the antibody raised against wild-type mBEII. Branching enzyme activity could not be detected in the D386N, E441Q, and D509E proteins by using any of the

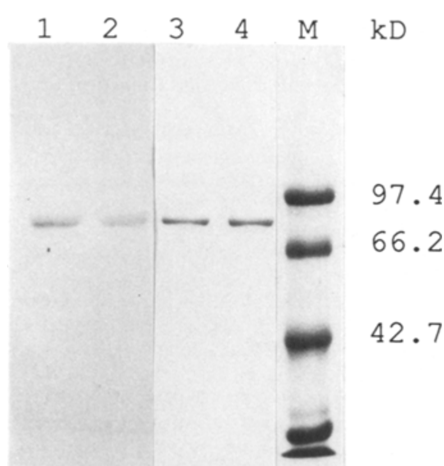

Fig. 5. SDS–PAGE of the purified wild-type and mutated mBEIIs. The purified protein samples (1 μg) were loaded onto a SDS–polyacrylamide gel, electrophoresed, and stained with Coomassie brilliant blue. Lanes: (1), wild type; (2) D386N; (3) E441Q; (4) D509E; (M) molecular markers.

three different assay methods even when the assay was performed with 500-times excess of the mutated proteins as compared with the assay for the wild-type enzyme (Table II).

3.5. EDAC Modification of mBEII

Wild-type mBEII was purified to homogeneity from *E. coli* cell extract as described previously (Guan *et al.*, 1994*b*) (Fig. 5) and the purified enzyme was used for EDAC modification. Inactivation of mBEII was both time and concentration dependent, and a plot of the logarithm of residual activity against time was linear down to 25% of the control, enabling the pseudo-first-order rate constant k_0' to be determined from the slope for each EDAC concentration (Fig. 6a). The order of reaction was determined from logarithmic plot of k_0' against EDAC concentration (Levy *et al.*, 1963), yielding a slope of 0.99 (Fig. 6b). This suggests that EDAC reacted with a single carboxyl group or some carboxyl groups which were equivalent. Maltohexaose (1–100 mM) slightly but constantly protected mBEII from modification by EDAC (1–5 mM) (data not shown).

Table II. Specific Activities of Wild-Type and Mutated BEIIs

	Specific activity (U/mg)		
Enzyme	Assay a	Assay b	Assay c
Wild type	1040	0.13	22
D386N	<1	<0.005	<0.1
E441Q	<1	<0.0005	<0.1
D509E	<1	<0.0005	<0.1

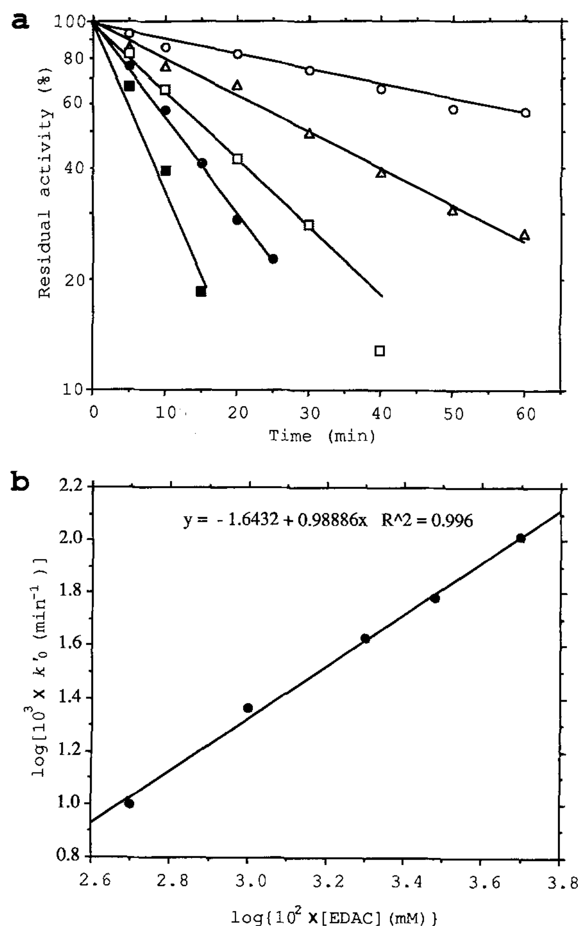

Fig. 6. Kinetics of inactivation of mBEII by EDAC. (a) Enzyme (final concentration 3.5 mM) was incubated in a buffer containing phenethylamine (25 mM) and EDAC at 5 mM (○), 10 mM (△), 20 mM (□), 30 mM (●), or 50 mM (■). At time intervals samples were withdrawn and diluted 20-fold, and residual BE activities were immediately determined by assay c. (b) The apparent order of reaction with respect to EDAC concentration. Observed pseudo-first-order rate constants k_0' were calculated from the slopes in (a).

4. CONCLUSIONS

We have tentatively identified amino acid residues involved in the active center of mBEII. To do so, we made eight different mutant enzyme genes targeting the five highly conserved amino acid residues and expressed these genes in *E. coli.* When either of the three residues Asp-386, Glu-441, or Asp-509 was replaced with its

respective amide form or the alternate acid form, the BE activity disappeared (Tables I and II). Conversely, a similar replacement of two of the highly conserved residues, Glu-408 and Glu-435, did not affect the BE activity (Table I).

The expression of wild-type and mutated mBEII genes was analyzed (Fig. 4). Cells containing mutant mBEII genes were able to synthesize full-size proteins. No significant difference was detected in the expression level of wild-type and mutated mBEII gene products (Fig. 4).

The importance of carboxyl amino residues was also shown by EDAC modification of mBEII. Based on these experimental results described here, the structure-prediction and hydrophobic-cluster analysis (Jespersen *et al.,* 1991), and the existence of four highly conserved regions (Baba *et al.,* 1991; Takata *et al.,* 1992; Guan *et al.,* 1994*b*), we conclude that Asp-386, Glu-441, and Asp-509 of mBEII most likely play an important role in catalysis.

Glu-408 of mBEII corresponding to Glu-373 of mBEI is located in the previously reported region 3 (Baba *et al.,* 1991; Guan *et al.,* 1994*b*). The residue corresponding to Glu-435 of mBEII is also conserved in mBEII, rice BEI, and BEIII (Guan *et al.,* 1994*b*) (Fig. 2). According to the results obtained in this work, region 3 of mBEII contained Glu-441, but neither Glu-408 nor Glu-435 (Fig. 2). The same location of region 3 as that described in this paper was suggested by site-directed mutagenesis in a glycogen branching enzyme from *Bacillus stearothermophilus* (Takata *et al.,* 1994). Jespersen *et al.* (1993), who used secondary structural analysis, suggested the same location for region 3.

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