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Cloning and sequencing of a gene for Mucor rennin, an aspartate protease from *Mucor pusillus*

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ABSTRACT

The aspartate protease of Mucor pusillus (Mucor pusillus rennin ; MPR) is a milk-clotting enzyme used in the cheese industry. The partial amino acid sequence of MPR was determined and oligonucleotide probes were synthesized for cloning of the MPR gene. A clone giving positive hybridization with the probes was selected from the cosmid library. Sequencing of the cloned DNA revealed an open reading frame of 1281 bp without introns which encodes 361 amino acids for the expected MPR with an NH_2-terminal extension of 66 amino acids. MPR seems to be synthesized as a prepro enzyme.

INTRODUCTION

The aspartate proteases extracellularly produced by the two closely related species of zygomycete fungus, Mucor pusillus (1) and Mucor miehei (2), are called Mucor rennins and are widely used as milk coagulants in cheese manufacture. Although some differences are observed between these two fungal proteases with respect to their peptide cleavage pattern and glycosylation (3), they share a common antigenic structure and almost identical enzymatic properties, i.e., high milk-clotting activity along with low proteolytic activity, which make it possible for them to be used as substitutes for calf chymosin in the cheese industry.

Inactivation of the Mucor rennins from M. pusillus (MPR) and M. miehei (MMR) by photo-oxidation and their detailed analysis by nuclear magnetic resonance spectroscopy have suggested that a single histidyl residue is involved in their catalytic function in addition to the two essential aspartate residues (4,5). Although the preliminary amino acid sequence of MMR shows a certain degree of homology with that of calf chymosin especially around the essential aspartate residues (6),

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no possible essential histidyl residue has been found at a corresponding site in the alignment. Confirmation of the sequence would be the first step toward further elucidation of the structure and function of these characteristic aspartate proteases.

In this paper, we report the cloning of the chromosomal MPR gene from M. pusillus using oligonucleotide probes synthesized according to the partial amino acid sequence of the enzyme. Sequencing of the cloned gene revealed that the MPR gene has no intron and that possible pre- and pro-sequences are present upstream of the mature MPR sequence. The total amino acid sequence of MPR deduced from the DNA sequencing shows high homology with that of MMR.

MATERIALS AND METHODS

Strains, plasmids and media

M. pusillus IFO4578(+) was used as the donor of the MPR gene for cloning. PDT agar medium containing 0.4% potato extract (Difco), 2% glucose, 2 mg/ml thiamine HCl and 1.5% agar, pH 7.0, was used for stock culture and YPD medium containing 0.3% yeast extract (Difco), 1% Bacto-peptone (Difco) and 2% glucose, pH 7.0, was used to obtain cells for DNA preparation.

Escherichia coli HB101 cultured in L-broth containing 0.2% maltose was used for cloning with a cosmid vector pJB8 (7). pBR322 was used for subcloning of the MPR gene.

Purification and partial amino acid sequencing of MPR

MPR was purified from Meito Rennet (Meito Sangyo, Tokyo, Japan) by the method described by Etoh et al. (3). The NH_2-terminal amino acid sequence of the purified MPR was determined with a Beckman Protein/Peptide Sequencer System 540D and the phenylthiohydantoin derivatives of the amino acids were analyzed using a high-performance liquid chromatograph (HPLC) equipped with a Shodex PROTEIN WS-803 column (Showa Denko, Tokyo, Japan).

Cleavage of MPR with CNBr and trypsin was performed mainly according to Koide et al. (8) as follows. One hundred milligrams of the purified MPR was carboxymethylated to protect cysteinyl residues and treated with CNBr (100 equivalents against the methionine content of the sample) dissolved in 70% formic acid

under an N_2 atmosphere for 24 h with shaking at room temperature in the dark. After removal of formic acid *in vacuo*, the resulting peptides were dissolved in acetonitrile:H_2O (1:1) containing 0.1% trifluoroacetic acid and separated by HPLC using a reverse-phase silica column. Among the eight peptides eluted separately, five were subjected to amino acid sequencing as described above. The carboxymethylated MPR (100 mg) was also digested with TPCK-trypsin (Sigma, 4%) in the presence of 2 M urea at pH 8.0 for 12 h at 25°C. The peptides were separated and 5 among 22 peptides were sequenced.

Synthesis of the oligonucleotide probes

Two kinds of 14-mer and two kinds of 17-mer mixed oligonucleotide probes were synthesized using the automated phosphoamidide method on a Nihon Zeon DNA Synthesizer (Nihon Zeon, Japan) and purified by HPLC. The synthesized probes were end-labelled with polynucleotide kinase and γ-^{32}P-ATP (New England Biochemicals).

Preparation of M. pusillus DNA

Spores of *M. pusillus* IFO4578(+) from one slant culture of PDT agar medium were inoculated into 1 liter of YPD medium in a 5-liter Erlenmeyer flask and cultured at 30°C for 3 days with shaking. The wet mycelia (about 100 g) were frozen in liquid nitrogen and pulverized with a power homogenizer. The broken cells were extracted with 600 ml of 0.5 M EDTA (pH 8.0) containing 0.5% SDS and 100 mg/l of Proteinase K at 50 C for 3 h. After removal of the residual cell debris, the extract was treated with phenol twice and nucleic acids were precipitated with ethanol. DNA was purified by CsCl equilibrium density gradient centrifugation.

Construction of the gene library of M. pusillus

The purified *M. pusillus* DNA was partially digested with *Sau*3AI and then treated with bacterial alkaline phosphatase. The DNA fragments larger than 25 kilobases (kb) in size collected by agarose gel electrophoresis were ligated with the left and right arms of cosmid pJB8 DNA and packaged into the phage particles using an *in vitro* packaging kit (Amersham). *E. coli* HB101 was infected with the phages and approximately 20,000 of the ampicillin resistant transformants were selected.

Cloning of the MPR gene

The gene library of M. pusillus in E. coli was screened by colony hybridization with the synthetic oligonucleotide probes according to the method of Wallace et al. (9). Hybridization temperatures were calculated according to the rules described by Suggs et al. (10) and hybridization was performed at 43°C with the 17-mer probes and at 28°C with the 14-mer probes, respectively. Plasmid DNAs were isolated by the method of Godson and Vapnek (10). Transformation of E. coli with the recombinant plasmids was performed by the method of Norgard et al. (12). Various restriction endonucleases and other enzymes were purchased from Takara Shuzo (Kyoto, Japan).

Southern blot analysis

Transfer of DNA fragments from agarose gel to nitrocellulose paper was performed as described by Southern (13). Hybridization conditions and labeling of DNA hybridization probes by nick translation were as described by Rigby et al. (14).

DNA sequencing

Specific restriction fragments of the cloned DNA were ligated into the appropriate M13 vectors mp10 or mp11 (15) and sequenced by the chain-terminating dideoxy method (16).

RESULTS

Partial amino acid sequencing of MPR and synthesis of DNA probes

We determined the partial amino acid sequence of MPR containing 185 amino acid residues (Fig. 4). These covered about half of the expected total sequence of the enzyme and also showed good coincidence with the corresponding sequences of MMR (87% homology).

Four parts of the MPR sequence were selected and the corresponding oligonucleotide mixtures, 14T-1, 17T-2, 14T-3 and 17T-4, were synthesized as the probes for cloning (Fig. 1). In 17T-4, the number of oligonucleotides was reduced by choosing the codons frequently used in both Saccharomyces cerevisiae (17) and E. coli (18).

Cloning of the MPR gene

The cosmid library of the M. pusillus genes in E. coli was screened with the labelled synthetic probes. Several colonies

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                    143                 147
Amino Acid          Met  Glu  Ala  Glu  Tyr

      mRNA      5'  AUG  GAA  GCN  GAA  UAU  3'
                           G         G

Probe 14T-1     3'  TAC  CTT  CGN  CTT  AT  5'
                           C         C


                    208                      213
Amino Acid          Tyr  Phe  Phe  Trp  Asp  Ala

      mRNA      5'  UAU  UUU  UUU  UGG  GAU  GCN  3'
                      C    C    C         C

Probe 17T-2     5'  TAT  TTT  TTT  TGG  GAT  GC  3'
                      C    C    C         C


                    326                 330
Amino Acid          Asn  Gln  Phe  Ile  Val

      mRNA      5'  AAU  CAA  UUU  AUA  GUN  3'
                      C    G    C    U
                                     C

Probe 14T-3     3'  TTA  CTT  AAA  TAA  CA  5'
                      G    C    G    T
                                     G


                    315                      320
Amino Acid          Thr  Cys  Met  Phe  Ile  Val

      mRNA      5'  ACN  UGU  AUG  UUU  AUA  GUN  3'
                           C         C    U
                                          C

Probe 17T-4     3'  TGN  ACA  TAC  AAG  TAA  CA  5'
                           G              G
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Figure 1 Oligonucleotide probes used and their corresponding amino acid- and mRNA sequences in MPR.

among 10,000 gave positive hybridization with 14T-1 and 14T-3 and one of them, No.11, which gave intense hybridization with 14T-3, 17T-2 and 17-4, was selected. Hybridization with 14T-1 was found to be relatively nonspecific.

A large plasmid containing a *Hin*dIII fragment of about 4.5kb in pJB8 was recovered from strain No.11 and designated pCT11. Each probe was hybridized with the isolated insert. The *Hin*dIII fragment was subcloned into pBR322 to give a recombinant plasmid

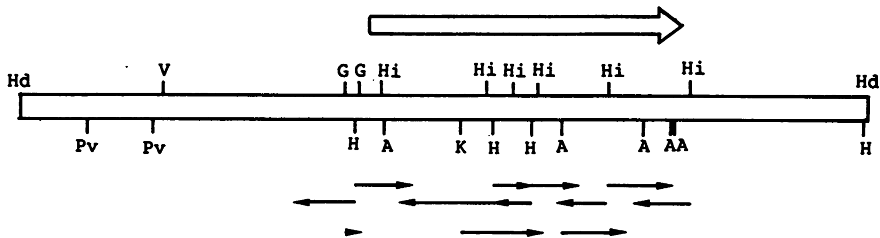

Figure 2 Restriction map of the insert DNA in pCT113 and sequencing strategy of the MPR gene. The clear arrow represents the approximate location of the gene. Restriction sites ; A(AluI), G(BglII), H(HaeIII), Hd(HindIII), Hi(HincII), K(KpnI), Pv(PvuII), and V(EcoRV).

pCT113 and the restriction enzyme pattern was determined (Fig. 2). Southern blot DNA-DNA hybridization of the HindIII digest of the total *M. pusillus* DNA with the ^{32}P-labeled 4.5kb fragment gave a single band of identical size (Fig. 3).

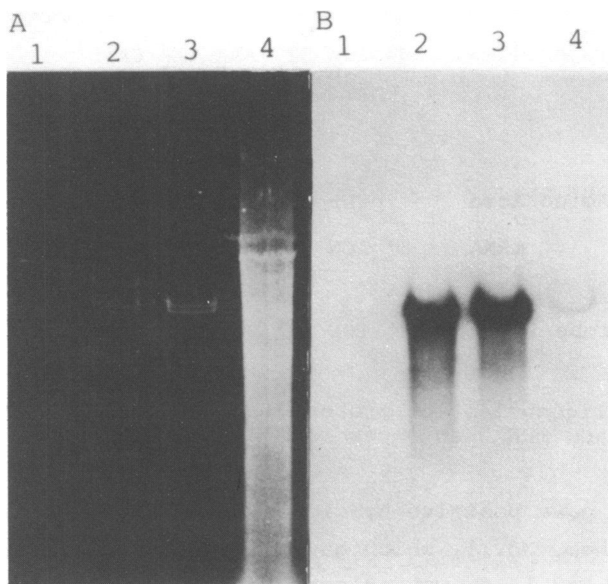

Figure 3 Southern blot DNA-DNA analysis of *M. pusillus* DNA. DNAs were electrophoresed in 0.8% agarose gel (A) and transferred and hybridized with the ^{32}P-labeled 4.5kb-HindIII fragment of pCT113 as a probe (B). Lane 1, λ-HindIII digest ; Lane 2, pCT11 digested with HindIII ; Lane 3, pCT113 digested with HindIII ; Lane 4, total DNA of *M. pusillus* IFO4578(+) digested with HindIII.

Table 1. Codon usage of MPR gene

Ala	30	Gly	45	Pro	19
GCU	13	GGU	14	CCU	9
GCC	9	GGC	23	CCC	6
GCA	7	CCA	8	CCA	4
GCG	1	GGG	0	CCG	0
Arg	8	His	3	Ser	42
CGU	2	CAU	0	UCU	14
CGC	4	CAC	3	UCC	14
CGG	0			UCA	0
AGA	0	Ile	19	UCG	3
AGG	0	AUU	7	AGU	2
		AUC	12	AGC	9
		AUA	0		
Asn	20			Thr	32
AAU	0			ACU	11
AAC	20	Leu	31	ACC	19
		UUA	0	ACA	2
		UUG	12	ACG	0
Asp	34	CUU	10		
GAU	18	CUC	6	Trp	2
GAC	16	CUA	1	UGG	2
		CUG	2		
				Tyr	19
Cys	4			UAU	2
UGU	0	Lys	22	UAC	17
UGC	4	AAA	7		
		AAG	15	Val	32
				GUU	13
Gln	17			GUC	14
CAA	7	Met	5	GUA	3
CAG	10	AUG	5	GUG	2
				TER	1
Glu	14	Phe	29	UAA	1
GAA	5	UUU	7	UAG	0
GAG	9	UUC	22	UGA	0

DNA sequencing of the cloned MPR gene

In order to identify the regions of the cloned DNA hybridizing with the probes, the 4.5kb-*Hind*III insert of pCT113 was digested with various restriction enzymes and the resulting fragments separated by agarose gel electrophoresis were examined with the labelled probe 17T-2. The 1.7kb-*Hae*III fragment was found to hybridize with the probe.

The fragment and the adjacent regions were sequenced according to the strategy shown in Fig. 2. A single open reading frame of 1281 bp without introns was found to encode a

polypeptide of 427 amino acids, which contained all the observed amino acid sequences of MPR at the appropriate positions (Fig. 4). An extra 66 amino acids were shown to be present upstream of the NH_2-terminal sequence of mature MPR. Codon usage in the reading frame is shown in Table 1. The presence of sequence related to the TATA box (TATAAA) was revealed at nucleotides -41 to -48 and also a sequence related to the CCAAT box (TCAAT) at nucleotides -108 to -112. Furthermore possible polyadenylation signal AATAAA was revealed 75-80 bp downstream of the coding sequence.

DISCUSSION

The gene cloned from *M. pusillus* gives the deduced amino acid sequence containing all the sequences observed in the MPR protein. The NH_2-terminal sequence of MPR was found to start from the 67th alanine in the deduced sequence following 360 amino acids which are highly homologous (83%) with those of MMR (6) as shown in Fig. 5. The molecular weight of this part is calculated to be 38600, which is almost identical to the observed value for MPR (3). Consensus sequences for transcription and polyadenylation exist at the appropriate positions upstream and downstream of the coding sequence. All these results strongly suggest that the cloned gene is the true gene of MPR.

The 66 NH_2-terminal amino acids in the deduced sequence might correspond to the prepro-sequence of the mature protease. The sequence from Met1 to Ala18, with a basic amino acid, lysine, at the 5th position, has distinctly high hydrophobicity and might act as a signal peptide for secretion. The following peptide from Lys19 to Phe66 is rich in basic amino acids which is a common feature of the processed part of the aspartate proteases (19,20,21). MPR preparations having glycine as an NH_2-terminal amino acid have been found from different strains of *M. miehei* (22), and these would be the processed product at the two adjacent glycines at the 61 and 62 position. It thus seems highly probable that MPR is synthesized in the form of a preproenzyme.

Most of the genes of *Saccharomyces cerevisiae* and several intracellular genes of *Neurospora crassa* (23,24) lack an intron,

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CGAGAGAAAGTGGAAAGAGTGGTGTTATGCATTAGATGTTACAAGTTCATGCCATAGGTGACACACACAATTTCTGCCT   79
TTTTATGACTTTGTCTGTTTAATCAATATGGTAATACACCCCGCGGTCCAAGGCAGTACCCACTCTTGCAAACACCTTT  158
CCAGGTAAAATCATACAATTATGGACTCTAACGTTTTTCAAAATGTCAATTTTGCAGACGAACGGCACACAGATCTTCT  237
                                             a
AGTGGCCAAACTGCGTAGATCCCTTTTTCATATAAAACCAGCCGGATGCGAGACTCTGAGACCTCATCAAATCCTCAAC  316
                             b
ATG CTC TTC TCC AAG ATC TCC TCT GCA ATC CTT TTG ACC GCT GCT TCT TTT GCA CTT ACC  366
MET LEU PHE SER LYS ILE SER SER ALA ILE LEU LEU THR ALA ALA SER PHE ALA LEU THR  -37

AGT CGT CGC CCA GTA TCC AAG CAA TCT GAT GCC GAT GAC AAG CTA TTG GCT CTT CCC TTG  426
SER ARG ARG PRO VAL SER LYS GLN SER ASP ALA ASP ASP LYS LEU LEU ALA LEU PRO LEU  -27

ACA TCC GTC AAC CGC AAA TAC TCT CAA ACC AAA CAC GGA CAG CAG GCT GCC GAA AAA TTG  486
THR SER VAL ASN ARG LYS TYR SER GLN THR LYS HIS GLY GLN GLN ALA ALA GLU LYS LEU   -7

GGC GGT ATC AAG GCG TTC GCT GAG GGA GAT GGT TCC GTT GAT ACA CCT GGC TTG TAC GAC  546
GLY GLY ILE LYS ALA PHE ALA GLU GLY ASP GLY SER VAL ASP THR PRO GLY LEU TYR ASP   14

TTT GAC TTG GAG GAG TAC GCC ATT CCA GTT TCC ATC GGT ACT CCT GGA CAA GAC TTT TAT  606
PHE ASP LEU GLU GLU TYR ALA ILE PRO VAL SER ILE GLY THR PRO GLY GLN ASP PHE TYR   34

CTT TTG TTC GAT ACC GGC AGT TCC GAT ACT TGG GTT CCC CAC AAA GGC TGC GAT AAC TCT  666
LEU LEU PHE ASP THR GLY SER SER ASP THR TRP VAL PRO HIS LYS GLY CYS ASP ASN SER   54

GAG GGC TGC GTT GGC AAA CGC TTC TTC GAT CCT TCC TCT TCT TCC ACC TTC AAA GAA ACC  726
GLU GLY CYS VAL GLY LYS ARG PHE PHE ASP PRO SER SER SER SER THR PHE LYS GLU THR   74

GAC TAC AAC CTG AAC ATC ACC TAC GGT ACC GGC GGT GCT AAC GGT ATC TAC TTC CGA GAC  786
ASP TYR ASN LEU ASN ILE THR TYR GLY THR GLY GLY ALA ASN GLY ILE TYR PHE ARG ASP   94

AGC ATT ACT GTC GGC GGT GCT ACC GTG AAG CAG CAA ACT TTG GCT TAC GTC GAC AAC GTC  846
SER ILE THR VAL GLY GLY ALA THR VAL LYS GLN GLN THR LEU ALA TYR VAL ASP ASN VAL  114

AGC GGC CCA ACT GCT GAG CAG TCT CCC GAC TCT GAA CTC TTC CTT GAT GGT ATG TTC GGC  906
SER GLY PRO THR ALA GLU GLN SER PRO ASP SER GLU LEU PHE LEU ASP GLY ILE PHE GLY  134

GCA GCC TAC CCT GAC AAC ACT GCC ATG GAA GCC GAA TAC GGA GAT ACT TAC AAC ACT GTC  966
ALA ALA TYR PRO ASP ASN THR ALA MET GLU ALA GLU TYR GLY ASP THR TYR ASN THR VAL  154

CAC GTT AAC CTC TAC AAG CAG GGC TTG ATC TCT TCT CCT GTC TTC TCT GTG TAC ATG AAC 1026
HIS VAL ASN LEU TYR LYS GLN GLY LEU ILE SER SER PRO VAL PHE SER VAL TYR MET ASN  174

ACC AAC GAC GGT GGC GGC CAA GTT GTC TTT GGT GGC GCT AAC AAC ACC CTT CTC GGA GGA 1086
THR ASN ASP GLY GLY GLY GLN VAL VAL PHE GLY GLY VAL ASN ASN THR LEU LEU GLY GLY  194

GAC ATT CAA TAC ACT GAC GTT TTG AAG AGC CGA GGC GGC TAC TTC TTC TGG GAT GCC CCT 1146
ASP ILE GLN TYR THR ASP VAL LEU LYS SER ARG GLY GLY TYR PHE PHE TRP ASP ALA PRO  214

GTC ACC GGT GTC AAA ATT GAT GGA GCT GAC GCT GTC AGC TTC GAC GGC GCC CAG GCA TTC 1206
VAL THR GLY VAL LYS ILE ASP GLY SER ASP ALA VAL SER PHE ASP GLY ALA GLN ALA PHE  234

ACC ATC GAT ACC GGC ACC AAC TTC TTC ATC GCA CCC TCC AGC TTT GCC GAG AAG GTT GTA 1266
THR ILE ASP THR GLY THR ASN PHE PHE ILE ALA PRO SER SER PHE ALA GLU LYS VAL VAL  254

AAG GCT GCA CTC CCC GAT GCT ACC GAG TCG CAG CAG GGT TAT ACT GTT CCT TGC TCC AAG 1326
LYS ALA ALA LEU PRO ASP ALA THR GLU SER GLN GLN GLY TYR THR VAL PRO CYS SER LYS  274

TAC CAG GAT TCC AAG ACC ACC TTC AGC CTT GTT CTG CAA AAG TCT GGT TCC AGC AGC GAT 1386
TYR GLN ASP SER LYS THR THR PHE SER LEU VAL LEU GLN LYS SER GLY SER SER SER ASP  294

ACC ATT GAC GTC TCG GTT CCT ATT AGC AAG ATG CTG CTT CCA GTC GAT AAG TCG GGC GAG 1446
THR ILE ASP VAL SER VAL PRO ILE SER LYS MET LEU LEU PRO VAL ASP LYS SER GLY GLU  314

ACT TGC ATG TTC ATC GTA CTT CCC GAT GGC GGT AAC CAG TTC ATT GTT GGC AAC CTC TTC 1506
THR CYS MET PHE ILE VAL LEU PRO ASP GLY GLY ASN GLN PHE ILE VAL GLY ASN LEU PHE  334

TTG CGC TTC TTC GTC AAC GTT TAC GAC TTT GGC AAG AAC CGT ATC GGC TTT GCA CCT TTG 1566
LEU ARG PHE PHE VAL ASN VAL TYR ASP PHE GLY LYS ASN ARG ILE GLY PHE ALA PRO LEU  354

GCT TCC GGA TAC GAG AAC AAC TAA AGGAATACTCCCTGTTCCCGACTTATCTACGTGTTACGGTACTGTAT 1673
ALA SER GLY TYR GLU ASN ASN TER

CTCTATCTTTACTTTTTAACTGTATTCAATAAATTATCTTGTTGTACTTTTACATGACTTTTGCGCTTGGTTAGCTCTT 1716
                         c
TGAAAGCATCAATGTCACATTTTTTTCTCAGACAACGAGGCACTTTTGCACTTTAGTCTGCCCGTATGCAAGTCGCAAA 1795

AGCAGCTTGACCTAGCTGACGGTATCATCGGTCAAGGATAAAAAGATCGCAACTTAGTAGACAATTCTGAAACATTTCA 1784

ACAATGAGCAAACAAACTTTGTTGGACGCATATATAACTCTGCCAAATATGAGATAATATATTCAAATGAACTCTCAAG 1953

TT                                                                              1955
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Figure 4 Nucleotide sequence coding for MPR. Underlined amino acid sequences were confirmed by sequencing of MPR protein. Amino acids missed in the sequencing are indicated with dotted lines. Double-underlined nucleotide sequences represent the consensus sequences for a, CCAAT box ; b, TATA box ; and c, polyadenylation signal, respectively.

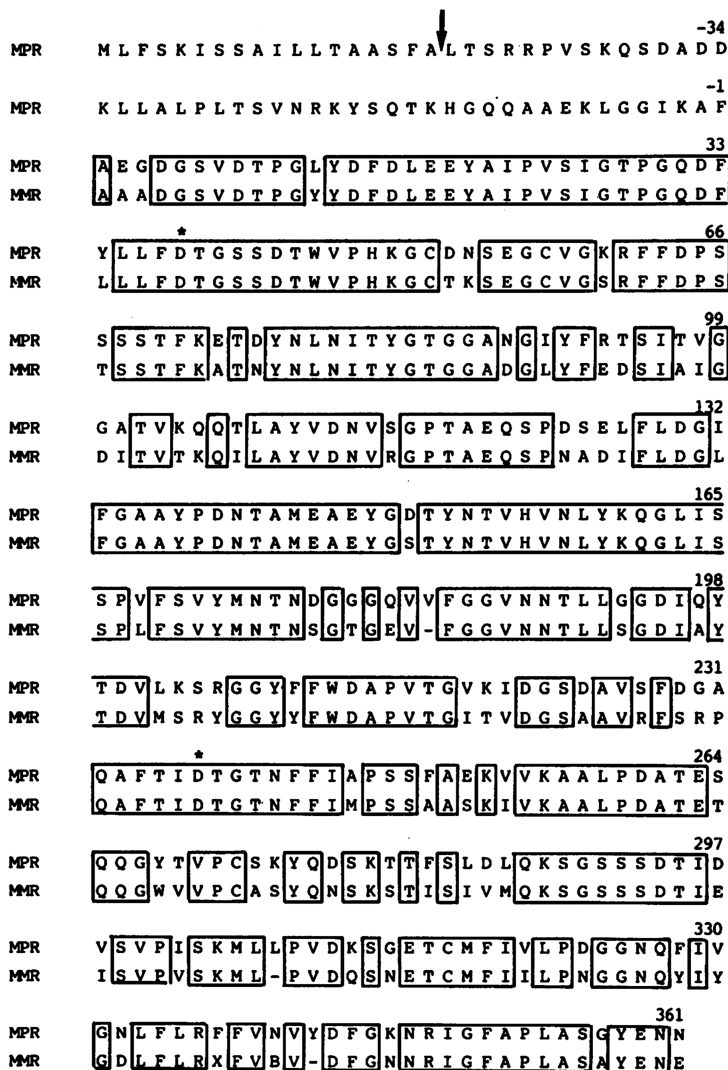

Figure 5 Alignment of the deduced amino acid sequence of MPR with that of MMR. Homologous sequences are boxed. The numbering is that of the mature MPR. The two essential aspartates are indicated by asterisks. The arrow represents the predicted signal peptide cleavage site.

although the genes of the extracellular glucoamylase from Aspergillus and Rhizopus fungi (25,26) and the extracellular cellulase from Trichoderma reesei (27) have been found to possess

intron sequences. M. pusillus and Rhizopus belong to the same class, the Zygomycetes, but the MPR gene lacks an intron in contrast to the glucoamylase gene of the latter. The pattern of codon usage in the MPR gene is similar to those in several filamentous fungi such as Aspergillus (25,28), Trichoderma (27) and Neurospora (23), but different from that in S. cerevisiae (17) especially with respect to the codons for Arg, Cys, Gln, Leu and Pro. Correlation of the gene structures with phylogenic groups will be a future problem in the taxonomy of filamentous fungi and yeasts.

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