

A New Class of Glutaminase from *Aspergillus oryzae*

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Abstract

The koji mold *Aspergillus oryzae* is able to produce glutaminase which converts glutamine to glutamic acid, one of the most important flavor components in soy sauce. We present here the isolation and the complete nucleotide sequence of the glutaminase-encoding gene from *A. oryzae* U212, an industrial strain used in Thailand. N-terminal and internal amino acid sequences were determined from purified glutaminase. A 700-bp fragment was amplified by PCR using oligonucleotide primers designed from partial amino acid sequences. This PCR fragment was used as a homologous probe for screening an *A. oryzae* genomic DNA library. RT-PCR showed that the gene contained seven short introns. Sequence analysis revealed an open reading frame that encodes a protein of 690 amino-acid residues with a predicted molecular mass of 76 kDa. The N-terminal and internal amino acid sequences of the deduced protein exactly matched the ones determined from the purified protein. Comparison of the amino acid sequence with glutaminase sequences from other origins showed that *A. oryzae* glutaminase shares little homology with those of bacteria, eukaryote and mammals. The *A. oryzae* glutaminase gene was expressed in *A. nidulans* to confirm the presence of a functional glutaminase gene in the cloned DNA. To our knowledge, this is the first reported glutaminase gene cloned from filamentous fungi.

Introduction

Soy sauce is a traditional seasoning of the orient, which has gained worldwide acceptance today. Its production involves solid state fermentation using *Aspergillus oryzae* in industrial and traditional koji fermentations for soy sauce

and other oriental fermented foods. This mould secretes a large variety of carbohydrases and proteases during the fermentation. There are a few enzymes which are crucial for soy sauce fermentation, including glutaminase which converts glutamine to glutamic acid, one of the most important flavor components in soy sauce. Since glutamine is also converted non-enzymatically to a flavorless compound, pyroglutamic acid (Sugiyama, 1984), increased expression of glutaminase could result in an increased amount of glutamic acid (Yamamoto and Hirooka, 1974), and an improved quality of soy sauce (Yano *et al.*, 1988).

Besides industrial application of soy sauce production, glutaminase has received much attention with respect to its therapeutic application for treatment of leukaemia (Roberts *et al.*, 1970). Strain improvements of koji molds for glutaminase enzyme have been reported by using mutation, protoplast fusion and haploidization (Ushijima *et al.*, 1987; 1990). Glutaminases from many organisms have been studied (Cook *et al.*, 1981; Soberon and Gonzalez, 1987; Duran *et al.*, 1995; 1996; Nagendra Prabhu *et al.*, 1997), and genes from several organisms have been cloned and characterized (Shapiro *et al.*, 1991; Wagayama *et al.*, 1996; Calderon *et al.*, 1999), though not from fungi. Molecular study of the enzymes involving in the soy sauce production has been reported. There was an attempt to use molecular techniques to improve the strain of *A. oryzae* for alkaline protease (ALP), a key enzyme in soy sauce production. Transformation of *A. oryzae* by the gene encoding ALP led to a five-fold increase in ALP activity (Cheevadhanarak *et al.*, 1991). The genetic basis of glutaminase overproduction in *A. oryzae* is of considerable interest if an approach using molecular techniques is to be applied to strain improvement. In this paper we describe the isolation and characterization the glutaminase-encoding gene from *A. oryzae*.

Results and Discussion

Purification of Glutaminase and Amino Acid Sequence Determination

After the glutaminase protein was resolved by non-denaturing polyacrylamide gel electrophoresis, it was found, as judged by SDS-PAGE, that the preparation contained single band of protein detected by Coomassie staining. The protein showed glutaminase activity, as it was able to hydrolyze L- γ -glutamyl-*p*-nitroanilide in the agarose gel giving a yellow color. The molecular weight of glutaminase on SDS-PAGE was estimated to be about 90,000. Amino acid sequences of the glutaminase were determined to design PCR primers for cloning of a glutaminase gene fragment. The N-terminal amino acid sequence was found to be ASTFSPARPPALPLA. Two internal amino acid sequences obtained from two peptides generated by a Glu-C endopeptidase digest of glutaminase were GExYxATDDQDGL and GKYPNT(Y/R)AMHDIT, where x represents ambiguous amino acid residues and Y/R is likely to be either tyrosine or arginine.

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Table 1. Oligonucleotides used for PCR cloning of glutaminase gene fragment

Amino acid sequence	Primer	Primer sense	Oligonucleotide sequence
GE \times Y \times ATDDQDGL	IS 1 IS 2	sense antisense	5'-ACN GA(T/C) GA(T/C) CA(A/G) GA(T/C) GG-3' 5'-CC (A/G)TC (T/C)TG (A/G)TC (A/G)TC NGT-3'
GKYPNT(Y/R)AMHDIT	P 1 P 2	sense antisense	5'-GCN ATG CA(T/C) GA(T/C) AT(ATC) AC-3' 5'-GT (TAG)AT (A/G)TC (A/G)TG CAT NGC-3'

Oligonucleotide primers were based on the underlined amino acid sequences.

Y/R is an amino acid residue that could not be identified.

x represents ambiguous amino acid residues.

Isolation of a Gene Fragment by Degenerate PCR

Oligonucleotides corresponding to N-terminal amino acid sequence consist of highly degenerate sequences. Thus four degenerate PCR primers were designed on the basis of two internal amino acid sequences (Table 1). PCR reactions were carried out on a template of *A. oryzae* genomic DNA with primer pairs IS1/P2 and IS2/P1, the former giving the most promising result. A DNA fragment approximately 700 bp long amplified using IS1 and P2 primers was cloned into *E. coli* and sequenced. The sequence of this fragment predicted peptide containing the same amino acid sequence, GKYPNT(Y/R), determined chemically from glutaminase protein. This amino acid sequence is located on the N-terminal side of the region corresponding to primer P2. This confirms that DNA amplified by PCR is a fragment of the gene encoding glutaminase we purified. The nucleotide sequence of this PCR fragment was also verified as tyrosine the ambiguous residue, Y/R.

Isolation of *A. oryzae* Glutaminase Encoding Gene

A 700-bp PCR fragment was used for high stringency screening of genomic DNA library of *A. oryzae*. Two positive clones were obtained. A 6-kb *Xba*I DNA fragment which hybridized to the probe was subcloned from one of the lambda clones into pGEM-7Zf(+).

Nucleotide Sequence Analysis of the Cloned Gene

DNA sequencing of this subclone was performed revealing regions which encoded the N-terminal and internal amino acid sequences of the known glutaminase peptides (Figure 1), thus confirming that the clone contained the glutaminase encoding gene. However these three known amino acid sequences were not in the same open reading frame, indicating that the coding region of the cloned gene was interrupted by introns. The intron-exon structure of the putative *A. oryzae* glutaminase gene was verified by RT-PCR revealing the presence of 7 introns. The glutaminase coding region spans 2454 nucleotides and was interrupted by 7 introns of 75, 58, 53, 51, 45, 55 and 46 bp. These introns exhibit the characteristics typical of filamentous fungal genes, such as consensus 5' and 3' splice-junction sequences and putative lariat-formation internal sequences (Gurr *et al.*, 1987). A potential TATA box, TATAAAT, and CAAT elements were located upstream of the initiation codon, and polyadenylation signal, AATAA, was located downstream from the stop codon. The coding region contained an open reading frame encoded for a protein of 690 amino acid residues with a predicted molecular mass

of 76 kDa. The N-terminal amino acid sequence of mature enzyme was found in the deduced amino acid sequence at residue positions 21-35. Furthermore the region consisting of the N-terminal 20 amino acid residues is highly hydrophobic, a common feature of signal sequences. This suggests that the enzyme is synthesized as a precursor with a putative signal peptide of 20 amino-acid residues, and then processed at a specific cleavage site between the Ala20 and Ala21 residues to form the mature enzyme, which is in accordance with von Heijne's rule (von Heijne, 1986). The amino acid sequences for the internal peptides were found at residues 208-221 and 417-429. A deduced amino acid residue at position 430 is glycine whereas the determined amino acid appeared to be threonine at this position. However this was confirmed as glycine by RT-PCR. The amino acid sequence of *A. oryzae* glutaminase contains eight potential N-linked glycosylation (Asn-X-Thr or Asn-X-Ser) sites. Sequence of *A. oryzae* glutaminase shows very low percentage of amino acid identity to those of bacteria (*Rhizobium etli*, *E. coli* and *Micrococcus luteus*), *C. elegans*, rat and human. This suggests that glutaminase from *A. oryzae* and glutaminases from other organisms did not evolve from a common ancestor. In spite of the fact that there is rather high sequence identity amongst previously characterised glutaminases from several organisms, including mammals and bacteria (Figure 2), the glutaminase from *M. luteus* exhibits very low similarity to glutaminases from other organisms.

Transformation of *A. nidulans* with Cloned Glutaminase Gene

For functional identification the cloned glutaminase gene from *A. oryzae*, which was subcloned into pGEM-7Zf(+), was introduced into *A. nidulans* 324 by cotransformation with pILJ16. Arginine prototrophs were selected and tested for glutaminase expression, and 3 out of 29 showed glutaminase activity (Table 2). Glutaminase activity was

Table 2. Glutaminase activities of *A. nidulans* transformants

Strain	Glutaminase activity (milli-units/mg protein)
<i>A. nidulans</i> 324	ND
<i>A. nidulans</i> transformed with pILJ16	ND
Transformant#17	10.36
Transformant#21	11.49
Transformant#24	11.02

ND : not detectable

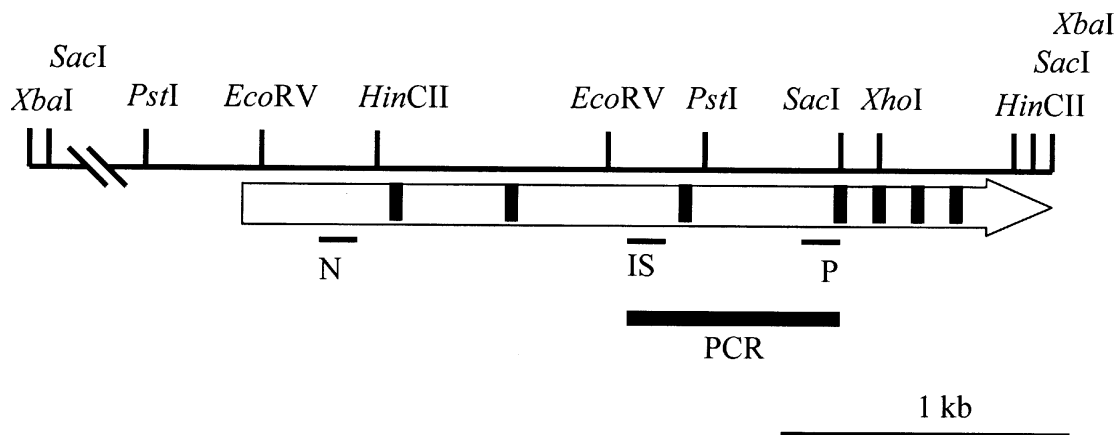


Figure 1. Physical map of 6-kb *Xba*I DNA fragment containing glutaminase gene, which is indicated by the arrow. The black boxes in the arrow represent intron regions. The 700-bp PCR fragment is indicated by thick line. Three thin lines indicate amino acid sequences determined from glutaminase enzyme. N, IS and P represent N-terminal amino acid and internal amino acid sequences of IS and P peptides, respectively. The nucleotide sequence of the glutaminase gene is available in the GenBank database (Accession No. AY005477).

not detected in the untransformed recipient, nor in strains transformed only with pLJ16. This suggests that the *A. oryzae* glutaminase gene is expressed in *A. nidulans*. A partial cDNA fragment isolated from mixed vegetative and conidiating mycelium of *A. nidulans* (http://www.genome.ou.edu/asper_blast.html) shows close sequence identity to the *A. oryzae* glutaminase gene, suggesting that *A. nidulans* does express a glutaminase gene under some conditions, though we could not detect enzyme activity in untransformed *A. nidulans* under the conditions used.

Recently, a sequence essentially identical to the one we have determined was deposited in Genbank (AB029552), but to our knowledge, supporting evidence that it encodes a glutaminase has not been presented. A genomic sequence corresponding to the *A. nidulans* cDNA clone has also been submitted (AB029553).

Conclusion

Generally, among the koji mold used for soy sauce and other oriental fermented food productions, the strains of *Aspergillus* with high protease productivity show low glutaminase activity, while high glutaminase producing strains show inadequate protease activity (Ushijima and Nakadai, 1987). Ushijima *et al.* (1987) noted that mutation of a protease hyper-producer to improve its glutaminase activity usually led to a decrease in the the protease activities of the resulting mutants. On the other hand, the glutaminase activities of protease-enriched mutants of a glutaminase hyper-producer were lower than that of parent. The relationship between these two enzymes in *A. oryzae* is of considerable interest for strain improvement of soy sauce producing *A. oryzae*, and molecular techniques could be now be applied to investigate the gene copy number and regulation of expression of the glutaminase and protease genes in different strains. Results from such studies may point to rational strategies for industrial strain improvement by gene manipulation to address the problem mentioned above.

Experimental Procedures

Organisms and Plasmid

A. oryzae U212, a mutant (Kalayanamitr *et al.*, 1987) which is an industrial strain used in Thailand, was used as a source of glutaminase enzyme and genomic DNA. This strain was maintained in malt extract agar (MEA) medium. *A. nidulans* strain 324 (*yA2 wA3 sC12 methH2 argB2 galA1*, an arginine auxotroph, Glasgow stock) was used as a recipient for transformation. Plasmid pLJ16 containing *argB* gene (Johnstone *et al.*, 1985) was used for transformation of *A. nidulans*. *A. oryzae* U212 was grown on wheat bran medium in 2000-ml flask, which contained 100 g of wheat bran moistened with 100 ml of distilled water, for glutaminase preparation.

Glutaminase Purification

A. oryzae U212 was cultivated on wheat bran culture at 28°C for 72 hours. Wheat bran koji cultures in 5 flasks were used. Crude enzyme was extracted from wheat bran with 10 mM potassium phosphate buffer (pH 7.2) for 12 h at 4°C. The glutaminase enzyme was purified by using ammonium sulfate precipitation (45-75%). The precipitate was dissolved in 10 mM potassium phosphate buffer (pH 7.2) and dialyzed against the same buffer, then applied to Q-Sepharose ion exchange column. Proteins were eluted with 0.1-0.5 M NaCl linear gradient in the same buffer. The fractions containing glutaminase activity were pooled and chromatographed on Sephacryl S-200 HR gel filtration column. The peak fractions of glutaminase activity were pooled and concentrated with Centriplus-50 concentrators (Amicon). Then the enzyme was resolved in non-denaturing polyacrylamide gel electrophoresis. After electrophoresis glutaminase protein was detected by laying the polyacrylamide gel on 1% agarose gel containing 5.0 mM L- γ -glutamyl-*p*-nitroanilide and 100 mM Tris-HCl buffer (pH 7.2) and incubating at 55°C until the yellow band was seen on the polyacrylamide gel (approximately 15 mins). The yellow staining band was excised and glutaminase protein

<i>A. oryzae</i>	VKFLSPITPDDLRRQSLVFSYLDVVDVESIDGKAHDIQVYADISAEWASGDRNAIAQWDY	180
<i>A. nidulans</i>	ITFLSPITPNDLRRQSLVFSYLDVSVTSLDGQSHSVQVYADISAEFASGDRSAIAQWNY	178
<i>M. luteus</i>	RHPIDPYLASLVTELGAVNPGETAQYIPVLAEAEDPRFGIALATPTGRLHCAGDADVEFT	61
<i>R. etli</i>	-----	0
<i>E. coli</i>	-----	0
<i>C. elegans</i>	FYRVNTVISDVKVCRSMSYDMCGNHQQEL--LFD-LYKDETTGKVYLP--RFFKALLESG	92
Human isoform C	GPGETDAFGNSEGKELVASGENKIKQGLLPSLEDLLFYTTAEGQEKIPVHKFITALKSTG	96
Human kidney	SEAAAQGRETPHSHQPHQDHDSESGMLSRGLDLLFYTTAEGQERTPIHKFTTALKATG	100
Rat hepatic	-----MLPRLGDLFYTTAEGQERIPVHKFTTALKATG	33
Rat kidney	SPGETDAFGNSEGKEMVAAGDNKVKQGLLPSLEDLLFYTTAEGQEKIPVHKFITALKSTG	172
<i>A. oryzae</i>	VTDDGVAYHKVYRQTQLLFSENTEQAEWGEWYWATDDQDGLSYQSGPDVDVIRGAFKNGK	240
<i>A. nidulans</i>	VTSDGVAYHKIYRQTPLLFSEHRDQAEWGDWYWATDNVAGLTYQAGPDVDVIRFAFRNGK	238
<i>M. luteus</i>	IQSASKFTYAAALVDRGFAAVDRQVGLNPSGEAFNELSLEAESHRPDNMINAGALAVH	121
<i>R. etli</i>	-----	0
<i>E. coli</i>	-----	0
<i>C. elegans</i>	IRKDD-E-RIDKMIQNIKDADLLDDFVWGTQHIYL-EKDTFKRYIGSSI-GVVTKALKKQ	148
Human isoform C	LRTSD-E-RL-KECM-DMLRLTLQTTSDG---VML-DKDLFKKCVQSNV-VLLTQAFRRK	147
Human kidney	LQTSDE-RL-RDCM-SEMHRVQESSSG---GLL-DRDLFRKCVSSSI-VLLTQAFRRK	151
Rat hepatic	LQTSDE-RL-QDCM-SKMQRMVQESSSG---GLL-DRELFQKCVSSNI-VLLTQAFRRK	84
Rat kidney	LRTSD-E-RL-KECM-DMLRLTLQTTSDG---VML-DKDLFKKCVQSNV-VLLTQAFRRK	223
<i>A. oryzae</i>	LANSDDKNYRAISTNWPVFAFSRDLGSKVTSAGTLFISGLAQDSAIQYSGKPEGTTVMPS	300
<i>A. nidulans</i>	LTNNNDVNYRAISNNWPVFGFAHDLGSISSSTKVLFSIGLTQREAIQYSGNSSLSPPLPA	298
<i>M. luteus</i>	QLLVGPEASRKRERLDRAVEIMSLLAGRRLSVDWETYESEMVAVDRNLSLAHMLRSYGVLO	181
<i>R. etli</i>	---M-AD-LQATLDSYTDILPRIGEKKVADYIPELAKIDPRQFGMAIVTVDGGVFRVGD	55
<i>E. coli</i>	--MLDANKLQAVDQAYTQ-FHSLNGGQADYIPFLANVPGQLAAVAIVTCDGNVYSAGD	57
<i>C. elegans</i>	MIIPDWERF-VSDMGEIFEVDRSHNEGDLATYIPQLSRVAEEDSWAMSVCTIDGQRKMWGD	207
Human isoform C	FVIPDFMSF-TSHIDELYE-SAKKQSGGKVADYIPQLAKFSFDLWGVSVCTADGQRHSITGD	206
Human kidney	FVIPDFEEF-TGHVDRIFE-DVKELTGGKVAAYIPQLAKSNFDLWGVSLCTVDGQRHSVGH	210
Rat hepatic	FVIPDFEEF-TGHVDRIFE-DAKELTGGKVAAYIPHLAKSNFDLWGVSLCTVDGQRHSVGH	143
Rat kidney	FVIPDFMSF-TSHIDELYE-SAKKQSGGKVADYIPQLAKFSFDLWGVSVCTVDGQRHSITGD	282
<i>A. oryzae</i>	LWKS YFSTATAALEFFHHDYAAAAALS KDLD DRISKDS IDAAGQDYLTITSLTVRQVFAA	360
<i>A. nidulans</i>	LWTSYFSTALDALDFHHDYQKSNLSDDLDRRIAQDSVAAAGHDYLTITSLSIRQAFAA	358
<i>M. luteus</i>	DSAEIVAGYVAQCAVLVTVKDLAVMGACLATGGIHPMTGERMLPSIVARRVSVMTSSG	241
<i>R. etli</i>	ADIAFISIQSISKVFMLTLALGKVG-EGLWKRVRGPEPSGSTFNSIVQLEHESGIPRNFPI	114
<i>E. coli</i>	SDYRFALIESISKVCTLALALEVDGPOAVQDKIGADPTGLPFNSVIALELHGKPLSPLVN	117
<i>C. elegans</i>	ALKPFCLQSVSKPFTYALVHDDIGPEELHAHVGOEPEPSGLRFN-DISL-DHNKPHNPLIN	265
Human isoform C	TKVPFCLQSCVKPLKYAIAVNDLGTVEYVHRYVVGKEPSGLRFN-KLFL-NEDDKPHNPMVN	264
Human kidney	TKVPFCLQSCVKPLTYAIAISVTLGTDYVHKFVGKEPSGLRYN-KLSL-DEEGI PHNPMVN	268
Rat hepatic	TKVPFCLQSCVKPLTYAIAISVTLGTDYVHKFVGKEPSGLRYN-KLSL-NEEGI PHNPMVN	201
Rat kidney	TKVPFCLQSCVKPLKYAIAVNDLGTVEYVHRYVVGKEPSGLRFN-KLFL-NEDDKPHNPMVN	340
<i>A. oryzae</i>	VQLTGTPEDPYIFMKEISSNGNMNTVDVIFPAHPIFLYTNPELLKLI LKPIYEIQENGY	420
<i>A. nidulans</i>	TQLCGPANDPYLFMKEISSNGNMNTVDVIFPAHPVFLYTNPALPKYLLRPHLEIQESGNY	418
<i>M. luteus</i>	MYDAAQWLADVGI PAKSGVAGGVLGALPGRVIGVFS PRLDEVGNSARVGLACRRLED	301
<i>R. etli</i>	AGAIATVTVVMAGHAPREAI GELLRFVRYLADDESITIDDKVARSETQTGYRNVALANFM	174
<i>E. coli</i>	AGAIATTSNLINAENV-EQRWQRI LHIQQQLAGEQ-VALSDEVNQSEQTTFHNRAIAWLL	175
<i>C. elegans</i>	AGAIIVASL LKNKLP LADRDFDMIHACRKFVSGYIGFDNSVFLSERETADRNYALSYYM	325
Human isoform C	AGAIIVTSLIKQGVNNAEKFDYVMQFLNKMAGNEYVGFNSATFQSERESGDRNFAIGYYL	324
Human kidney	AGAIIVSSLIKMDCKNAEKFDVFLQYLNKMAGNEYMGFSNATFQSEKETGDRNYAIGYYH	328
Rat hepatic	AGAIIVSSLIKMDCKNAEKFDVFLQYLNKMAGNEFMGFSNATFQSEKETGDRNYAIGYYL	261
Rat kidney	AGAIIVTSLIKQGVNNAEKFDYVMQFLNKMAGNEYVGFNSATFQSERESGDRNFAIGYYL	400
<i>A. oryzae</i>	PNTYAMHDIGTHYPNATGHPKGDDEKMPLEECGNMVIMALAYAKAKDYDYLSDHYPIILN	480
<i>A. nidulans</i>	PNSYAMHDIGAHYPNATGHPDGNDEPMPLEECGNMVIMALAYAKAGDTAYLESHTYITL	478
<i>M. luteus</i>	FRLHLMGDGSLGGTAVRFVEREGDRVFLHLQGVIRFGGAEAVLDALDRLRTGAEKPGTGW	361
<i>R. etli</i>	RAYRNLDPVDHVLGV--YFHQC-ALAMSCQLARAGLFLAARCSNPMTGHSVSPKRRAR	231
<i>E. coli</i>	YSAGLYCDAMEACDV--YTRQC-STLLNTIELATLGATLAAGVNP LTHKRVLQADNVP	232
<i>C. elegans</i>	REHKVFPKDLNLQDTLDLYFQIC-SIETNCDSLAVMAATLANGVNPMMGERIVNRRACR	384
Human isoform C	KEKKCFPEGTDMVGI LDFYFQLC-SIEVTCESASVMAATLANGGFCPI TGERVLSPEAVR	383
Human kidney	EKKCFPKGVDMMAALDLYFQLC-SVEVTCESGSVMAATLANGGICPI TGESVLSAEAVR	387
Rat hepatic	KEKKCFPNPVDMMALDLYFQLC-SVEVTCESGSVMAATLANGGICPI TGESVLSAEAVR	320
Rat kidney	KEKKCFPEGTDMVGI LDFYFQLC-SIEVTCESASVMAATLANGGFCPI TGERVLSPEAVR	459

Figure 2. Deduced amino acid sequence alignment of *A. oryzae* glutaminase with glutaminases from other organisms. Shaded backgrounds indicate identity of amino acid residues.

<i>A. oryzae</i>	KWTTYLVEDSIYSANQISTDDFAGSLANQTNLALKGIIGIQAMAVISNTT-GHPDDASNH	539
<i>A. nidulans</i>	RWTDYLIEDSLYPANQISTDDFAGPLANQTNLALKGIIGIEAMSVIASLT-GSDDDKMNL	537
<i>M. luteus</i>	DAAVYPRWQEA-AADRAALSAATGGRAVHEAAAAARDENDGPIRTVVLNLAARVDRIDDDV	420
<i>R. etli</i>	RINALMLTTCGHYDGGSDFAFHVGLPGKSGVGGGIFAVAPGIASIAVWSPGLNKVGNSQLG	291
<i>E. coli</i>	YIIAEMMEGLYGRSGDWAYRVGLPGKSGVGGGILAVVPGVMGIAAFSPPLDEDGNSVRG	292
<i>C. elegans</i>	DTLSLMYSCGMYDWSGQFAFHVGLPAKSGVSGDMIIVIPNVMGIALYSPRLDCLKNTVVRG	444
Human isoform C	NTLSLMHSCGMYDFSQQFAFHVGLPAKSGVAGGILLVVPNVMMGCWSPPLDKMGNSVKG	443
Human kidney	NTLSLMHSCGMYDFSQQFAFHVGLPAKSAVSGAILLVVPNVMMGMCLSPPLDKLGNSHRG	447
Rat hepatic	NTLSLMHSCGMYDFSQQFAFHVGLPAKSAVSGAILLVVPNVMMGMCLSPPLDKLGNSHRG	380
Rat kidney	NTLSLMHSCGMYDFSQQFAFHVGLPAKSGVAGGILLVVPNVMMGCWSPPLDKMGNSVKG	519
<i>A. oryzae</i>	SSIAKDYIARWQTLGVAHDANPP-HTTLSYGANETHGLLYNLYADRELGLNLVPPQSVYDM	598
<i>A. nidulans</i>	TNYAHDYIEKWLILGIARNTTYP-HTTLSYGSNESHGLLYNLYADRELGLNLVPPQSVYDM	596
<i>M. luteus</i>	GRRLIAEGVRRLLQADGVRVEVEDPERILPLLEEAGH-----	456
<i>R. etli</i>	AVALEMLAARTGWSVFGD-----	309
<i>E. coli</i>	QKMVASVAKQLGYNVFKG-----	310
<i>C. elegans</i>	VKFAEQLVQKYNFNHNDLSVY-LKIENSK--VNL-FEFSFEISYGGGAERIS-FRNRPG	499
Human isoform C	IHFCHDLVSLCNFNHNYDNLRHFAKKLDPREGGQQR-HSFGPLDYESLQQEL-ALKETVW	501
Human kidney	TSFCQKLVSLFNFNHNYDNLRHCAKRLDPREGAEIRNKTVVNLFAAYSQDVSALRRFAL	507
Rat hepatic	ISFCQKLVSLFNFNHNYDNLRHCAKRLDPREGGEVRNKTVVNLFAAYSQDVSALRRFAL	440
Rat kidney	IHFCHDLVSLCNFNHNYDNLRHFAKKLDPREGGQQRVKSVINLLFAAYTGDVSALRRFAL	579
<i>A. oryzae</i>	QNTFYPTVKEKYGVPDTRHVYTKADWELF-TAAVASES-VRDMFHQALATWINETPTNR	656
<i>A. nidulans</i>	QSNFYPTIKGQYGVPLDTRHQYTKGDWELF-TAAVASVS-TRDMFIKLLAQWINETPTNR	654
<i>M. luteus</i>	-----	456
<i>R. etli</i>	-----	309
<i>E. coli</i>	-----	310
<i>C. elegans</i>	NALHGKMKMLRPRMTGLVRYL-AAKPYATEI-----	529
Human isoform C	KKVSPESNE-DISTTV-VYRMESLGEKS-----	527
Human kidney	SAMDMEQKDYDSRTALHVAAAEGHIEVVKELIEACKVNPFAKDRWGNIPLDDAVQFNHLE	567
Rat hepatic	SAVDMEQKDYDSRTALHVAAAEGHIDVVKELIEACKVNPFAKDRWGNIPLDDAVQFNHLE	500
Rat kidney	SAMDMEQRDYDSRTALHVAAAEGHVEVVKELIEACKVNPFAKDRWNNTPMDEALHFHGHD	639

Figure 2 (continued).

was eluted from the polyacrylamide gel by adding 10 mM potassium phosphate buffer pH 7.2 and incubating at 4°C overnight. The final preparation, contained 1.34 units of glutaminase (2.8 units/mg of protein), was stored at -80°C. The glutaminase activity from *A. oryzae* was purified 582-fold. Proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Determination of Glutaminase in *A. nidulans*

10⁸ spores were inoculated in 50 ml of YEPD liquid medium. The culture was incubated at 30°C with shaking at 200 rpm for 24 h. Mycelia were harvested by filtration through Whatman 1MM filter paper and washed with sterile 0.9% (w/v) NaCl. The mycelia were then frozen with liquid nitrogen and ground to fine powder. The ground mycelia were resuspended in 10 mM potassium phosphate buffer (pH 7.2) and incubated at 4°C for 12 h. The cell debris was then removed by centrifugation (9000 rpm, 4°C 15 min). The supernatants were collected for glutaminase assay.

Enzyme Activity and Protein Assays

Glutaminase activity was determined by a modification of the assay described by Tomita *et al.* (Tomita *et al.*, 1988). The reaction mixture (final volume 1.0 ml), containing 5.0 mM L- γ -glutamyl-*p*-nitroanilide (L- γ -GpNA), 100 mM Tris-HCl buffer (pH 7.2) and the enzyme solution, was incubated at 55°C for 1 hr. Two ml of 1.5 N acetic acid was added to stop the enzyme reaction and then the absorbance was measured at 410 nm. One unit of glutaminase activity was defined as the amount of enzyme that catalyzed the

formation of 1 μ mol of *p*-nitroaniline from the substrates per minute under the conditions mentioned above. Protein concentrations were determined spectrophotometrically by the Bradford method (Bradford, 1976) with bovine serum albumin as a standard.

PAGE

Denaturing SDS-PAGE was carried out as described by Laemmli (Laemmli, 1970).

N-Terminal and Internal Amino Acid Sequence Analysis

Glutaminase protein was electrophoresed (SDS-PAGE) and blotted onto polyvinylidene difluoride (PVDF) membrane (ProBlott™ Membrane, Applied Biosystems) and visualized by staining with Coomassie blue R-250. Protein bands were excised and analyzed for amino acid sequence. Internal peptide fragments were generated by in-gel protein digestion, as described by Rosenfeld *et al.* (1990), of glutaminase using *Staphylococcus* V8 protease (Glu-C endopeptidase). The fragments were separated by Tricine-SDS-PAGE (Schagger and von Jagow, 1987) and transferred onto PVDF membrane as described above. Automated Edman degradation for amino acid sequence analysis was performed on the blot with a solid-phase protein sequencer (model 476A; PE Applied Biosystems).

DNA Manipulations

All recombinant DNA procedures were performed according to standard protocols (Sambrook *et al.*, 1989). Restriction enzymes were purchased from Boehringer

Mannheim (Mannheim, Germany). Modifying enzymes and Prime-A-Gene labeling kit were obtained from Promega (Madison, WI). T7 Sequenase version 2 DNA sequencing kit and radioactive [α - 32 P]dCTP (3000 Ci/mmol) were from Amersham.

Amplification of Glutaminase Genomic DNA Sequence by PCR

Total DNA from *A. oryzae* was used as template for PCR amplification of partial glutaminase gene. Oligonucleotides were designed (Table 1) based on the amino acid sequences and used as primers. PCR reactions were carried out by using IS1 and P2 primer pair or IS2 and P1 primer pair. PCR fragments were purified from agarose gel by GeneClean™ Kit and cloned into pTAg (The LigATor PCR cloning vector, R&D systems) by using the manufacturer's TA cloning protocol.

Construction of Genomic Library and Screening

The *A. oryzae* genomic library was constructed in λ FIX II partial filled-in *Xho*I half site arms (Stratagene) as described in the instruction manual. Recombinant plaques of the *A. oryzae* genomic library were immobilized onto nylon membranes (Hybond-N, Amersham International plc.). This library was screened for the glutaminase gene by plaque hybridization using the homologous 700-bp PCR fragment as probe, following a standard method (Sambrook *et al.*, 1989).

Reverse Transcription-PCR

Total RNA of *A. oryzae* was isolated by using TRIzol™ reagent from GIBCO BRL (Life Technologies) as described by Clarke *et al.* (1997). First strand cDNA was synthesized using SuperScript II RNase H⁻ Reverse transcriptase (GIBCO BRL, Life Technologies) according to the manufacturer's manual. Two RT-PCR reactions were performed. Primer RT1 (5'-TCAACATTCTCCCCTGCG-3') and primer RT2 (5'-CATGGCGTATGTGTTGGG-3') were used for determination the first three introns. The presence of four other introns were confirmed by 3'RACE (Rapid Amplification of cDNA Ends)-PCR technique as described by Froshmam (1990) using the gene specific primer RT3 (5'-GAGAACGGAAAGTATCCC-3') and 3'RACE primer (5'-GAGGACTCGAGCTCAAGC-3'). The oligo (dT)₁₇ adapter primer for first strand cDNA synthesis was extended with the sequence of 3'RACE primer.

Fungal Transformation

A. nidulans 324 was maintained, cultivated and transformed as described previously (Balance and Turner 1985). Transformants were selected on minimal medium for arginine prototrophy.

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