

identity of the isolated cDNA clones. Thus, the mature form of RBE3, as deduced from the cDNA sequence, is a 760-residue protein with a molecular mass of 86,376 Da. This value is consistent with that obtained experimentally by SDS-PAGE (Fig. 13).

Comparison of Transit Peptide Sequence of the RBE3 Precursor with Several Amyloplastic Enzymes --- The 65-residue sequence preceding the amino terminus of the mature RBE3 shares no similarity in the residue composition and predicted hydrophobicity with a typical signal peptide sequence that is required for translocation of a newly synthesized protein across a membrane. GBSS and ADP-glucose pyrophosphorylase, which are exclusively localized in the amyloplast of developing endosperms, are initially synthesized as the precursors with transit peptides at the amino termini^{30,60,61}. The sequence of the amino-terminal extension of RBE3 is rich in Ala, Gly, Ser, Val, and Arg and is poor in acidic amino acids, as has been found in the transit peptides of other members of translocated proteins^{62,63}. It is therefore most likely that the amino-terminal leader sequence of the RBE3 precursor appears to function as a transit peptide necessary for targeting and transporting this protein to the amyloplast. There is a motif of Gly-Ala-Val-Arg in the transit peptide sequence of the RBE3 precursor, which is repeated two or three times at residues -1 to -4, -12 to -15, and -45 to -48 (Fig. 17). It is interesting that the RBE1 precursor also contains the sequences similar to the repetitive motif at residues -14 to -17, -30 to -33, and -43 to -46, although the similarities of the primary and secondary structures predicted are low in the transit peptides between the RBE3 and RBE1 precursors.

Comparison of Amino Acid Sequences of Mature Proteins between RBE1 and RBE3 --- As shown in Fig. 18, the mature RBE3 has a significant sequence identity with RBE1. The central portions between these two proteins are highly conserved, whereas there is a low degree of the sequence identity in the amino- and carboxyl-terminal regions; RBE3 possesses an almost 70-residue extra sequence at the amino terminus and lacks an approximately 50-residue sequence at the carboxyl terminus, as compared with RBE1^{14,57}). However, the consensus sequences of four regions, which form the catalytic site of amylolytic enzymes^{33,34}), are conserved between these two branching enzymes (Fig. 18). Thus, RBE3 as well as RBE1 belongs to a family of amylolytic enzymes. The overall sequence identity is 49 % between RBE3 and RBE1. The sequence alignment of RBE3 with *E. coli*¹³) and yeast branching enzymes⁶⁵) shows a low degree of identity (not shown). The mature proteins of RBE3 and RBE1 contain 5 and 9 cysteines, respectively (Fig. 18). Four out of 5 cysteine residues in the RBE3 sequence are located at the same positions in the RBE1 sequence.

Expression Pattern of the RBE3 Gene --- To assess expression of the RBE3 gene in developing rice seeds of wild type and the *amylose-extender* mutants, Northern blot analysis was carried out using a cDNA fragment of RB32 as a probe (Fig. 19). A 3.0-kilobase mRNA signal was found in the wild type, EM72, EM129, and EM189. The expression levels of the RBE3 gene were almost equal among the wild type and three mutant lines. EM10 and EM145 lacked or possessed only a slightly hybridized transcript with the same size. It is not certain at present that the faint signals correspond to the true RBE3 message. As a control experiment, the Northern blot was reprobed by the cDNA fragments encoding rice RBE1 and GBSS. The 2.8- and 2.4-kilobase

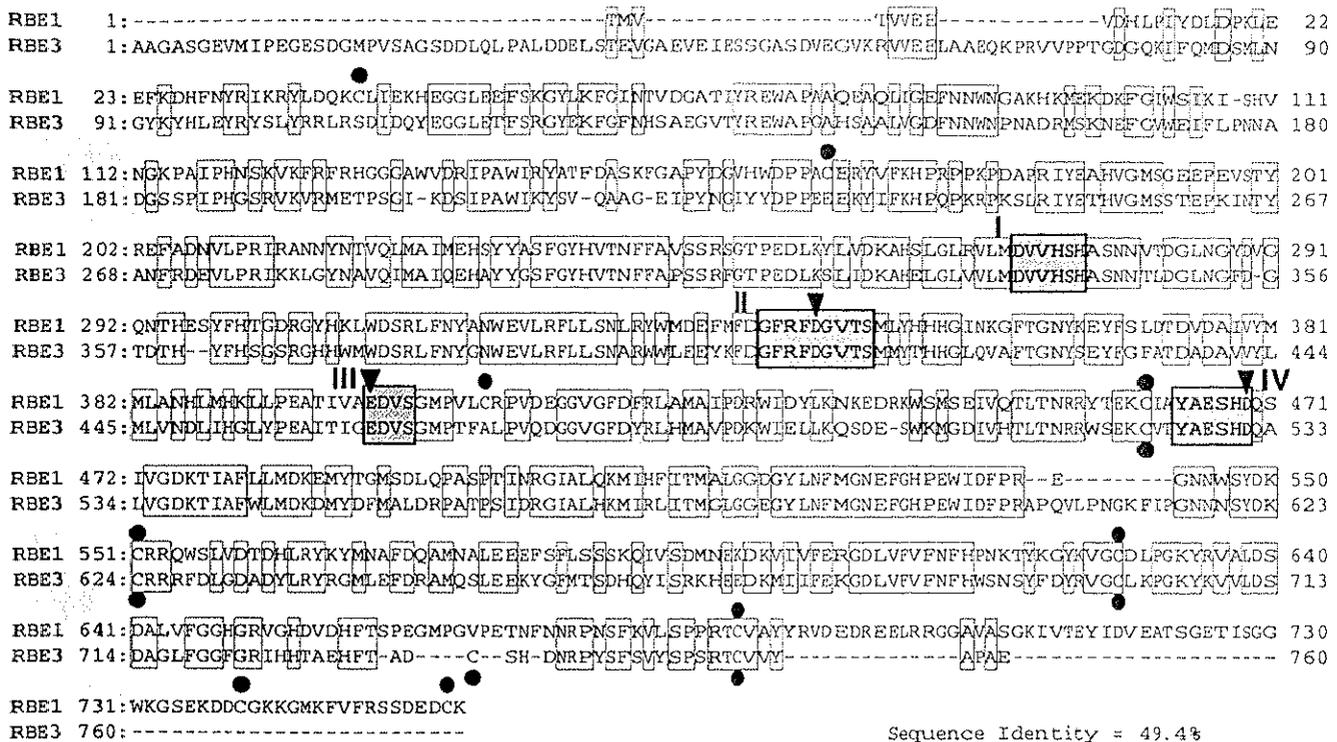


Fig. 18. Sequence alignment of two forms of rice branching enzyme, RBE3 and RBE1. The mature proteins of RBE3 and RBE1 of 760 and 756 residues, respectively, are compared. Amino acids are represented by the standard one-letter code. Identical residues are indicated by *open boxes*. *Dashes* represent gaps introduced to maximize the alignment. The conserved sequences of RBE3 with the four regions of catalytic sites of amylolytic enzymes (regions I, II, III, and IV) and the probable catalytic residues of the amylolytic enzymes for cleavage of α -1,4 linkages are indicated by *shaded boxes* and *arrows*, respectively. The locations of cysteine residues in the sequences of RBE3 and RBE1 are indicated by *closed circles*.

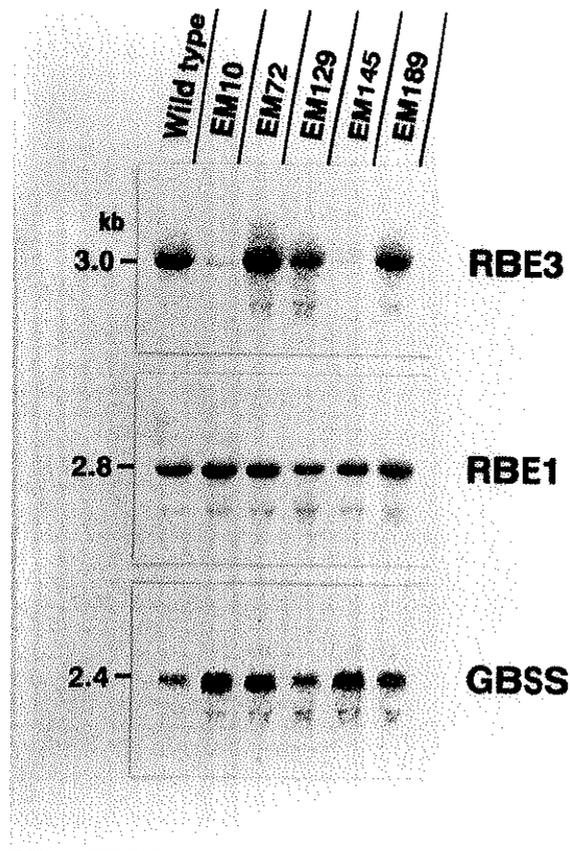


Fig. 19. The RBE3 gene is not expressed in two out of five *amylose-extender* mutants of rice. Total cellular RNAs from developing seeds (13-15 days after flowering) of wild type and five *amylose-extender* mutants were subjected to Northern blot analysis, using the ³²P-labeled cDNA fragments encoding RBE3, RBE1, and GBSS as probes. *kb*, kilobase(s).

transcripts of the RBE1 and GBSS genes, respectively, were detected equally in all RNA samples, including those of EM10 and EM145. Thus, these data are consistent with that obtained by Western blot analysis (Fig. 13).

Northern blot analysis of total cellular RNAs from various tissues of rice plants demonstrates that the RBE3 gene is specifically expressed in developing seeds (Fig. 20). Although the RBE1 gene is mainly expressed in developing seeds, a little transcript was detected in leaf and stem (Fig. 20). Moreover, the RBE3 mRNA is abundantly present between 5th and 15th days of seed development (Fig. 21). This expression pattern of the RBE3 gene is almost identical to those of the RBE1 and GBSS genes.

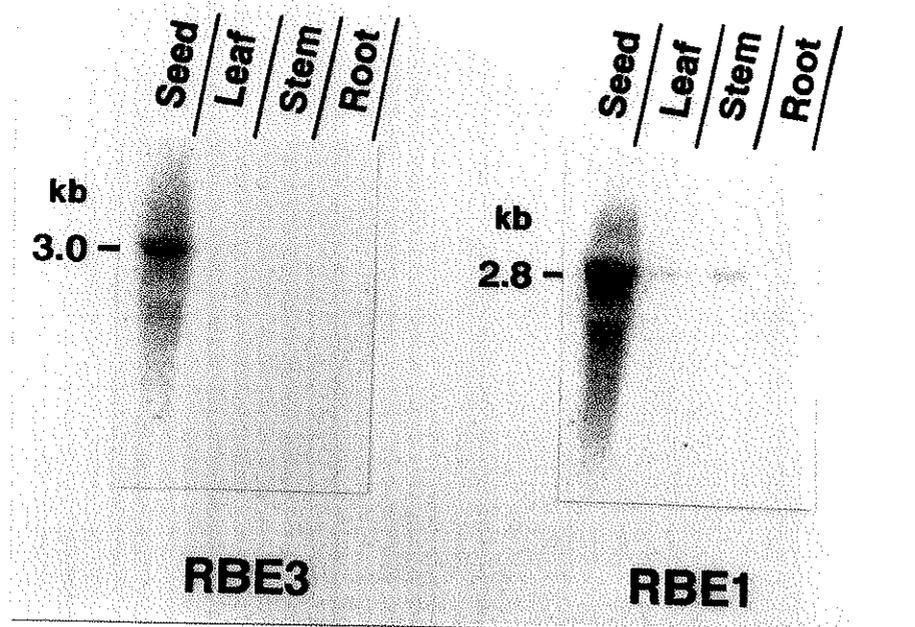


Fig. 20. Distribution of the RBE3 mRNA in rice tissues. Total cellular RNAs from seed, leaf, stem, and root of normal rice plant at 10-15 days after flowering were subjected to Northern blot analysis. The blot was probed by the ^{32}P -labeled cDNA encoding RBE1, and then reprobbed by the labeled RBE3 cDNA. *kb*, kilobase(s).

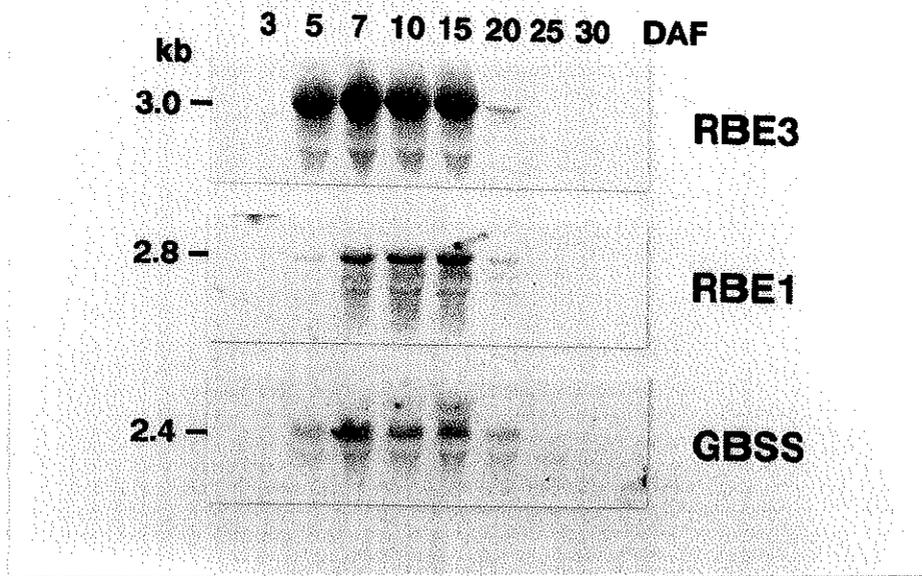


Fig. 21. Changes of the RBE3 mRNA accumulation during seed development. Total cellular RNAs from rice seeds at various days after flowering (*DAF*) were subjected to Northern blot analysis. The blots were probed by the ^{32}P -labeled cDNA fragments encoding RBE3, RBE1, and GBSS. *kb*, kilobase(s).

Discussion

The data represented here strongly suggest that formation of abnormal starch components in *amylose-extender* mutants of rice is due to the lack of an isoform of starch branching enzyme, RBE3. Two lines of the rice endosperm mutants, EM10 and EM145, possess no RBE3 molecule (Fig. 13), and a negligible amount of a transcript similar to that of RBE3 is detectable in these two mutants (Fig. 19). Characterization of the cDNA clones encoding RBE3 demonstrates that this branching enzyme isoform is distinguishable in the protein structure from another form of rice branching enzyme, RBE1 (Figs. 16 and 18).

Recently, the *r* (*rugosus*) locus of pea, which is a determinant for the wrinkled-seeded character, has been cloned by Battacharyya *et al.*⁶⁶⁾, who have also shown that the wrinkled (*rr*) seeds lack a 114-kDa isoform of branching enzyme present in the normal, round (*RR* or *Rr*) seeds. The 114-kDa branching enzyme gene is at the *r* locus and is interrupted by an 800-nucleotide transposon-like insertion, resulting in the failure of the enzyme production⁶⁶⁾. Starch from the wrinkled-seeded pea as well as from the *amylose-extender* mutants of rice, maize, and barley is characterized by the presence of two abnormal components: an amylopectin with long inner and outer branches and a low molecular weight intermediate material⁴³⁾, as described before. Therefore, it is most likely that rice RBE3 plays the same function in starch (branched glucan) synthesis *in vivo*, as in the case of the 114-kDa branching enzyme isoform of pea. A possibility that rice RBE3 corresponds to maize BE-II (or BE-IIb) is also acceptable, since the *amylose-extender* mutant of maize lacks the activity of the branching enzyme isoform^{5,10,56)}.

Three lines of rice *amylose-extender* mutants, EM72, EM129, and EM189, contain the 87-kDa protein molecules that immunoreact with

affinity-purified anti-RBE3 antibody (Fig. 13). Northern blot analysis indicates that RBE3 gene expression in these three mutants occurs at the same level as in the wild type (Fig. 19). These results are intriguing, since the rice *amylose-extender* mutants were produced by the chemical-mutagen method^{54,55}; where in the genes, including the RBE3 gene, have the nucleotide modifications been generated? The reason that the RBE3 genes of the above three mutants are apparently expressed like the wild type (Figs. 13 and 19) may be explained by the following possibility.

The amino acid sequences of the RBE3 molecules from EM72, EM129, and EM189 may have been modified by a nucleotide substitution(s) of the RBE3 gene sequence. The substituted amino acid residue(s) appears to be related to the enzyme activity. Although expression of the genes for RBE1 and RBE3 is normal in EM72, EM129, and EM189, a lower level of branching enzyme activity than found in the wild type is evident in these three mutants (Figs. 13, 14, and 19). Since the expression levels of the RBE3 gene are negligibly low in EM10 and EM145, it is likely that the RBE3 activity contributes to 30-40 % of total branching enzyme activity (Fig. 14). Therefore, RBE3 in EM72, EM129, and EM189 must be inactive and is presumably mutated at a critical residue(s) for the activity. The lack or the very low level of RBE3 gene expression in EM10 and EM145 (Fig. 19) implies that the promoter sequence of the RBE3 gene is modified by nucleotide substitution(s). Identification of the substitutions in the two mutants may find a clue for elucidation of the *cis*-element(s) required for the gene expression specific for the endosperm tissues, since RBE3 is specifically present in the endosperm of developing seeds (Fig. 20).

The deduced amino acid sequence of RBE3 indicates that this protein is initially synthesized as an 825-residue precursor including a 65-residue transit peptide at the amino terminus (Figs. 16 and 17). A

great number of the sequences of the transit peptides, which are required for translocation of proteins into various cellular organelles, including mitochondria and chloroplast, have been reported, although the precise roles of the transit sequences in the import and maturation of the precursors have not been resolved^{62,63}. The transit sequences are usually rich in basic and hydroxylic amino acids and poor in acidic amino acids, but the consensus sequence as a signal of the precursor import has not yet been established. In this study, a sequence motif of Gly-Ala-Val-Arg is repeated two or three times in the sequence of the transit peptide of the RBE3 precursor (Fig. 17). The sequences similar to the motif sequence are also found in the transit peptide of the RBE1 precursor. It is interesting to suppose that these 4-residue sequences act as a targeting and/or transport signal for the precursors of branching enzymes translocated into the amyloplast.

The conservation of four consensus sequences, which constitute the catalytic site of amylolytic enzymes, including α -amylase^{33,34}, in the RBE3 sequence (Figs. 16 and 18) demonstrates that RBE3 as well as RBE1 and maize BE-I^{12,14} belongs to a family of the amylolytic enzymes (Fig. 22). This finding supports the probability that branching enzyme possesses two enzymatic functions such as cleavage of α -1,4-glucosidic linkages and transfer of the newly formed reducing ends to other α -1,4-linked chains^{14,32,67,68}. These two reactions must be conducted simultaneously, since branching enzyme has been found to have no starch-hydrolyzing activity. It was reported that branching enzyme requires free sulfhydryl groups for the activity³². Although the arrangement of 5 cysteines in the RBE3 molecule is not clear, the conservation of 4 cysteine residues between RBE3 and RBE1 (Fig. 18) appears to be important for the activities of these enzymes. Thus, at least these 4 cysteines may possess free sulfhydryl groups.

	Region I	Region II	Region III	Region IV
Branching enzyme				
Rice (RBE1,RBE3)	²⁷¹ DVVHSH ³³⁷	¹⁴¹ GFRFDGVTS ⁴⁰⁴	⁴⁰⁰ EDVS ⁴¹³	⁴⁶⁴ YAESH ⁵²⁷ D
Maize	²⁷⁷ DVVHSH	¹⁴⁷ GFRFDGVTS	¹⁷² EDVS	⁴⁷⁰ YAESH ⁵²⁷ D
<i>E. coli</i>	³³⁵ DWVPGH	⁴⁰¹ ALRVDAVAS	⁴¹⁴ EEST	⁵²¹ LPLSH ⁵²⁷ D
α-Amylase				
<i>A. oryzae</i>	¹¹⁷ DVVANH	²⁰² GLRIDIVKH	²¹⁰ EVL D	²⁹² FVENH ²⁹⁷ D
<i>B. subtilis</i>	⁹⁷ DAVINH	¹⁷³ GFRFDAAKH	²⁰⁴ EILQ	²⁶⁴ WVESH ²⁹⁷ D
Barley	¹⁰¹ DIVINH	¹²⁷ DGRLDWGPH	²¹⁸ EVWD	²⁹⁹ FVDNAD ³⁰⁴ D
Human	⁹⁹ DAVINH	¹³⁴ GFTIDFVRG	²¹⁴ EVID	²⁹⁸ FVDNAD ³⁰⁴ D
G4-forming amylase				
<i>P. saccharophila</i>	¹¹³ DVVENH	¹⁹⁹ GFRFDVVRG	²¹⁹ ELWK	²⁸⁹ FVDNHD ²⁹⁴ D
CGTase				
<i>K. pneumoniae</i>	¹³⁰ DYAPNH	²¹⁹ AIRIDAIKH	²⁵⁷ EWFG	³²⁸ FMDNHD ³³³ D
Pullulanase				
<i>K. aerogenes</i>	⁶⁰⁰ DVVYNH	⁶⁷¹ GFRFDLMGY	⁷⁰⁴ EGWD	⁸²⁷ YVSKHD ⁸³² D
Isoamylase				
<i>P. amyloclavata</i>	²⁹¹ DVVYNH	³⁷⁰ GFRFDLASV	⁴⁵⁴ EWSV	⁵⁰² FIDVHD ⁵⁰⁷ D
Neopullulanase				
<i>B. stearothermophilus</i>	²⁴¹ DAVENH	³²⁴ GWRLDVANE	³⁵⁷ EIWH	⁴¹⁹ LLGSHD ⁴²⁴ D

Fig. 22. Comparison of the deduced amino acid sequence of rice RBE1 and RBE3 with typical amylolytic enzymes. Amino acid residues are numbered from the amino-terminus of the mature protein. The sequences of rice RBE1 at 271-276, 341-349, 400-403, and 464-469, and of rice RBE3 at 337-342, 404-412, 463-466, and 527-532, are compared with those of typical amylolytic enzymes, as described by Svensson⁶⁹. The possible catalytic residues for cleavage of α -1,4 linkages^{33,34} are indicated by *bold letters*.

Although the genes encoding RBE1 and RBE3 are expressed at the same stages of seed development (Fig. 20 and 21), the biological roles of these two isoenzymes in starch synthesis are not clear at present. Recently, Takeda *et al.*⁷⁰⁾ have reported branching of amylose molecules by three forms of maize branching enzyme, BE-I, BE-IIa, and BE-IIb; BE-I preferentially transfers long chains, whereas the other two isoenzymes transfer short chains⁷⁰⁾. Our present and previous studies^{12,14)} demonstrate that rice RBE1 and RBE3 probably correspond to maize BE-I and BE-IIa (or BE-IIb), respectively, as described above. Thus, RBE3 may be distinguished from RBE1 by the preferential formation of the short-chain branches. This possibility appears to be supported by the fact that the loss of RBE3 activity in rice *amylose-extender* mutants is correlated with production of abnormal branched glucans (amylopectin and intermediate material) that possess a lower proportion of short-chain fraction than found in normal amylopectin^{48-53,55)}. It is intriguing that the amino- and carboxyl-terminal sequences are highly divergent between the mature RBE1 and RBE3 (Fig. 18). This structural difference may be related to the specificity of the enzyme function. Establishment of expression systems for both RBE1 and RBE3 is helpful to elucidate the structure/function relationships of these branching enzymes.

Chapter 3

Molecular Characterization of Recombinant Branching Enzymes

Introduction

As described in Chapters 1 and 2, the cDNA cloning of two isoforms of branching enzyme, RBE1 and RBE3, in developing rice seeds demonstrates that RBE1 shares almost 50% of sequence identity with RBE3 especially at the central part of the protein molecule. There are two noticeable differences in the primary sequences of these two isoforms: the presence of an extra sequence at the amino- or carboxyl-terminus and an approximately 10-residue sequence specifically present in RBE1 or RBE3 (Fig.31). However, little is known of the difference of the enzymatic functions between these two isoforms of rice branching enzyme. In addition, it still remains unclear how the structural differences affect the enzyme activity.

In this chapter, to elucidate the structure/function relationships of RBE1 and RBE3, I have established the expression system for these two isoforms in *E. coli*. Using this expression system, mutant proteins, including chimeric enzymes between RBE1 and RBE3, have been produced and characterized. The results described in this chapter demonstrate that the enzymatic function of RBE1 is distinguishable from that of RBE3 probably due to the structural difference in the amino- and carboxyl-terminal regions.

Materials and Methods

Constructions of Expression Plasmids for RBE1 and RBE3 --- An expression plasmid of the cDNA fragment encoding the mature RBE1 was constructed in a pET-23d vector (Novagen, Madison, WI) containing the bacteriophage T7 promoter. The RBE1 cDNA in pUC19 was digested by *NcoI* and *BamHI* to obtain a DNA fragment carrying the sequence of nucleotides 213 to 694. The DNA fragment was introduced into the pET23d vector at the *NcoI* and *BamHI* site. The resulting plasmid was digested by *BamHI* and *EcoRI*, and the *BamHI/EcoRI* fragment encoding the 3'-end sequence of the RBE1 cDNA, nucleotides 694 to 2739, was inserted at the same sites. For expression of mature RBE3 in *E. coli*, the cDNA insert of a RBE3 cDNA clone, RB33, was digested by *AccII* to obtain a DNA fragment carrying the sequence of nucleotides 331 to 686. The DNA fragment was ligated to a phosphorylated *NcoI* linker, 5'-CAGCCATGGCTG-3' (Takara), digested by *NcoI* to remove the express linkers, and introduced into a pTV119N vector at the *NcoI* site. The resulting plasmid was digested by *ClaI* and *EcoRI*, and replaced by the *ClaI/EcoRI* fragment of RB33 encoding the 3'-end sequence of the RBE3 cDNA. The *NcoI/EcoRI* fragment newly generated was then introduced into the pET23d vector at the *NcoI* and *EcoRI* sites.

Expression Plasmids for Mutated RBE1 --- A pTV119N vector was digested by *EcoRI*. The *EcoRI* site was blunt-ended by treatment with klenow fragment, and the resulting vector was ligated to a phosphorylated stop linker, 5'-CTAACTAATTAAAGCTTTAATTAG-TTAG-3', termed pTV119N-ST. The RBE1 cDNA in pUC19 was digested by *NcoI* and *BamHI* to obtain a DNA fragment carrying the

sequence of nucleotides 213 to 694. The DNA fragment was introduced into pTV119N-ST at the *NcoI/BamHI* site. The resulting plasmid was digested by *BamHI* and *EcoRI*, and replaced by the *BamHI/EcoRI* fragment of RB13 encoding the 3'-end sequence of the RBE1 cDNA, nucleotides 694 to 2739 (termed pTV-RBE1).

For constructions of RBE1-deletion mutants from the amino- and carboxyl-terminus of RBE1, pTV-RBE1 was digested by *NcoI* and *EcoRI*, *NcoI* and *BamHI*, *NcoI* and *PmaCI*, *AflIII* and *HindIII*, *SphI* and *HindIII*, *XhoI* and *HindIII*, *SmaI* and *HindIII*, *DdeI* and *HindIII*, or *BanIII* and *HindIII*. These plasmids digested were blunt-ended by treatment with klenow fragment or T4 DNA polymerase, and then self-ligated. Seven oligonucleotides, 5'-GACCAGAAGTCCTTGATTG-3', 19-mer, (replacement of Cys-40 by serine residue; termed C1S), 5'-GGATCCTCCAGCTTCTGAAAGG-3', 22-mer, (replacement of Cys-166 by serine residue; termed C2S), 5'-CAGTGCTTTCCCGGCCAGT-TGA-3', 22-mer, (replacement of Cys-409 by serine residue; termed C3S), 5'-TACAGAGAAATCCATTGCCTAT-3', 22-mer, (replacement of Cys-461 by serine residue; termed C4S), 5'-TATGATAAATCGAGACGTCAGT-3', 22-mer, (replacement of Cys-551 by serine residue; termed C5S), 5'-ACAAGGTCGGATCTGACTTGCC-3', 22-mer, (replacement of Cys-628 by serine residue; termed C6S), and 5'-CCGTACCTCTGTGGCTTAC-3', 20-mer, (replacement of Cys-688 by serine residue; termed C7S) were synthesized using Applied Biosystems DNA synthesizer Model 381. Site-directed mutagenesis was carried out by the method of Kunkel *et al.*⁷¹⁾, and the mutations were verified by sequencing analysis. These mutant plasmids were expressed in *E. coli* JM109.

Expression Plasmids for RBE1-RBE3 Chimeric Enzymes --- To introduce a new *SacI* or *PstI* site into a RBE1 cDNA, corresponded to nucleotides 2130 *SacI* site or 2502 *PstI* site for RBE3 cDNA, a RBE1 cDNA was amplified by polymerase chain reaction (PCR). And to introduce new *BamHI* site into a RBE3 cDNA, corresponded to nucleotides 694 *BamHI* site for RBE1 cDNA, a RBE3 cDNA was amplified by PCR. The reaction program consisted of 35 cycles of 94 °C for 1 min, 55 °C for 2 min, and 72 °C for 3 min in a SANTHERMO PCR reactor (Sanko Junyaku Co., Ltd.). Seven expression plasmids for RBE1-RBE3 chimeric enzymes were constructed, as described below:

1. 1Pst3

The RBE1 cDNA was introduced new *PstI* site by PCR, nucleotides 2172, corresponded to *PstI* site of the RBE3 cDNA, nucleotides 2502. The RBE1 specific region (RBE1-SR) locates near down stream region of the new *PstI* site of the RBE1 cDNA. Primers BE1-Sac2 (5'-AGAGCTCGCAACAACACTGGAGCT-3', 22mer) and BE1-Pst1 (5'-TCTGCAGCATGGCCAACTCTTCCA-3', 24mer) corresponded to nucleotides 1832 to 1853 in the cDNA sequence and nucleotides 2155 to 2178 in the complementary sequence. BE1-Sac2 was designed to contain a new *SacI* site and BE1-Pst1 was designed to contain a new *PstI* site, with several mismatching bases. The amplified DNA fragment, termed BE1-SP340, was digested by *XhoI* and *PstI* to obtain a DNA fragment carrying the sequence of nucleotides 1943 to 2172. The resulting fragment and the *PstI/KpnI* fragment of RBE3 cDNA, nucleotides 2502 to 2854, were introduced into a pET-RBE1 at the *XhoI/KpnI* sites. The resulting plasmid was termed pET-1Pst3.

2. 1Xho3

A RBE3 cDNA clone, RB33, was digested by *XhoI* and *KpnI* to obtain a DNA fragment carrying the sequence of nucleotides 2273 to

2854. The DNA fragment was ligated to a pET-RBE1 at the *XhoI/KpnI* sites. The resulting plasmid was termed pET-1Xho3.

3. 1Sac3

The RBE1 cDNA was introduced new *SacI* site by PCR, nucleotides 1833, corresponded to *SacI* site of the RBE3 cDNA, nucleotides 2130. The RBE3 specific region (RBE3-SR) locates near down stream region of the *SacI* site of the RBE3 cDNA. Primers BE1-dom2 (5'-CTTTCGATTTAATGGAGTTAC-3', 21mer) and BE1-Sac1 (5'-GCGAGCTCTTGAAAGTCAATCC-3' 23mer) corresponded to nucleotides 1234 to 1254 in the cDNA sequence and nucleotides 1818 to 1840 in the complementary sequence. BE1-Sac1 was designed to contain a new *SacI* site with several mismatching bases. The amplified DNA fragment was digested with *SphI* and *SacI* to obtain a DNA fragment carrying the sequence of nucleotides 1422 to 1833. The DNA fragment and the *SacI/KpnI* fragment of RBE3 cDNA, nucleotides 2130 to 2854, were introduced into a pET-1Xho3 at the *SphI/KpnI* sites. The resulting plasmid was termed pET-1Sac3.

4. 1Bam3

The RBE3 cDNA was introduced new *BamHI* site by PCR, nucleotides 1003, corresponded to *BamHI* site of the RBE1 cDNA, nucleotide 694. Primers BE3-Bam (5'-GAATATATTGGGATCCTCCT-3', 20mer) and BE3-313R (5'-CTAAACCAAGCTCATGAGCT-3' 20mer) corresponded to nucleotides 993 to 1012 in the cDNA sequence and nucleotides 1300 to 1319 in the complementary sequence. BE3-Bam was designed to contain a new *BamHI* site with several mismatching bases. The amplified DNA fragment was digested with *BamHI* and *KpnI* to obtain a DNA fragment carrying the sequence of nucleotides 1003 to 1224. The DNA fragment was ligated into a pET-RBE1 at the *BamHI* and *KpnI* sites. The resulting plasmid was digested by *XhoI* and

KpnI, and then blunt-ended the *XhoI* site by treatment with klenow fragment. The digested plasmid was introduced into the *KpnI*/brunt-ended *StyI* fragment of RB33 encoding the 3'-end sequence of the RBE3 cDNA. The resulting plasmid was termed pET-1Bam3.

5. 3Xho1

A pTV-RBE1 was digested by *XhoI* and *EcoRI* to obtain a DNA fragment carrying the sequence of nucleotides 1943 to 2739. The DNA fragment was ligated to a pET-RBE3 at the *XhoI/EcoRI* sites. The resulting plasmid was digested by *XhoI*, and introduced into a *XhoI* fragment of RBE3 cDNA carrying the sequence of nucleotides 1511 to 2273. The orientation of the *XhoI* fragment was verified by sequencing analysis. The resulting plasmid was termed pET-3Xho1.

6. 3Sac1

The DNA fragment of BE1-SP340, described above, was digested by *SacI* and *XhoI* to obtain a DNA fragment carrying the sequence of nucleotides 1833 to 1943. The resulting fragment and the *XhoI/EcoRI* fragment of RBE1 cDNA, nucleotides 1943 to 2739, were introduced into a pET-RBE3 at the *SacI/EcoRI* sites. The resulting plasmid was termed pET-3Sac1.

7. 3Pst1

The RBE1 cDNA was introduced new *PstI* site by PCR, nucleotides 2172, corresponded to *PstI* site of the RBE3 cDNA, nucleotides 2502. The RBE1 specific region (RBE1-SR) locates near down stream region of the new *PstI* site of the RBE1 cDNA. Primers BE1-Pst2 (5'-GCTGCAGATCACTTCACGTCTC-3' 22mer) and BE1-CT (5'-GTCCAAGGCAAGCCAGGAT-3' 19mer) corresponded to nucleotides 2171-2192 in the cDNA sequence and nucleotides 2536 to 2553 in the complementary sequence. BE1-Pst2 was designed to contain a new *PstI* site with several mismatching bases, and BE1-CT was designed to contain

a *StyI* site. The amplified DNA fragment was digested by *PstI* and *StyI* to obtain a DNA fragment carrying the sequence of nucleotides 2172 to 2546, and then blunt-ended the *StyI* site by treatment with klenow fragment. The resulting fragment and the *SacI/PstI* fragment of RBE3 cDNA, nucleotides 2130 to 2502, were introduced into a pET-RBE3 at the *SacI/HincII* sites. The resulting plasmid was termed pET-3Pst1.

Production of Recombinant Proteins in E. coli --- A single colony of transformants was cultured at 37°C overnight in Luria broth containing 0.1 mg/ml ampicillin (10 ml, LA) with constant shaking. A portion (6 ml) of the bacterial culture was added to a fresh LA (400 ml), and incubated at 37°C for 3h with shaking. The production of the recombinant RBE1 and RBE3 was induced by addition of 1 ml of 0.1 M IPTG, and the cell growth was continued at 30°C for 8 h. Cells were harvested by centrifugation at 3,000 rpm for 10 min, washed with 20 ml of 10 mM Tris/HCl, pH 8.0, containing 1 mM EDTA and 150 mM NaCl (TES), and suspended in 30 ml of 50 mM Tris/HCl, pH 7.5, containing 5 mM EDTA and 5 mM 2-mercaptoethanol (TEM). The cell suspension was frozen at -80°C, thawed, thoroughly sonicated, and then centrifuged at 10,000x g for 10 min at 4°C. The resulting supernatant was used as crude extracts of the recombinant enzymes.

Purification of Recombinant Proteins --- The recombinant proteins were purified by modification of the procedure used to purify branching enzymes from developing rice seeds, as described previously (see Chapter 1). Crude extracts (30 ml) from *E. coli* cell paste were applied to a DEAE-cellulose column (1.9 x 45 cm) previously equilibrated with TEM. Proteins were eluted with a linear gradient of 0-0.3 M KCl (1 liter). Fractions containing branching enzyme activity

were pooled and concentrated by ammonium sulfate precipitation with a 0-70% saturation. The precipitate was dissolved and dialyzed against TEM. The dialyzed solution was applied to an ω -aminooctyl Sepharose 4B column previously equilibrated with TEM. After the column was washed with 60 ml of TEM, proteins were eluted from the column with a linear gradient of 0-0.5 M KCl (200 ml). Fractions containing the enzyme activity were dialyzed and stored at 4°C.

Assay for Enzyme Activity --- Two conventional methods were used for measurements of branching enzyme activity. **Assay A**; Branching enzyme activity was measured in the direction of stimulation of α -D-glucose 1-phosphate catalyzed by rabbit-muscle phosphorylase α , as described in Chapter 1. **Assay B**; Branching enzyme activity was also assayed by the decrease in absorbance of D-glucan-iodine complex^{5,72}. The reaction mixture consisted of 0.1 M sodium citrate, pH 7.0, 200 μ g of potato amylose or potato amylopectin, and enzyme in a final volume of 0.2 ml. Aliquots (50 μ l) sampled at time intervals were mixed with an iodine solution (2.95 ml). The absorbance of the D-glucan-iodine complex was measured at 660 nm using a Simadzu UV-240 spectrophotometer. One unit of activity was defined as a decrease in absorbance of 1.0 at 660 nm per min.

SDS-PAGE and Western Blot Analysis --- PAGE in the presence of 0.1 % SDS (SDS-PAGE) was carried out by the method of Laemmli¹⁹. Proteins were stained with Coomassie brilliant blue R-250 (Sigma). For Western blot analysis²⁰, proteins were separated by SDS-PAGE, transferred onto Immobilon-P PVDF membranes (Millipore), using a Sartorius Semi-Dry blotter. The blots were blocked with 1 % skim milk, probed by affinity-purified antibody against RBE1 or RBE3 at

room temperature for 1 h, and then incubated with goat anti-rabbit IgG horseradish peroxidase conjugate (Jackson ImmunoResearch Laboratories). The immunoreactive bands were detected by incubation of the blots in a mixture of 20 mM Tris/HCl, pH 7.5, containing 0.15 M NaCl, 0.05% 4-chloro-1-naphthol, and 0.0003% hydrogen peroxide.

Gel Filtration of Branched Glucans Produced by Branching Enzymes --- Branching reaction of potato amylose by branching enzyme was conducted at 30°C for 20 h in a mixture (20 ml) containing 20 mM sodium citrate, pH 7.0, 20 mg of potato amylose, and branching enzyme (1 unit by Assay B). The reaction was terminated by heating at 65 °C for 5 min, and the mixture was lyophilized. The branched products were suspended in 0.5 ml of water, dissolved by addition of 1 M NaOH (0.5 ml), and neutralized by addition of 1 M HCl (0.5 ml). To the glucan solution, 0.5 M sodium acetate/HCl, pH 3.5 (5 ml), and isoamylase (590 units) were added. After incubation at 42°C for 20 h, the mixture was lyophilized, and the debranched products were suspended in water (0.6 ml) and dissolved by addition of 5 M NaOH (0.2 ml). The resulting solution was made up to 2 ml with water. The glucan solution (2 ml) was then applied onto a column (1.2 x 95 cm) of Sephadex G-100 previously equilibrated with 0.05 M NaOH, and eluted with the same solution at room temperature. Fractions (1.2 ml) were collected at a flow rate of 2.5 ml/h and neutralized with 1 M HCl. Amounts of total carbohydrates and reducing-end groups were measured by the phenol-sulfuric acid method⁷³⁾ and the Somogyi-Nelson procedure⁷⁴⁾, respectively, using maltose as a standard. The value of average chain length was calculated by the ratio of the carbohydrate equivalent to glucose to the reducing terminal. Absorption spectra of D-glucan-iodine complex were recorded on a Shimadzu UV-160 spectrophotometer over the range

of 400 to 700 nm according to the procedure of Krisman⁷²⁾ with minor modifications.

Analytical Procedures --- Protein concentration was determined by the method of Hartree²⁴⁾ or Bradford²⁵⁾. Nucleotide sequence analysis was carried out by the dideoxy chain-termination method²⁶⁾, using a *BcaBEST* dideoxy sequencing kit from Takara Shuzo (Kyoto). Computer-aided analysis of nucleotide and protein sequences was carried out using a GENETYX program (Software Development, Tokyo).

Results and Discussion

Establishment of Expression System in E. coli --- To obtain recombinant proteins, expression systems for the cDNA fragments encoding RBE1 and RBE3 were established in *E. coli* BL21 (DE3), using a pET-23d vector (Fig. 23), as described in maize branching enzymes(). The cDNA sequences coding for the transit peptides of the RBE1 and RBE3 precursors were removed, so that the recombinant proteins were designed to correspond to the mature enzymes. An ATG codon for Met present between two amino-terminal amino acids of the mature RBE1 was used as an initiator. In RBE3, the nucleotide sequence, GCGGCGGGC, encoding the amino-terminal 3-residue sequence, Ala-Ala-Gly, of the mature RBE3 was changed into an ATGGCT sequence by addition of an *Nco*I linker. Thus, the recombinant RBE3 was designed to lack second and third amino-terminal amino acids, Ala and Gly. The recombinant proteins were produced in soluble extracts of the transformed *E. coli* cells. For a large-scale preparation, 400-ml cultures were prepared, and the cell paste was collected. After sonication, the soluble extracts were subjected to ion-exchange chromatography on a column of DEAE-cellulose (Fig. 24) followed by hydrophobic chromatography on an ω -aminooctyl Sepharose 4B column (Fig. 25). By the two-step purification procedure, the recombinant RBE1 and RBE3 were purified to nearly homogeneity on SDS-PAGE (Fig. 26) with 84 and 36% yields, respectively (Table 2). The specific activities of the purified recombinant RBE1 and RBE3 were 417 and 25.1 units/mg of protein under Assay A, respectively.

Implication of Cysteine Residues on Branching Enzyme Activity ---

Since plant branching enzyme requires free sulfhydryl groups for the

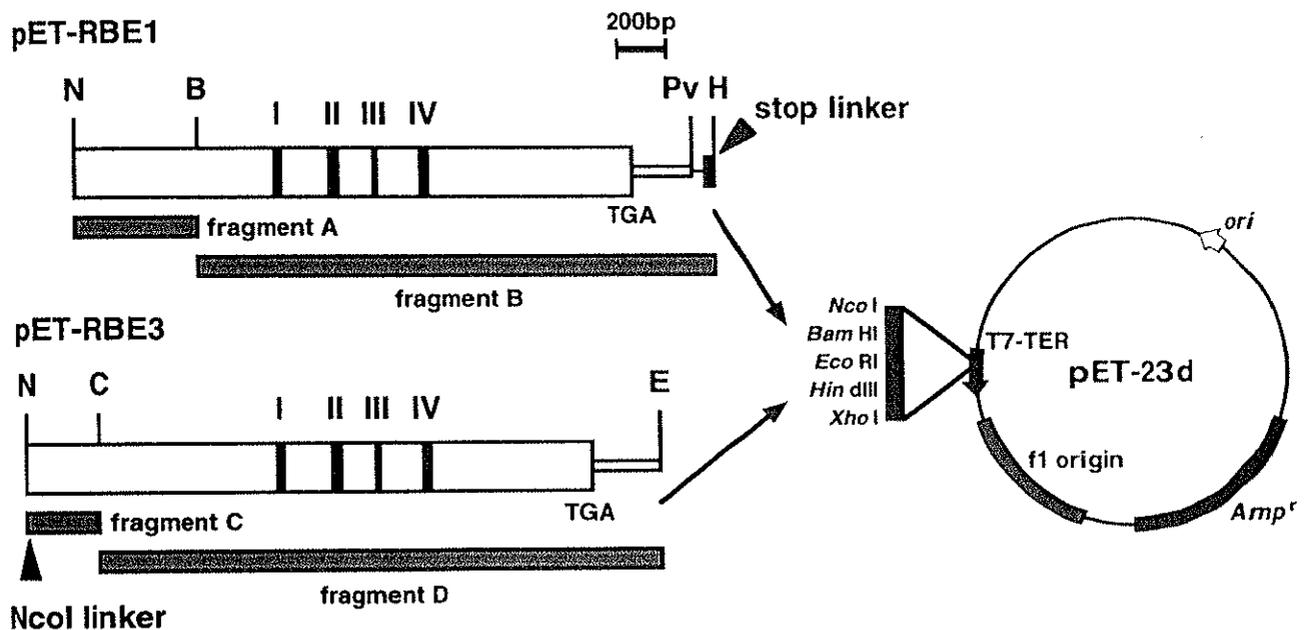


Fig. 23. Construction of *E. coli* expression plasmids for RBE1 and RBE3. Only relevant restriction sites are indicated; *Nco*I (N), *Bam*HI (B), *Pvu*II (Pv), *Hin*dIII (H), *Cla*I (C), and *Eco*RI (E). An *Nco*I linker was introduced into 5'-terminal end of RBE3 cDNA. A stop linker was introduced into 3'-terminal of RBE1 cDNA. Open boxes indicate the coding region of the mature branching enzymes. The conserved sequences of the RBE1 and RBE3 with the four regions of amyolytic enzymes are I to IV (indicated by closed boxes).

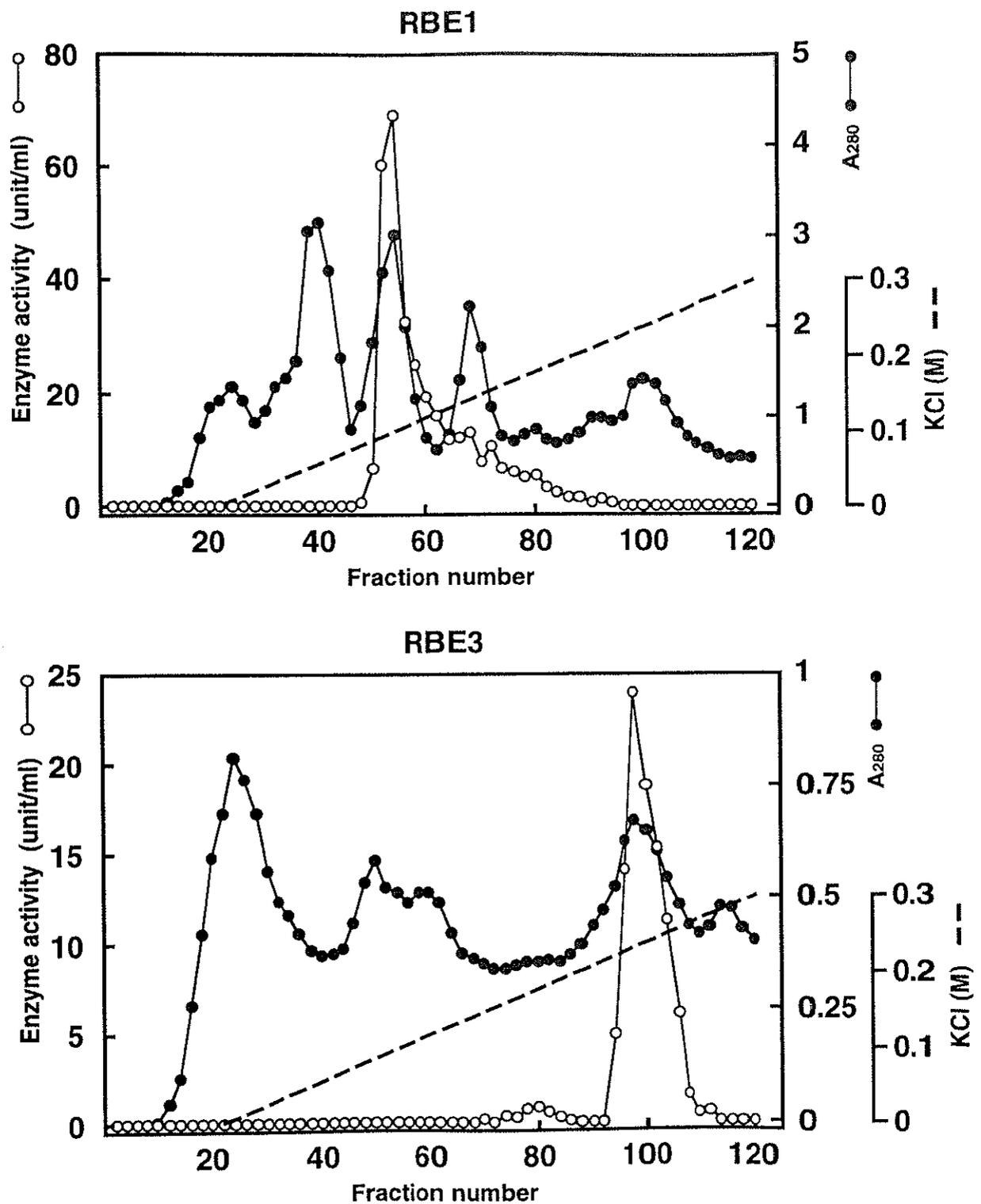


Fig. 24. Chromatography of recombinant branching enzymes from *E. coli* soluble extracts on DEAE-cellulose. Crude extracts from transformed cell pastes were applied to a DEAE-cellulose column (1.9 x 40 cm). Proteins were eluted from the column with a linear gradient of 0-0.3 M KCl (----, 1 liter). Fractions (10 ml) were collected and assayed for the enzyme activity (○) and absorbance at 280 nm (●).

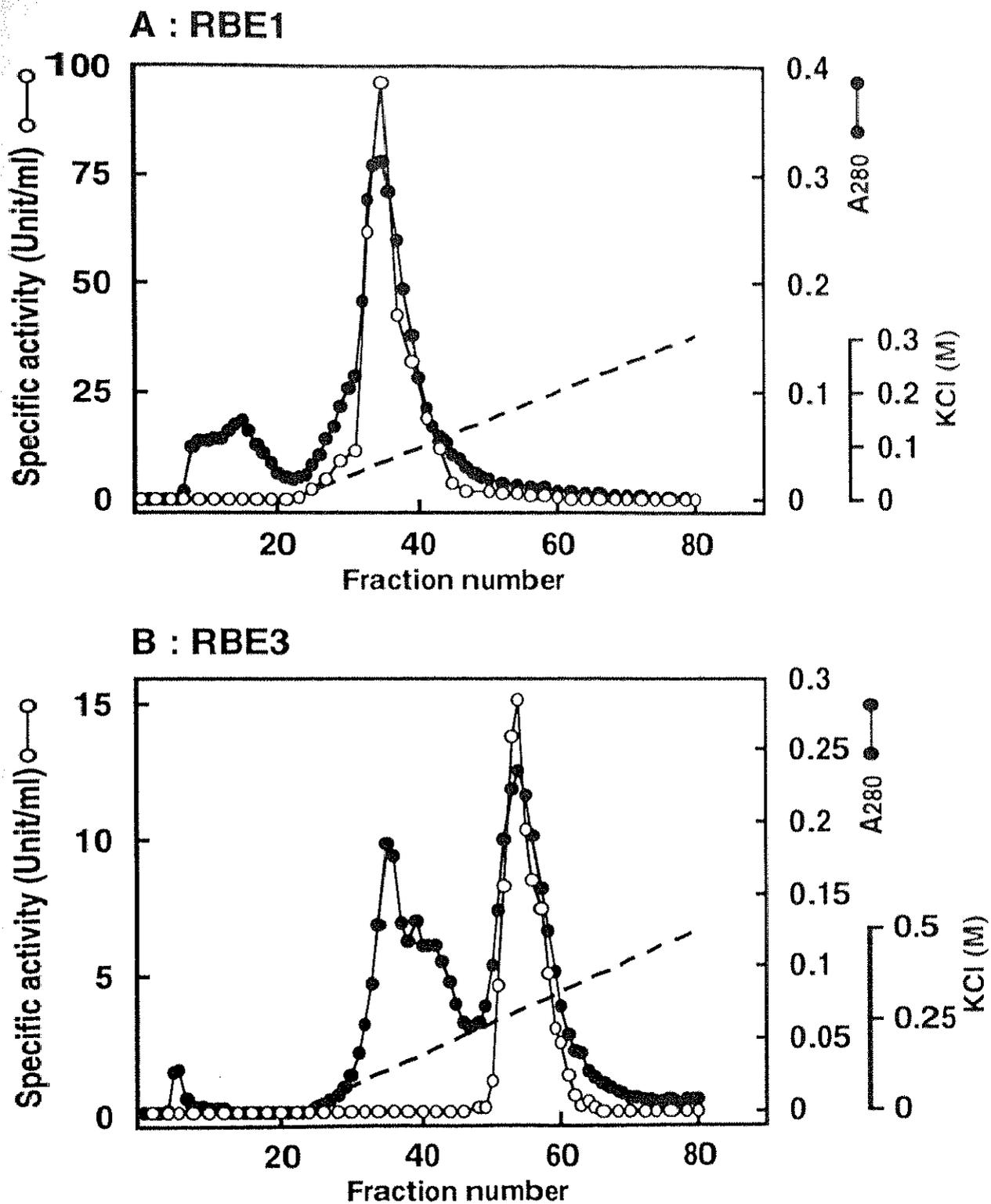


Fig. 25. Purification of recombinant branching enzyme by ω -aminoethyl Sepharose 4B column chromatography. After DEAE-cellulose column chromatography, each of the recombinant branching enzymes was applied to an ω -aminoethyl Sepharose 4B column. Proteins were eluted from the column with a linear gradient of KCl. Fractions were collected and assayed for the enzyme activity (\circ) and absorbance at 280 nm (\bullet).

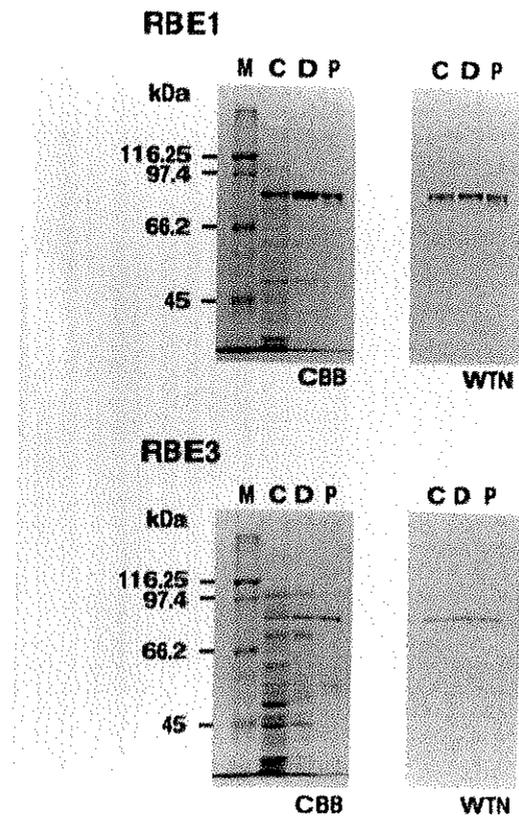


Fig. 26. SDS-PAGE and Western blot analysis of purified recombinant RBE1 and RBE3. Proteins from soluble extracts of *E. coli* cell paste (*C*), from DEAE-cellulose column chromatography (*D*), and from ω -aminooctyl Sepharose 4B column chromatography (*P*) were subjected to SDS-PAGE (left panels) followed by Western blot analysis (right panels). The gel was stained with Coomassie brilliant blue (left panels). The blots were probed by affinity-purified anti-RBE1 antibody (upper right panel) and by affinity-purified anti-RBE3 antibody (lower right panel).

Table 2. Purification of recombinant branching enzymes produced in *E. coli*

A : RBE1

Fraction	Volume (ml)	Total protein (mg)	Total activity (unit)	Specific activity (unit/mg)	Purification (fold)	Yield (%)
Crude extracts	32	428.8	10973	25.6	1	100
DEAE-cellulose	151	132.3	8572	64.8	2.5	78
ω -Aminoethyl Sepharose	134	22.1	9228	417	16.3	84

B : RBE3

Fraction	Volume (ml)	Total protein (mg)	Total activity (unit)	Specific activity (unit/mg)	Purification (fold)	Yield (%)
Crude extracts	33	497.3	547.1	1.06	1	100
DEAE-cellulose	164	145.6	449.9	3.09	2.9	82
ω -Aminoethyl Sepharose	73.5	7.94	199.3	25.1	23.7	36

The enzyme activity was measured by phosphorylase stimulation assay (Assay A).

activity⁶⁹), effects of monoiodoacetic acid (MIA) and *p*-chloromercuribenzoic acid (PCMB) on purified recombinant RBE1 and RBE3 were examined (Fig. 27, Panel A and B). The RBE1 activity was completely inhibited by the presence of 10 mM MIA or 90 μ M of PCMB, whereas 10 mM MIA or 20 μ M of PCMB inactivated RBE3. When the enzymes inactivated by PCMB were mixed with 5 mM 2-mercaptoethanol, the enzyme activity was recovered (Fig. 27, Panel C). Thus, these data demonstrate that free sulfhydryl group(s) is essential for rice branching enzymes.

The mature form of RBE1 contains 9 cysteine residues (5 residues for RBE3), four of which are conserved between RBE1 and RBE3 (Fig. 18). To examine which cysteine residue(s) is implicated in the activity of branching enzyme, each or two of 7 cysteine residues in the mature RBE1 molecule, except for the two residues located in the carboxyl-terminal region, were replaced into serine residue by point mutagenesis. The reason for the exception was that deletion of the carboxyl-terminal 55-residue sequence of RBE1 containing eighth and ninth cysteines did not affect the enzyme activity, as described below. Expression plasmids carrying the point mutations in pTV-RBE1 were introduced into *E. coli* cells, and the mutant proteins, termed C1S, C2S, C3S, C4S, C5S, C6S, C7S, and C67S, were produced in soluble extracts of the transformed cells (Fig. 28). The molecular masses of the recombinant proteins produced, including wild-type RBE1, were all estimated to be 85 kDa. When the enzyme activity was measured by Assay A, C3S, C6S, and C7S exhibited approximately 40% of the activity found in the wild-type (Fig. 29). Moreover, the activity of C67S, which had the replacements of both sixth and seventh cysteines into serines, was very low (almost 10%). No significant difference of the activity was observed among the wild type, C1S, C2S, C4S, and C5S. Thus, these results suggest that the

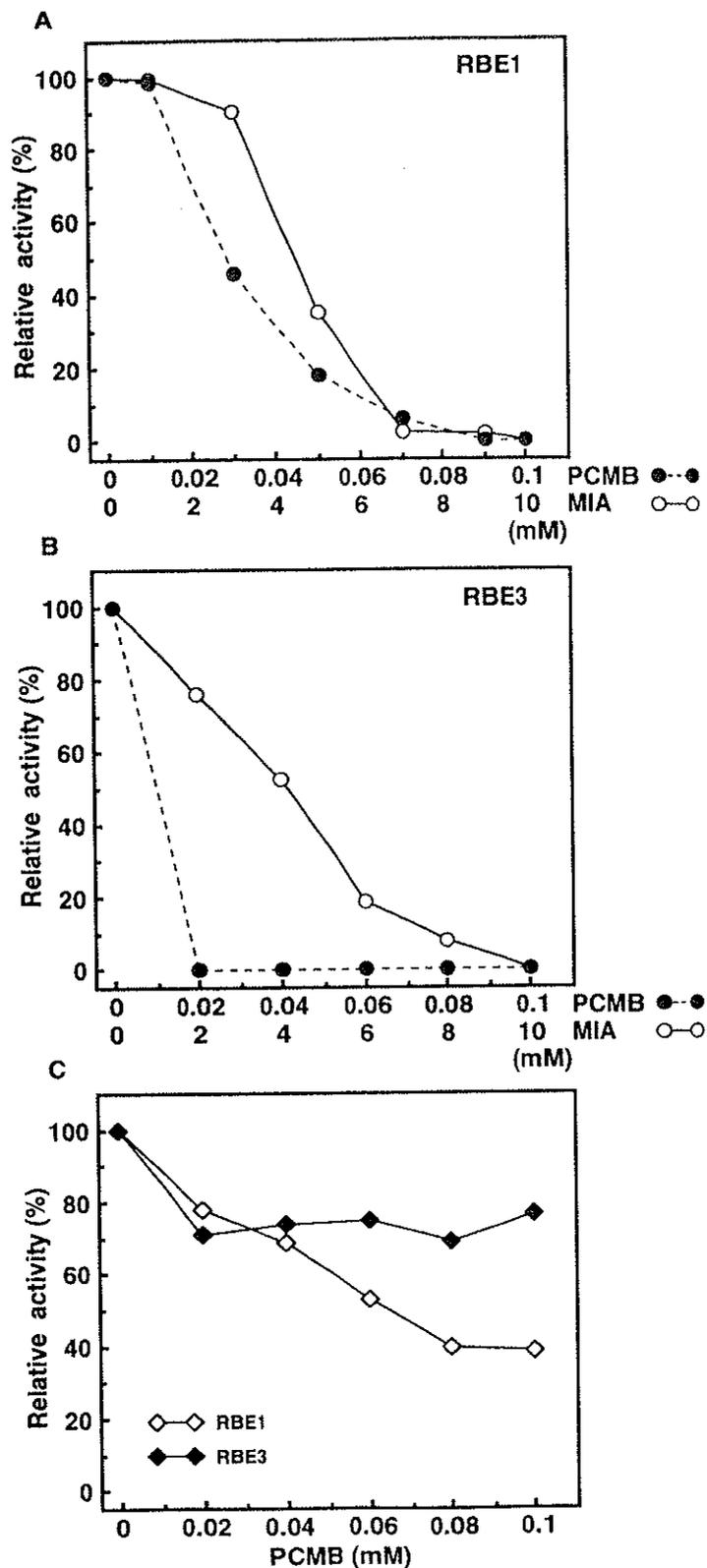


Fig. 27. Effect of SH-inhibitors on branching enzyme activity. The enzyme solution (0.05 units/ml) was incubated at 30°C for 1 h with potato amylose (1 mg/ml) in 0.1 M citrate buffer containing various concentration of SH-inhibitors (PCMB ●, MIA ○; panel A and B). After the enzymes inactivated by PCMB were mixed with 5 mM 2-mercaptoethanol, the reaction mixture was incubated at 30°C for 1 h (Panel C). The enzyme activity was measured by Assay B.

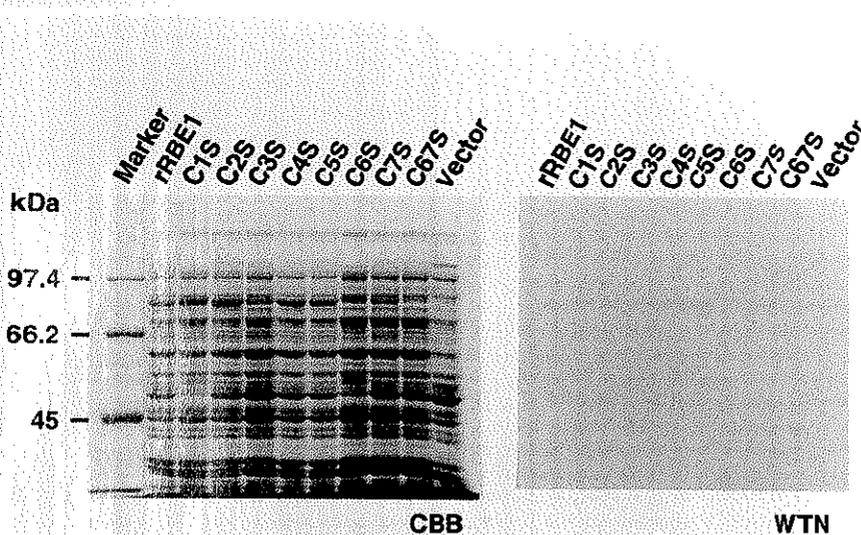


Fig. 28. SDS-PAGE and Western blot analysis of wild-type and mutant RBE1s. Soluble protein fractions from *E. coli* cell paste were subjected to SDS-PAGE (left panel) followed by Western blot analysis (right panel). The gel was stained with Coomassie Brilliant Blue (left panel). The blot was probed by affinity-purified anti-RBE1 antibody (right panel).

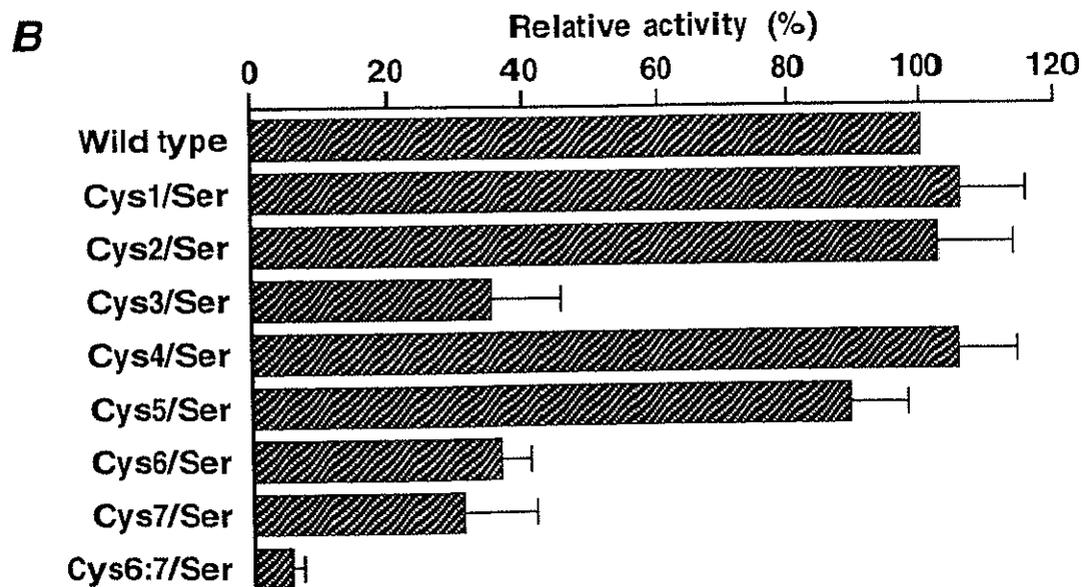
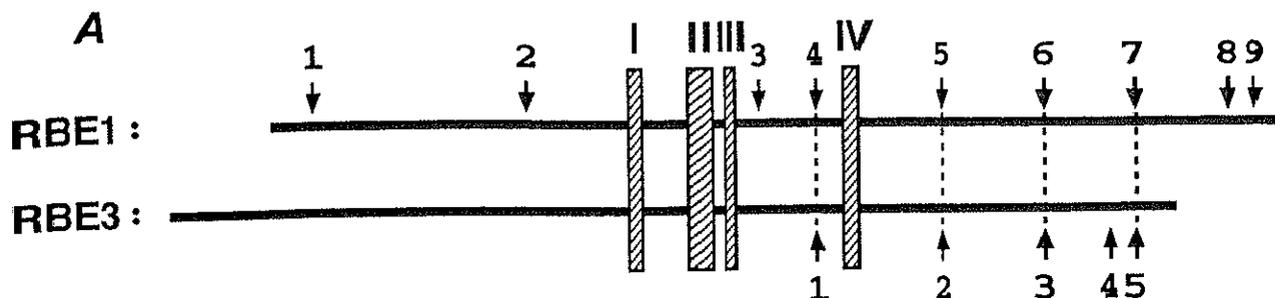


Fig. 29. Effect of cysteine replacement on RBE1 activity. The location of cysteine residues both RBE1 and RBE3 is represented by panel A. Panel B; the enzyme activity was measured by Assay A. The activities are indicated by relative activity (wild type=100%). C3S, C6S, C7S, and C67S show a lower activity than RBE1.

RBE1 activity requires that third, sixth, and seventh cysteines in this molecule remain free sulfhydryl groups. Since two out of these three residues, sixth and seventh residues, are conserved between RBE1 and RBE3, the conserved residues may be also important for the activity of RBE3.

Roles of Amino- and Carboxyl-Terminal Sequences in RBE1 Activity --- To examine how the amino- and carboxyl-terminal sequences of RBE1 are implicated in the enzyme activity, mutant proteins lacking various lengths of the sequences from the amino- and carboxyl-termini, termed deletion mutants, were produced in *E. coli* and characterized (Fig. 30). Of 9 different deletion mutants, only two mutants, *dC39* and *dC55*, lacking the 39- and 55-residue sequences from the carboxyl terminus, respectively, exhibited the enzyme activity. Other deletion mutants, including the mutants deficient in the amino-terminal sequences (*dN47*, *dN161*, and *dN237*), had no activity. The level of the enzyme activity in *dC55* was almost twice higher than that in the wild type. However, the reason for this observation is unknown at the present time. At any rate, my data indicate that the amino-terminal sequence of RBE1 is essential for the enzyme activity, and that the carboxyl-terminal sequence of at least 55 residues is not implicated in the activity.

Characterization of Chimeric Enzymes between RBE1 and RBE3 Produced in E. coli --- As described before, the amino acid sequence of RBE1 shares a noticeable degree of identity (49%) with the RBE3 sequence (Fig.18). However, in addition to the difference of the sequences in the amino- and carboxyl-terminal regions, there is an extra sequence specifically present either in RBE1 or in RBE3 (Fig. 31).

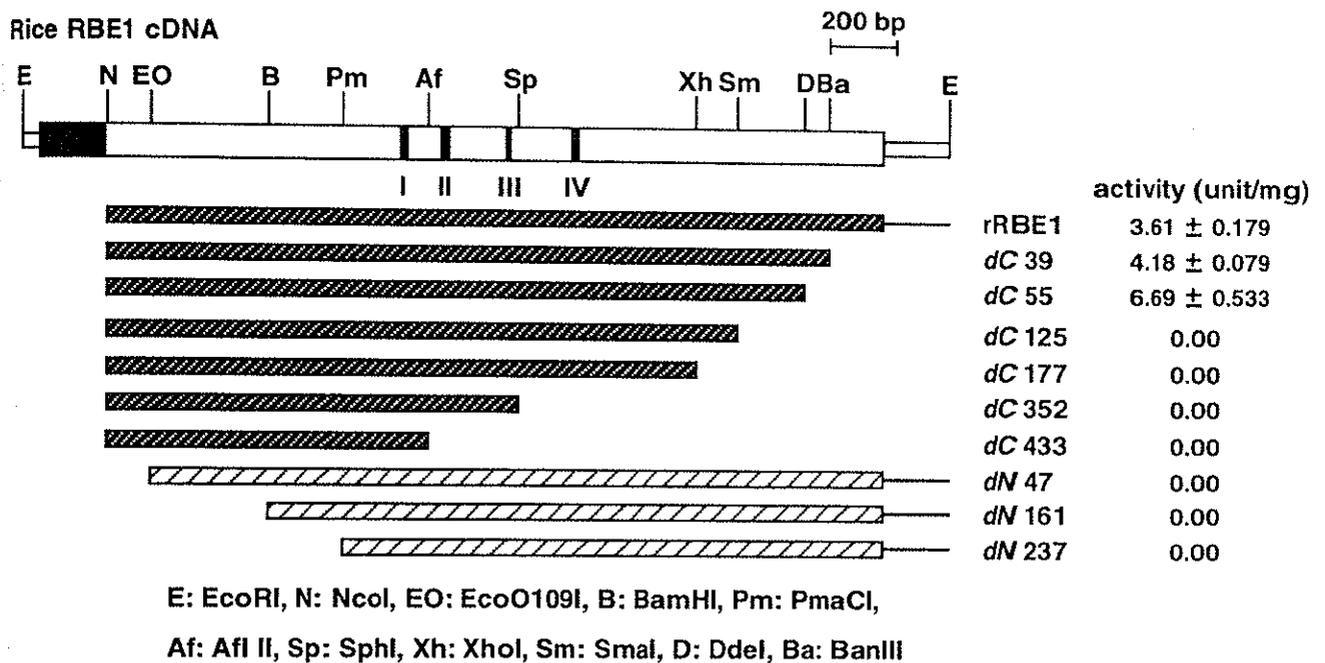


Fig. 30. Constriction of bacterial expression plasmids for RBE1-deletion mutants, and the activity of those mutants. Only relevant restriction site are indicated. As shown in upper diagram, the coding region of transit peptide is indicated by *closed box*, and *open box* indicates the coding region of the mature RBE1. The conserved sequences of the RBE1 with the four regions of amylolytic enzymes are *I to IV* (*closed thin boxes*). The activity of soluble extracts from *E. coli* cell paste was measured by Assay A.

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RBE1 1:-----TMM-----TVVE-----VHLEFYDLPKLE 22
RBE3 1:AAGASGEVMIPEGESDGMFVSAGSDDLQLPALDDELSLENYGAEVETIESSGASDVEGVKRVVVEELAAEQKPRVVPPTGGQKIFQMSMLN 90

RBE1 23:EFKDHFNRIKRYLDQKCLLEKHEGGLEEFKGYLKFGLNIVDQATIYREWAPAQEAQICEFNWNGAKHKMEKDFGIWSIKI-SHV 111
RBE3 91:GYVHLEFYRSLYRRLRSLLDQVEGGLETFSRGYEKFGFNHSAEGVITYREWAPCAHSAALVGDFFNNWNPNADRMSKNEFGVWEIFLPNNA 180

RBE1 112:NCKPATPHNSKVKFRFRHGGGAWVDRIIPAWIRYATFDASKFGAPYDGVHWDPPACERYVEFKHPRPKPPDAFRIYEAHVGMSSGEPEVSTY 201
RBE3 181:DGSSPIPHGSRVKVVMETPPSGI-KDSIPAWIKYVSV-QAAG-EIPYNGIYYDPPPEEKYIFKHPOKPKRPSLRIRIYETHVGMSSSTEPKINTY 267

RBE1 202:REFADNVLPRIIRANNYNTVQLMAIMEHSYYASFGYHVTNFFAVSSRSGTPEDLKYLVDKAHSLGLRVLMDVVHSHASNNVTDGLNGYDVG 291
RBE3 268:ANFRDEVLPRIKKLGYNVQIMATQEHAYYGSFGYHVTNFFAVSSRSGTPEDLKSLIDKAHELGLVLMDDVVHSHASNNITDGLNGFD-C 356

RBE1 292:QNTHESYFHTCDRGYHKLWDSRLFNYPANWEVLRFLLSNLRVWMDFFMFDGFRFDGVTSMLYTHHGINKGFTGNVYKESLDTDVAIVYM 381
RBE3 357:TDTH--YFHSCSRGHVWWSRLFNYPANWEVLRFLLSNLRVWMDFFMFDGFRFDGVTSMYTHHGLOVAFTGNVSEYEGFATDADAVVYL 444

RBE1 382:MLANHLMLKLLPEATIVAEDVSGMPVLCRPVDEGGVGFDFRLAMALPDRWIDYLNKEDRKMMSSEIVQLTNRRYTEKCIYAESHDQS 471
RBE3 445:MLVNDLTHCLYPEAITICEDVSGMPFFALPVODGGVGFDFRLAMAPDKWIELLKQSDS-SWKMGDIVPLTNRWSEKCVTYAESHDQA 533

RBE1 472:IVGDKTIAFLLMDKEMTCMSDLOPASPTINRGIALQKMTHTFTMALGGDGYLNFMGNEFGHPEWIDFPR--E-----GNNMSYDK 550
RBE3 534:LVGDKTIAFWLMDKMYDFMALDRPATPSIDRGIALHKMIRLITMGLGEGYLNFMGNEFGHPEWIDFPRAPQVLENGKFLPGNNMSYDK 623

RBE1 551:CRRWSLVDTLHRLRYKYNAFDQAMNALEEFSELSKQIVSDMNEKDKMIVFERGDLVVFVNFHPNKIYKGYKVGCDLPGKYRVALDS 640
RBE3 624:CRRRFDLGDADYLRVRCMLEFDRAHQSLSEKYGFMTSQHQYISRKHEEDKMIIFERGDLVVFVNFHWSNSYFDVVGCLKPGKYRVALDS 713

RBE1 641:DALVFGGHGRVGHVDVHFTSPECMFGVPEYFNFRNSFKVLSPPRTCVAVYRVEDREELRRGGAVASGKIVTEYIDVEATSGETISGG 730
RBE3 714:DAGLFGGFGRHHTAETHFT-AD---C--SH-DNREYSEFSVSESRTCVM-----APAE----- 760

RBE1 731:WKGSEKDDCGKKGMKVFVFRSSDEDCK
RBE3 760:-----

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Fig. 31. Sequence alignment of two forms of rice branching enzyme, RBE1 and RBE3. The mature proteins of RBE1 and RBE3 of 756 and 760 residues, respectively, are compared. Identical residues are indicated by *open boxes*. Dashes represent gaps introduced to maximize the alignment. The conserved sequences with the four regions of catalytic sites of amylolytic enzymes (regions I, II, III, and IV) are represented by *shaded boxes*. The RBE1- and RBE3-specific regions (BE1-SR and BE3-SR, respectively) are indicated by *hatched boxes*.

Since the difference of the sequences of RBE1 and RBE3 may explain their distinct functions in starch biosynthesis, it is important to examine the functional domains of these two major isoforms of rice branching enzyme. Thus, I constructed seven expression plasmids for chimeric enzymes between RBE1 and RBE3, and characterized these proteins produced in *E. coli* (Fig. 32).

SDS-PAGE and Western blot analysis indicated that two out of seven constructs, termed 1Sac3 and 1Bam3, were not effectively expressed in soluble extracts of the transformed cells (Fig. 33). The molecular masses of produced chimeric proteins, 1Pst3, 1Xho3, 3Sac1, 3Xho1, and 3Pst1, on SDS-PAGE were consistent with the calculated molecular masses. 1Xho3 and 3Xho1 were purified from the soluble extracts by two-step chromatography using columns of DEAE-cellulose and ω -aminooctyl Sepharose (Figs. 34 and 35), as in the case of RBE1 and RBE3 (Figs. 24 and 25). Two other chimeric proteins, 1Pst3 and 3Sac1, were purified by batch-wise chromatography (data not shown). The purities of these chimeric enzymes so obtained exceeded 90% based upon SDS-PAGE, by which the molecular masses of 1Pst3, 1Xho3, 3Sac1, and 3Xho1 were found to be 80, 80, 92, and 94 kDa, respectively (not shown).

Activities of purified recombinant enzymes, including wild-type RBE1 and RBE3 (termed rRBE1 and rRBE3, respectively), and chimeric proteins, were measured by both Assays A and B (Fig. 36). Potato amylose and amylopectin were used as substrates in Assay B. rRBE1 had an approximately 2.5-fold higher activity than rRBE3 in Assay A (Fig. 36, Panel A). The activities of 1Pst3 and 1Xho3 were almost 1.5 and 2.0 times greater than that of rRBE1, whereas a lower level of the activity than found in rRBE3 was observed in 3Sac1 and 3Xho1. Since the structural difference among rRBE1, 1Pst3, and 1Xho3 is both the

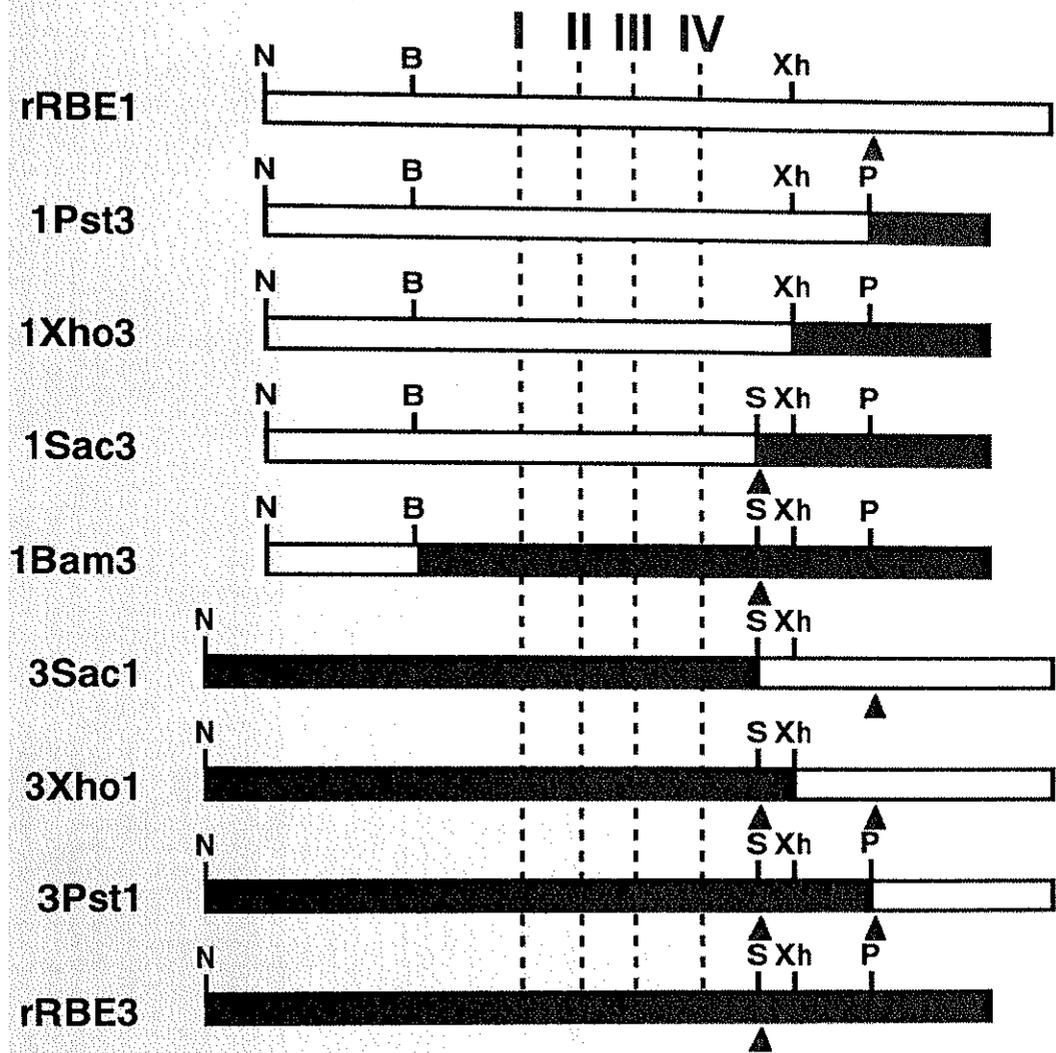


Fig. 32. Construction of bacterial expression plasmids for the chimeric enzyme between RBE1 and RBE3. Only relevant restriction sites are indicated; *Nco*I (N), *Bam*HI (B), *Xho*I (Xh), *Pst*I (P), and *Sac*I (S). *Sac*I and *Pst*I sites were introduced into RBE1 cDNA by PCR. RBE1- or RBE3-specific regions are indicated by arrowheads. The conserved sequences of the RBE1 and RBE3 with the four regions of amyolytic enzymes are I to IV.

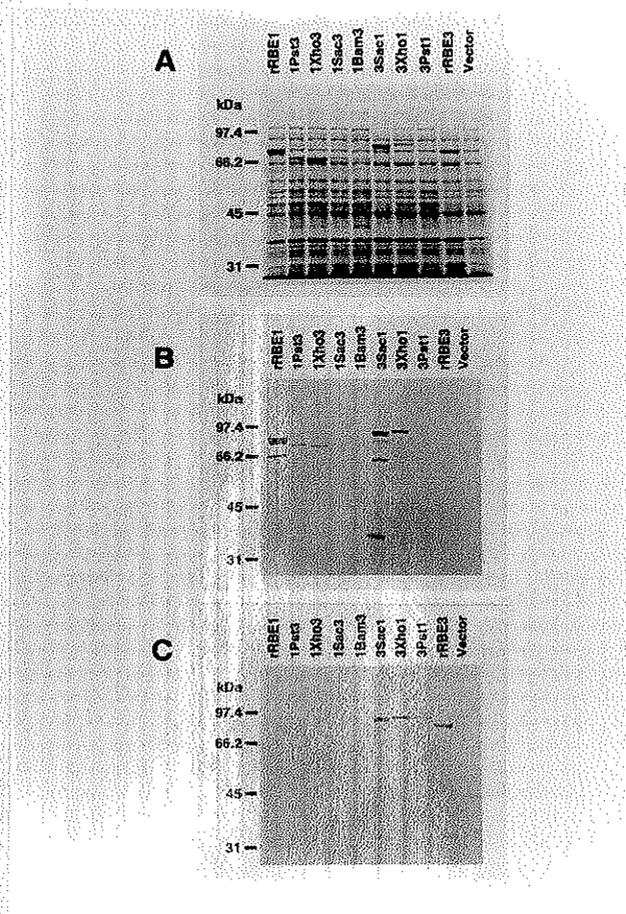


Fig. 33 SDS-PAGE and Western blot analysis of crude extracts of chimeric enzymes. Proteins from soluble extracts of *E. coli* cell paste were subjected to SDS-PAGE (panel A) followed by Western blot analysis (panels B and C). The gel was stained with Coomassie brilliant blue (panel A). The blots were probed by affinity-purified anti-RBE1 antibody (panel B) and by affinity-purified anti-RBE3 antibody (panel C).

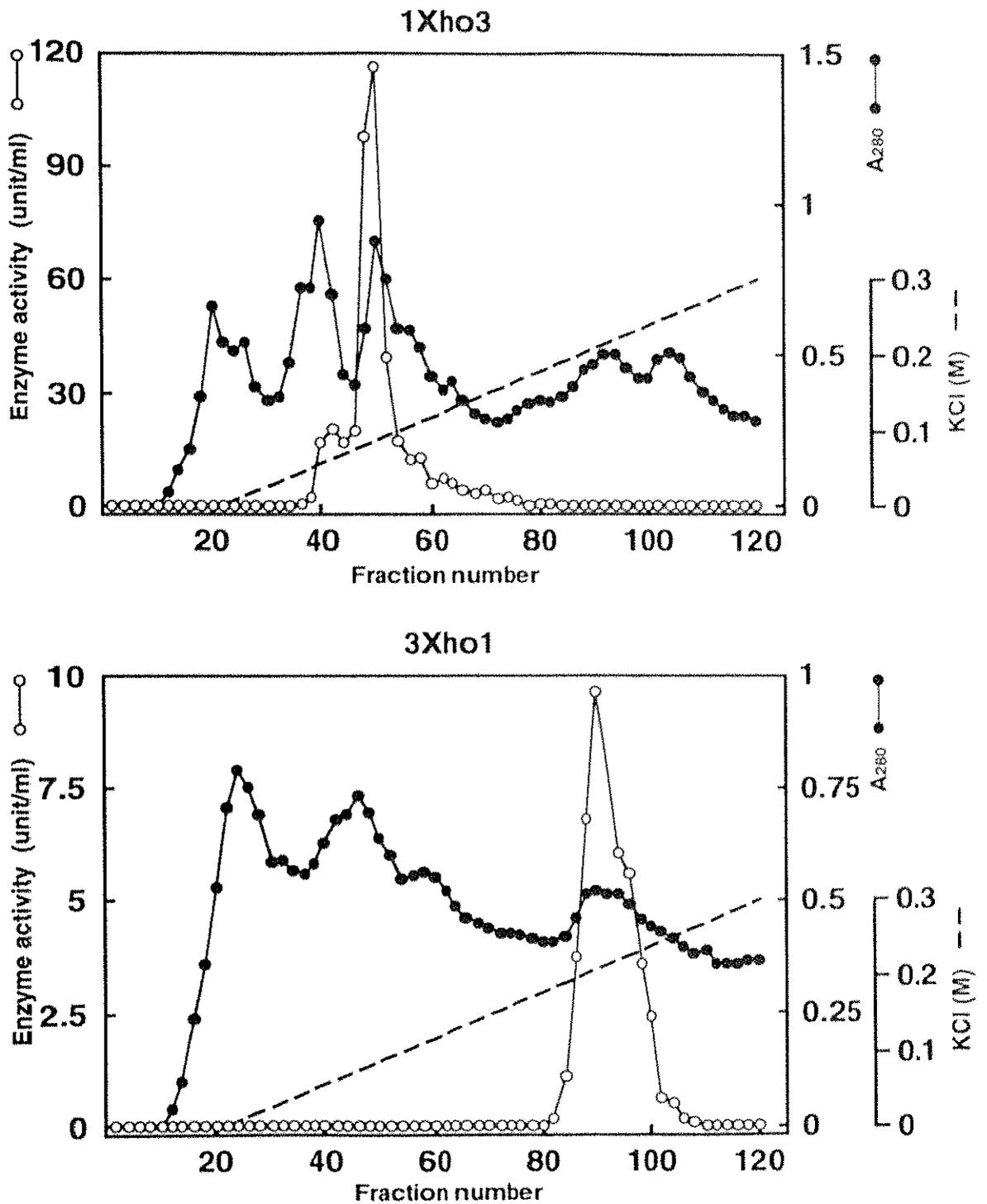


Fig. 34. Chromatography of chimeric enzymes from *E. coli* soluble extracts on DEAE-cellulose. Crude extracts from transformed cell pastes were applied to a DEAE-cellulose column (1.9 x 40 cm). Proteins were eluted from the column with a linear gradient of 0-0.3 M KCl (----, 1 liters). Fractions (10 ml) were collected and assayed for the enzyme activity (○) and absorbance at 280 nm (●).

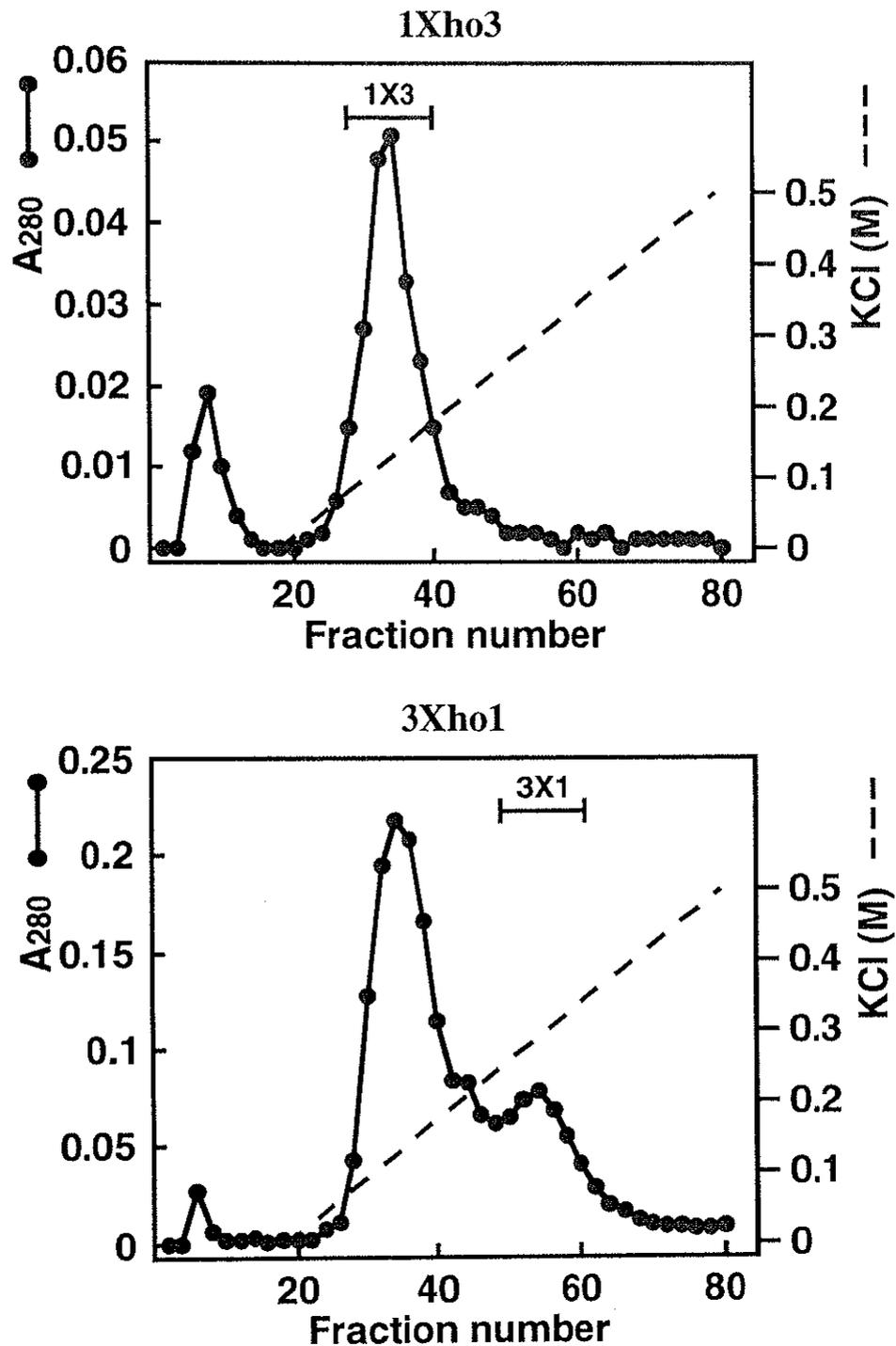


Fig. 35. Purification of chimeric enzymes by ω -aminooctyl Sepharose 4B column chromatography. After DEAE-cellulose column chromatography, each of the chimeric enzymes was applied to an ω -aminooctyl Sepharose 4B column. The enzymes were eluted from the column with a linear gradient of 0-0.5 M KCl. Fractions were collected and assayed for the enzyme activity and absorbance at 280 nm (●). 1X3 and 3X1 indicate the fractions containing chimeric enzymes.

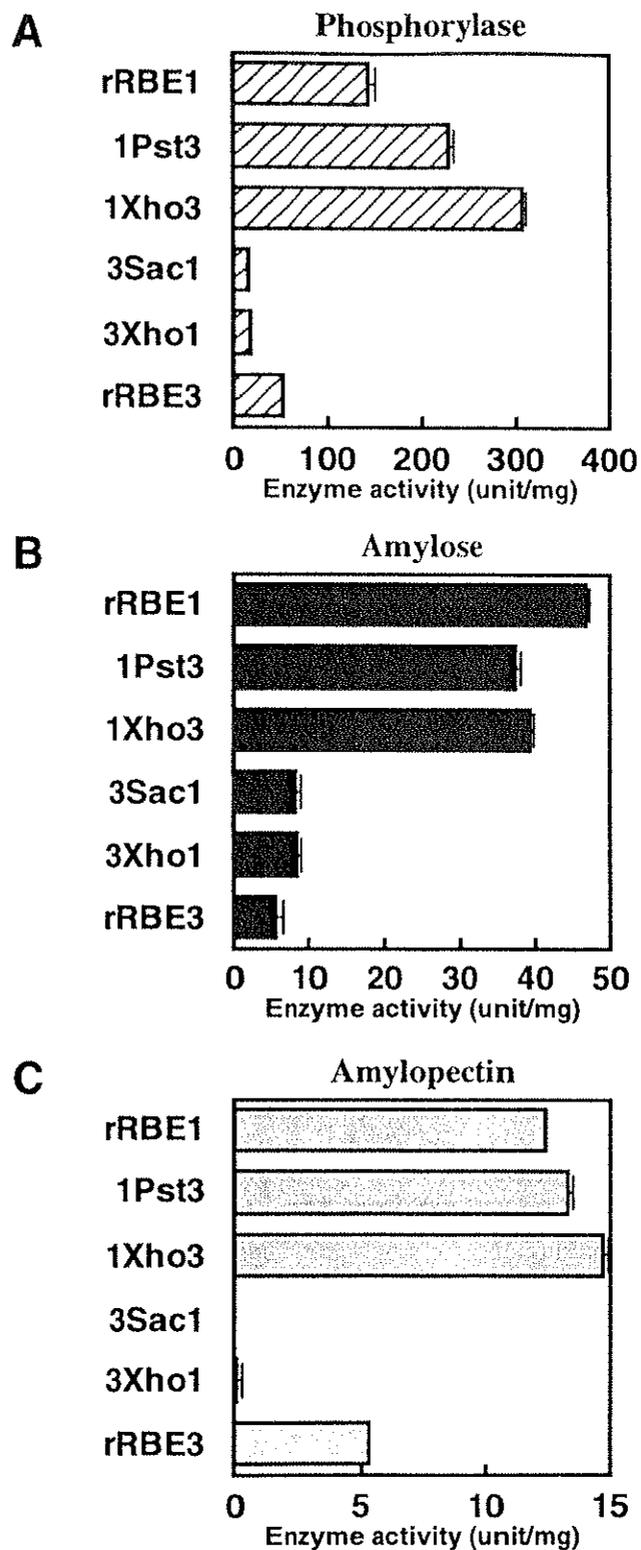


Fig. 36. Assay of branching enzyme activity in RBE1, RBE3, and chimeric proteins. The enzyme activity was measured by Assay A (panel A), Assay B using amylose for substrate (panel B), and Assay B using amylopectin for substrate (panel C). As shown in panel C, 3Sac1 and 3Xho1 little exhibited the enzyme activity toward amylopectin.

replacement of the carboxyl-terminal sequence of RBE1 into that of RBE3, and the lack of the RBE1-specific 14-residue sequence in these two chimeric proteins (Figs. 31 and 32), it is possible that the carboxyl-terminal 200-residue sequence of RBE1, including the RBE1-specific sequence, is not important to branch elongating linear-chains catalyzed by starch synthase. This sequence appears to be implicated in preventing RBE1 from production of the branch points on the elongating chains.

When amylose was used as a substrate, rRBE1 exhibited the largest activity (Fig. 36, Panel B). The level of the activity in 1Pst3 and 1Xho3 was almost 80% of that in rRBE1, while only 10-20% of the rRBE1 activity was found in 3Sac1, 3Xho1, and rRBE3. These results may suggest that the reaction of already synthesized amylose chains by branching enzyme does not require the carboxyl-terminal 200-residue sequence of RBE1, as in the case of branch formation in the elongating chains. However, in both reactions, rRBE3 exhibited only a much lower activity than rRBE1. Thus, the amino-terminal extra sequence of almost 70 residues in the RBE3 molecule may be the hindrance to exhibit the enzyme activity, since the sequence of the amino-terminal and central regions of RBE3, excluding the amino-terminal 70-residue sequence, are highly conserved with that of RBE1 (Fig. 31).

The level of the rRBE3 activity was similar to those of 1Pst3 and 1Xho3 when amylopectin was used as a substrate (Fig. 36, Panel C). The most intriguing observation was that 3Sac1 and 3Xho1 little exhibited the enzyme activity toward amylopectin. rRBE3 still had almost 40% of the rRBE1 activity. Since rRBE3 is structurally distinguished from 3Sac1 and 3Xho1 by the replacement of the carboxyl-terminal sequence of approximately 200 residues in RBE1, the corresponding sequence of RBE3 is likely to be essential for branching amylopectin. At any rate, the difference of the amino- and carboxyl-

terminal sequences of RBE1 and RBE3 may explain the fact that RBE1 exhibits a much greater activity than RBE3 in all cases, because the sequences in the central regions of these two enzymes are highly conserved, as described before (see Fig. 31). Moreover, it appears that the short stretches of the sequences specifically present in RBE1 and RBE3 are not important for the enzyme activity.

Analysis of Branched Products from Amylose --- To examine the fine structures of branched glucans produced from potato amylose by purified rRBE1, 1Xho3, 3Xho1, and rRBE3, amylose was incubated with each of these four enzymes (1 unit by Assay B), and the products were debranched by isoamylase, and then filtered through a Sephadex G-100 column (Fig. 37). The elution patterns of the products without the isoamylase treatment indicated that 1Xho3 and rRBE3 produced a branched glucan with a lower molecular weight than found in rRBE1 and 3Xho1. When the amylose substrate was filtered through the same column, the elution pattern was the same as those of rRBE1 and 3Xho1 (data not shown). However, λ_{\max} of the glucan/iodine complex in the peak fractions of rRBE1, 1Xho3, 3Xho1, and rRBE3 was 510, 508, 540, and 541 nm, respectively. Thus, these data suggest that the products of 1Xho3 and rRBE3 may be loosely branched glucans.

Unit-chain profiles of products debranched by isoamylase indicated that rRBE1 produced a single population of branches with the average length of approximately 6 glucose units (Fig. 37). In three other enzymes, the lengths of the produced branches varied from 5 to 100 glucose units, showing the unclear elution patterns. These data appear to be consistent with the facts that rRBE1 has a much higher level of the activity toward amylose and amylopectin than rRBE3, and that 3Xho1 little exhibits the activity toward amylopectin (Fig. 36). However, there

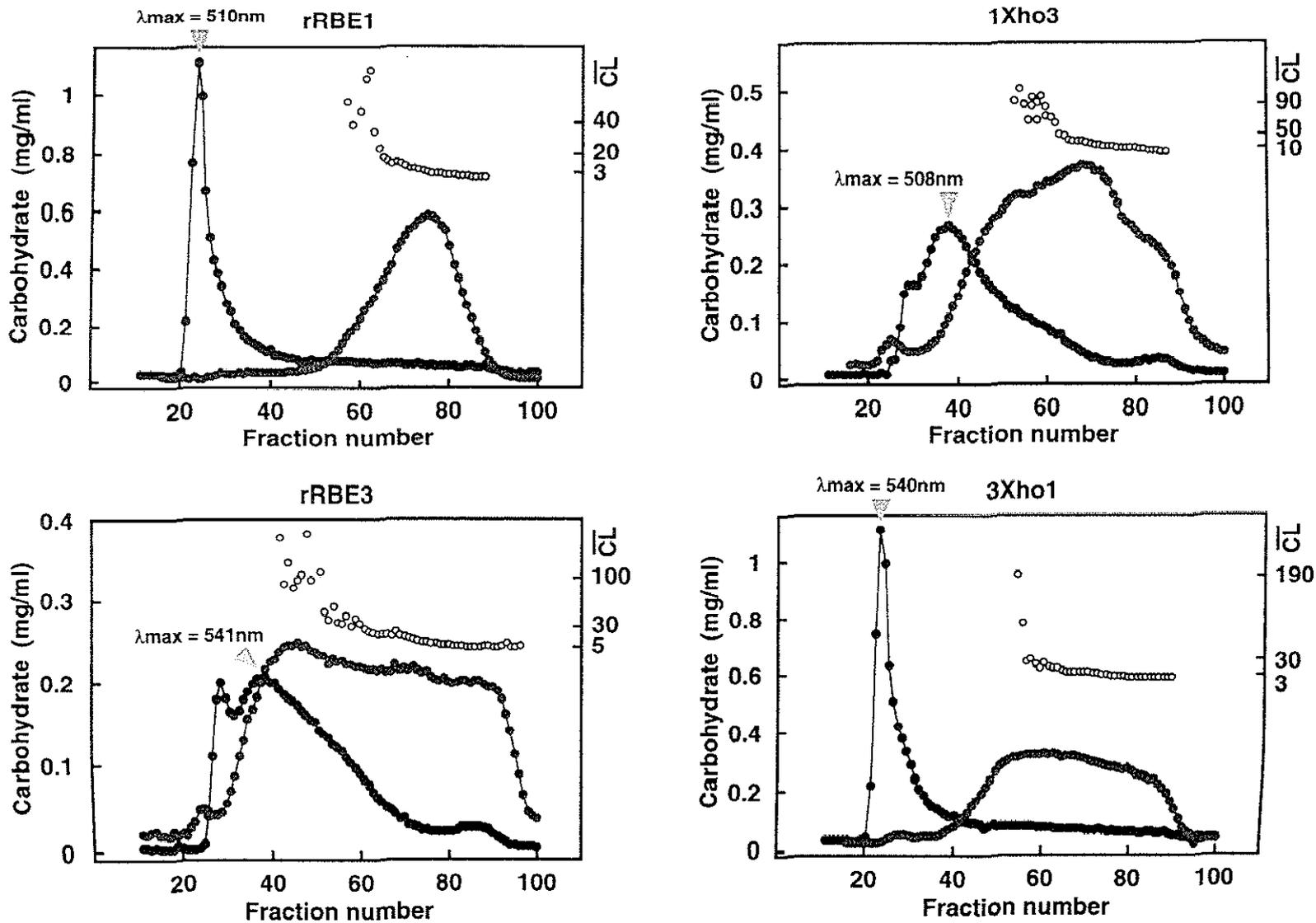


Fig. 37. Sephadex G-100 chromatography of the products by recombinant branching enzymes. The products from potato amylose by recombinant branching enzymes are indicated by closed circles. The products debranched by isoamylase are indicated by shaded circles. Average chain length is represented by open circles.

is a discrepancy that the unit-chain profile of the debranched product in 1Xho3 is totally different from that in rRBE1 (Fig. 37), in spite that no significant difference of the enzyme activity toward amylose and amylopectin was observed between rRBE1 and 1Xho3 (Fig. 36). The reason for this discrepancy is unknown at the present time. Although further experiments are necessary to elucidate the structure/function relationships of RBE1 and RBE3, my data represented here may conclude that at least the extra sequences of RBE1 and RBE3 located in the amino- and carboxyl-terminal regions are implicated in the functional differences of these two isoforms of rice branching enzyme.

General Conclusion

Multiple forms of starch branching enzyme, RBE1, RBE2, RBE3, and RBE4, were purified from developing rice seeds by chromatography on DEAE-cellulose and Toyopearl HW-55F columns. Western blot analysis indicated that RBE1 was immunologically identical to RBE2, and distinguishable from RBE3 and RBE4. The amino-terminal amino acid sequence of RBE1 was identical to that of RBE2, but highly divergent to those of RBE3 and RBE4. These results suggest that RBE1 and RBE2 are products from the same gene. The cDNA clones encoding RBE1 and RBE3 were identified from a developing rice seed cDNA library. The precursor proteins of RBE1 and RBE3 contain the transit peptide sequences, which probably function as a transport signal of nuclear-encoded proteins into the amyloplast, at the amino-terminus. The consensus sequences of four regions, which form the catalytic sites of amylolytic enzymes, are conserved in the amino acid sequences of RBE1 and RBE3. These sequences are also conserved in other starch branching enzymes, including maize endosperm^{12,77}, potato tuber^{35,36}, and *E. coli*¹³. Thus, branching enzyme belongs to a family of amylolytic enzymes^{69,78}.

To elucidate the structure/function relationships of RBE1 and RBE3, I established the expression systems for these two enzymes in *E. coli*. The recombinant RBE1 was capable of branching amylose and amylopectin more rapidly than the recombinant RBE3. Thus, RBE1 is functionally different from RBE3. The activities of RBE1 and RBE3 were inhibited by the presence of MIA or PCMB. Three mutant proteins of RBE1, which contained serine residue instead of cysteine at residue 409, 628, or 688, exhibited almost 40% of the activity of the

wild-type RBE1. These results demonstrate that several SH-groups of cysteine residues are important for the activity of branching enzyme.

As shown in Fig. 31, RBE3 possesses an almost 70-residue extra sequence at the amino-terminus and lacks an approximately 50-residue sequence at the carboxyl-terminus, as compared with RBE1. Analysis of chimeric enzymes between RBE1 and RBE3 suggest that the extra sequences of RBE1 and RBE3 located in the carboxyl- and amino-terminal regions, respectively, are implicated in the functional differences of these two isoforms. Although the roles of RBE1- and RBE3-specific sequences in the enzymatic functions are still unknown, the RBE1-specific sequence leads to the possible presence of an α -helical structure in the corresponding region of the RBE1 molecule, as judged by computer-aided analysis. Alternatively, a β -turn structure is possibly present in RBE3 due to the RBE3-specific sequence. A proposed secondary structure of branching enzyme, which is based upon the three-dimensional structure of several amylolytic enzymes^{42,79,80}, is shown in Fig. 38. Bacterial branching enzyme is noticeably different from plant enzymes in the size of eighth $\beta \rightarrow \alpha$ loop. This fact may be related to the variation in spacing of branches between glycogen and amylopectin⁸⁰. Since only RBE3-specific sequence is present in the eighth $\beta \rightarrow \alpha$ loop of the RBE3 molecule, the size of the $\beta \rightarrow \alpha$ loop differs between RBE1 and RBE3. Thus, these differences may be implicated in the distinct functions of the two isoforms of rice branching enzyme.

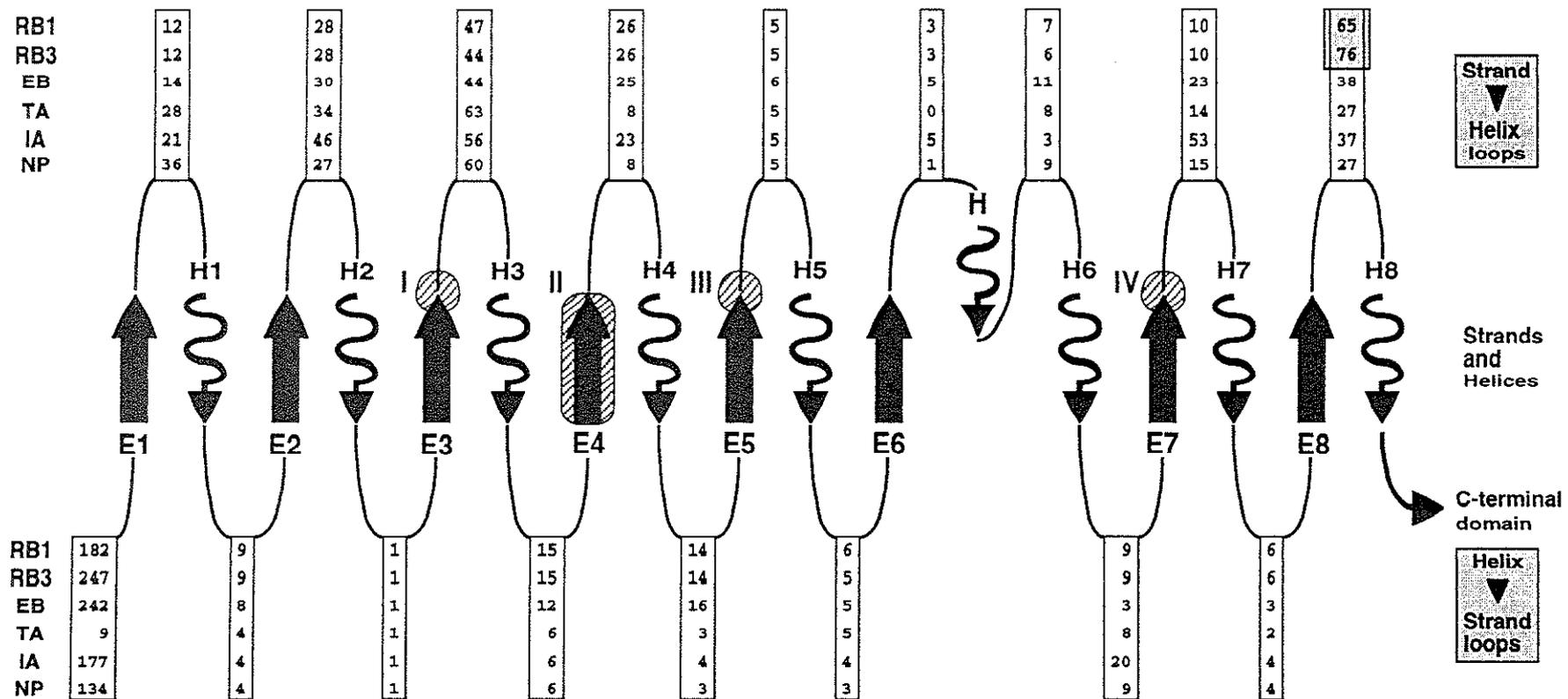


Fig. 38. Numbers of amino acid residues in β -strand \rightarrow α -helix loops and α -helix \rightarrow β -strand loops of the $(\beta/\alpha)_8$ -barrel of amylolytic enzymes. Strands (E1-E8) and helices (H1-H8 and H) are shown between the loops. The conserved sequences of the RBE1 and RBE3 with the four regions of amylolytic enzymes are I to IV (hatched circles). Numbers of amino acid residues in eighth $\beta\rightarrow\alpha$ loop of RBE1 and RBE3 were indicated by shaded box. Abbreviations used for the enzymes are: RB1, rice RBE1; RB3, rice RBE3; EB, *E. coli* branching enzyme; TA, Taka-amylase; IA, Isoamylase; NP, Neopullulanase.