Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Molecular Catalysis B: Enzymatic 23 (2003) 87–100
JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC
www.elsevier.com/locate/molcatb

Review

Microbial glutaminase: biochemistry, molecular approaches and applications in the food industry

Renu Nandakumar [a], Kazuaki Yoshimune [b], Mamoru Wakayama [c], Mitsuaki Moriguchi [b,*]

[a] *Department of Chemical and Biochemical Engineering, University of Maryland Baltimore County, 1000 Hilltop Circle, Baltimore, MD 21250, USA*
[b] *Department of Applied Chemistry, Faculty of Engineering, Oita University, Dannoharu 700, Oita 870-1192, Japan*
[c] *Department of Bioscience and Biotechnology, Faculty of Science and Engineering, Ritsumeikan University, Noji, Kusatsu, Shiga 525-8577, Japan*

Received 7 February 2003; accepted 2 May 2003

Dedicated to Professor Dr. Kenji Soda in honor of his 70th birthday

Abstract

Glutaminase is widely distributed in microorganisms including bacteria, yeast and fungi. The enzyme mainly catalyzes the hydrolysis of γ-amido bond of L-glutamine. In addition, some enzymes also catalyze γ-glutamyl transfer reaction. A highly savory amino acid, L-glutamic acid and a taste-enhancing amino acid of infused green tea, theanine can be synthesized by employing hydrolytic or transfer reaction catalyzed by glutaminase. Therefore, glutaminase is one of the most important flavor-enhancing enzymes in food industries. In this review, subsequent to a discussion on the definition of glutaminase, the enzymatic properties, applications of glutaminase in the food industry, and occurrence and distribution of the enzyme are described. We then illustrate the gene cloning, primary structure, and 3D-structure of glutaminase. Finally, to facilitate the future applications of glutaminase in food fermentations, the mechanisms of action of salt-tolerant glutaminase are briefly discussed.

Keywords: Glutaminase; Glutamic acid; Glutamine; Amidohydrolase

1. Introduction

L-Glutaminase (L-glutamine amidohydrolase EC 3.5.1.2) catalyzes the hydrolysis of L-glutamine to L-glutamic acid and ammonia (Fig. 1). In recent years, glutaminase has attracted much attention with respect to proposed applications in both pharmaceuticals and food industries. L-Glutaminase, in combination with or as an alternative to asparaginase, could be of significance in enzyme therapy for cancer especially acute lymphocytic leukemia [1,2].

It is well known that most of the basic flavor components of fermented condiments are amino acids produced by the enzymatic degradation of proteins contained in the raw materials and among them, L-glutamic acid is a widely acclaimed flavor-enhancing amino acid. For example, the unique flavor of fermented soy sauce is attributed mainly to glutamic acid (concentrations of 0.7 to 0.8% per total nitrogen). The activity of glutaminase, which is responsible for

* Corresponding author. Tel.: +81-97-554-7891; fax: +81-97-554-7890.
E-mail address: mmorigu@cc.oita-u.ac.jp (M. Moriguchi).

1381-1177/$ – see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/S1381-1177(03)00075-4

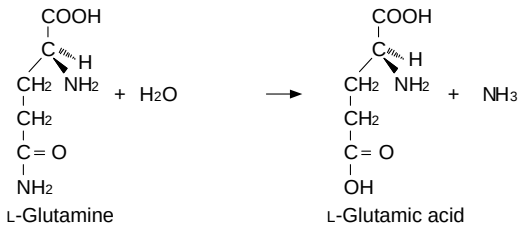


Fig. 1. Hydrolysis catalyzed by glutaminase.

the synthesis of glutamic acid, makes it an important additive during soy sauce fermentation. Attempts to increase the glutamate content of soy sauce using salt- and thermo-tolerant glutaminases have drawn much attention.

In the context of increased practical applications for glutaminase, we present here an overview of the biochemical aspects of microbial glutaminase and the molecular approaches commonly employed to enhance their exploitation as a flavor enhancer in the food industry. In this review, we refer to an enzyme as glutaminase if it is classified as glutaminase by the individual authors, even though the enzyme has high asparaginase activity.

2. Types of glutaminase reaction

The family of amidohydrolase that catalyzes the deamination of glutamine contains two classes. The first class includes glutaminase, which is highly specific for glutamine and catalyzes the hydrolysis of glutamine to glutamic acid. The second class contains the enzyme that is less specific and catalyzes the hydrolysis of glutamine to glutamic acid and asparagine to aspartic acid with similar efficiency. Glutaminase–asparaginase (EC 3.5.1.38), a class of amidohydrolase that catalyzes the hydrolysis of both glutamine and asparagine with similar efficiency and a wide substrate specificity, has received considerable attention as some of them are being used in the treatment of leukemia. The ratio of glutaminase to asparaginase activity was approximately 1.5:1.0 in the case of enzyme from *Pseudomonas boreopolis* [3].

Because of the similarity between the substrates of glutaminase and glutaminase–asparaginase, the amino acid sequences of glutaminase are indistinguishable from those of glutaminase–asparaginase. The sequence homology between glutaminase and glutaminase–asparaginase is indicative of a common mechanism for deamination. In order to differentiate the glutaminase from the glutaminase–asparaginase in the genome sequences, both the glutaminase and asparaginase activities of the gene products have to be measured.

Many of glutaminases catalyze γ-glutamyl transfer from such γ-glutamyl donor (donor) as glutamine or glutathione to γ-glutamyl acceptor (acceptor) as catalyzed by D-glutamyltransferase (EC 2.3.2.1) and γ-glutamyltransferase (EC 2.3.2.2). Hartman [4] proposed the categorization of glutaminases and γ-glutamyltransferases, which catalyze the cleavage of the γ-acyl bond in glutamine, and classified these enzymes into four groups based on the catalytic characterization: (a) only hydrolysis, (b) hydrolysis prior to transfer reaction with some acceptors, (c) transfer reaction prior to hydrolysis (hydrolysis is largely suppressed by suitable acceptor in favor of transfer reaction), (d) only transfer reaction. Glutaminases from mammalian origin, *P. putrefaciens* [5], and *Micrococcus luteus* K-3 [6] catalyze only hydrolysis

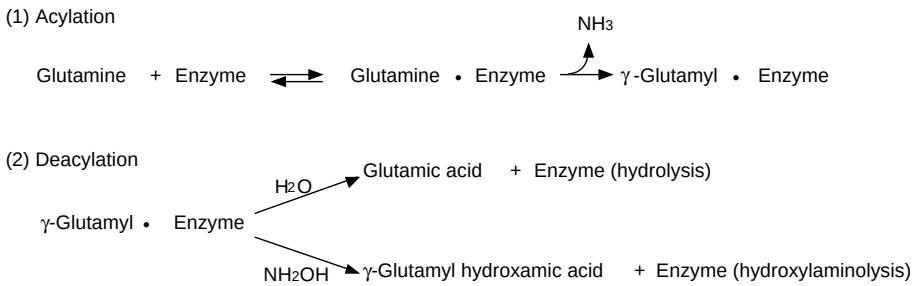


Fig. 2. Proposal of the reactions catalyzed by glutaminase.

reaction. The second group is the glutaminase which uses water as a preferential acceptor, and hydrolyze glutamine prior to the transfer reaction. Glutaminases from *Escherichia coli* [7], and *P. aeruginosa* [8] belong to this category. Glutaminase from *P. nitroreducens* has both group b (hydrolysis of glutathione prior to γ-glutamyltransfer to glycylglycine) and group c (γ-glutamyl transfer to ethylamine or methylamine with repressed hydrolysis of glutamine) [9]. These reactions catalyzed by glutaminase are proposed to proceed through the formation of an acyl enzyme derivative in a two-step acylation–deacylation sequence analogous to chymotrypsin and papain [10] (Fig. 2).

3. Occurrence and distribution

Glutaminase is ubiquitous in microorganisms including bacteria, yeast and fungi. Table 1 is a representative list of the major genera of microorganisms reported to produce glutaminase. More comprehensive

Table 1
Major genera of microorganisms reported to produce glutaminase

Microbial species	References	Microbial species	References
Bacteria			
Acetobacter liquefaciens	[13]	*Micrococcus luteus*	[6]
Achromobacter sp.	[36]	*Nocardia* sp.	[13]
Acinetobacter glutaminasificans	[56,57]	*Proteus morganii*	[64]
Aerobacter aerogenes	[58]	*Proteus vulgaris*	[11]
Aeromonas hydrophila	[11]	*Pseudomonas aeruginosa*	[8,10,65]
Agrobacterium tumefaciens	[13]	*Pseusomonas aurantiaca*	[66]
Alcaligenes faecalis	[13]	*Pseudomonas aureofaciens*	[13]
Azotobacter agilis	[13]	*Pseudomonas boreoplois*	[3]
Bacillus circulans	[59]	*Pseudomonas fluorescens*	[16]
Bacillus flavum	[13]	*Pseudomonas ovalis*	[67]
Bacillus licheniformis	[17]	*Pseudomonas schuylkilliensis*	[13]
Bacillus megaterium	[13]	*Pseudomonas* sp.	[68]
Bacillus pasteurii	[22]	*Rhizobium etli*	[69]
Bacillus subtilis	[60]	*Rhodopseudomonas sphaeroides*	[13]
Brevibacterium ammoniagenes	[13]	*Serratia marcescens*	[60]
Clostridium welchii	[61]	*Spirillum lunatum*	[13]
Corynebacterium equi	[13]	*Staphylococcus aureus*	[13]
Enterobacter cloacae	[58]	*Streptomyces californicus*	[13]
Erwinia aroideae	[58]	*Streptomyces netropsis*	[13]
Erwinia carotovora	[13]	*Streptomyces olivochromogenes*	[13]
Escherichia coli	[7,19,62,63]	*Vibrio cholerae*	[15]
Flavobacterium flavescens	[13]	*Vibrio costicola*	[15]
Micrococcus glutamicus	[13]	*Xanthomonas juglandis*	[58]
Micrococcus lysodeikticus	[13]		
Yeast			
Cryptococcus albidus	[46]		
Candida scottii	[13]		
Cryptococcus nodaensis	[70]		
Cryptococcus sp.	[13]		
Debaryomyces sp.	[18]		
Hansenula	[13]		
Saccharomyces cerevisiae	[20]		
Fungi			
Aspergillus oryzae	[45,52]		
Tilachlidium humicola	[15]		
Verticillium malthousei	[13]		

lists are compiled by Wade et al. [11], Nelson et al. [12], and Imada et al. [13]. In general, glutaminases from *E. coli*, *Pseudomonas* spp., *Rhizobium etli*, *M. luteus* K-3, *Bacillus* spp., *Clostridium welchii* and *Aspergillus oryzae* have been isolated and well studied. As far as the occurrence of glutaminase producers with respect to the environment is concerned, so far the majority of microorganisms identified to possess glutaminase activity were isolated from soil except for reports from aquatic (marine) environment [6,14,15].

Considering the potential in vitro applications of glutaminase, the extracellular nature of these enzymes is of much significance from the view point of direct attack upon raw substrate materials and due to the ease of purification. However, only a limited number of reported microbial glutaminases are extracellular enzymes. *A. oryzae* is observed to secrete glutaminase both extracellularly and intracellularly [45]. Among bacteria, *P. fluorescens*, *Vibrio costicola*, and *V. cholerae* are observed to produce extracellular glutaminases, and the extracellular secretion is about 2.6–6.8 times higher than intracellular production [16]. Glutaminase is secreted extracellularly also by strains of *Bacillus subtilis* and *B. licheniformis* [17], *Debaryomyces* sp. [18], and *Beauveria* sp. [15].

As far as the cellular locations of glutaminase are concerned, observations tend to vary among the microbial species studied. Neither glutaminase A nor B of *E. coli* is located in the periplasmic place as the enzyme was not released by osmotic shock [19]. Glutaminase A of *Saccharomyces cerevisiae* is membrane bound, while glutaminase B is cytoplasmic [20]. Two forms of glutaminase, a free form and a binding form, are known to exist in *A. oryzae* [21]. Recently, a cytoplasmic, phosphate-activated glutaminase was isolated from *B. pasteurii* [22].

4. Properties of microbial glutaminase

The molecular weight, optimal pH, and substrate specificity of glutaminases from various microbes have been summarized in Table 2. As far as the temperature dependence of glutaminases is concerned, many of the glutaminases reported have both an optimal and stable temperature of around 40–50 °C. However, the temperature stability of glutaminase I (*Micrococcus* glutaminase) of *M. luteus* could be increased by the addition of 10% NaCl [6]. *A. oryzae* glutaminase was optimally active in a temperature range of 37–45 °C and was stable at up to 45 °C but lost its activity almost completely at 55 °C [23].

It is interesting that the exposure of *E. coli* glutaminase B to cold resulted in a reversible inactivation of enzymatic activity, while subsequent warming to 24 °C restored the activity. There was no difference in the molecular weight of the active species between the cold inactivated enzyme and the warm activated enzyme. The conformational changes which probably occur upon exposure to cold probably, would result from a weakening of the interaction among hydrophobic groups in the protein [19].

Glutaminase from the *koji* mold (*A. oryzae*), which is commonly used for soy sauce fermentation, is markedly inhibited by the high-salt concentration used in the fermentation process (about 3 M sodium chloride) [21]. Therefore, salt-tolerant glutaminase may play potentially significant roles in industrial processes that require high-salt environments. Glutaminases from marine isolates *M. luteus* K-3 and *B. subtilis* are shown to be highly salt-tolerant: tolerant to up to 16 and 25% of NaCl, respectively [6,24,25]. Among the two isozymes of glutaminase I and II from *M. luteus* K-3, glutaminase I were stable and exhibited about 1.3 fold higher activity in the presence of 8–16% NaCl than in the absence of NaCl, while glutaminase II was not stabilized or activated [6]. Detailed information on salt tolerance from microbial glutaminase is summarized in Table 3.

5. Molecular cloning and gene sequencing of glutaminase

Gene encoding glutaminase from several microorganisms have been cloned and overexpressed. For the purpose of increasing the amount of glutamic acid produced during soy sauce fermentation, glutaminase gene from *A. oryzae* was cloned and expressed in *A. nidulans* [26] and in *A. oryzae* [23]. The glutaminase gene was also isolated from *A. nidulans* [23]. *Micrococcus* glutaminase is a salt-tolerant enzyme which is active both in the absence and in the presence of 3 M NaCl, and is distinct from a halophilic proteins

Table 2
Characteristics of glutaminases from different microorganisms

Microorganism	Molecular mass (kDa)		Optimum pH	Optimum temperature (°C)	Substrate		References
	Native	Subunit			Hydrolysis (K_m)	Hydroxylaminolysis (K_m)	
Bacteria							
Acinetobacter glutaminasificans	132	33	7.0		L-Glutamine (5.8 ± 1.5 μM) D-Glutamine L-Asparagine (4.8 ± 1.4 μM) D-Asparagine γ-L-Glutamyl hydroxamate	γ-L-Glutamyl hydroxamate	[56,71]
Bacillus pasteurii	100	55	9.0	37	L-Glutamine (9.5 mM) D-Glutamine (83 mM) L-Asparagine (24 mM)	L-Glutamine (17 mM) L-Glutamate (~500 mM) γ-L-Glutamyl hydroxamate (15 mM)	[21]
Bacillus subtilis	55		6.0	50	L-Glutamine (0.64 mM) D-Glutamine		[60]
Escherichia coli							
A	110	28	5.0		L-Glutamine (0.42 mM) γ-L-Glutamyl methylamide (3.3 mM) γ-L-Glutamyl hydrazide (12 mM) γ-L-Glutamyl hydroxamate (5.1 mM) γ-L-Glutamyl methoxyamide (5.0 mM) γ-Methyl L-glutamate (64 mM) γ-Ethyl L-glutamate (64 mM) γ-Thio methyl L-glutamate (10 mM) γ-Thio ethyl L-glutamate (23 mM)		[19,61,62] [72,73]
B	90	35	7.1–7.9		L-Glutamine γ-L-Glutamyl hydroxamate (5.1 mM)		[7]
Micrococcus luteus							
I	86	43	8.0	50	L-Glutamine (4.4 mM) γ-Methyl L-glutamate γ-Ethyl L-glutamate γ-L-Glutamyl hydroxamate γ-Benzyl L-glutamate γ-L-Glutamyl-L-glutamate	γ-L-Glutamyl hydroxamate	[6]
II	86		8.5	50	L-Glutamine (6.5 mM)		
Pseudomonas aeruginosa							
A	137	35	7.5–9.0		L-Glutamine (0.12 mM) D-Glutamine (0.85 mM) L-Asparagine (0.068 mM) D-Asparagine (0.105 mM)	L-Glutamine D-Glutamine L-Asparagine D-Asparagine	[8,10]
B	67		8.5		L-Glutamine (0.18 mM) D-Glutamine (0.23 mM) L-Theanine (0.71 mM) D-Theanine (0.92 mM) γ-L-Glutamyl hydrazide γ-L-Glutamyl-*p*-nitroanilide	L-Glutamine D-Glutamine L-Theanine D-Theanine	

Table 2 (*Continued*)

Microorganism	Molecular mass (kDa)		Optimum pH	Optimum temperature (°C)	Substrate		References
	Native	Subunit			Hydrolysis (K_m)	Hydroxylaminolysis (K_m)	
Pseudomonas nitroreducens	40		9.0		L-Glutamine (6.5 mM) D-Glutamine DL-Theanine Glutathione γ-L-Glutamyl-*p*-nitroanilide (0.03 mM)	L-Glutamine (4 mM) D-Glutamine γ-L-Glutamyl-*p*-nitroanilide γ-L-Glutamyl methylamide Glutathione	[9]
Pseudomonas aurantiaca	148	37	6.8–8.0		L-Glutamine (0.53 mM) L-Asparagine (0.57 mM)	L-Theanine	[13]
Rhizobium etli	106.8	26.9			L-Glutamine (1.5 mM)		[74]
Yeast							
Debaryomyces	115	50, 65	8.5	40	L-Glutamine (4.5 mM)		[18]
Fungi							
Aspergillus oryzae MA-27-IM							
Intracellular	113		9.0	45	L-Glutamine (0.091 mM) D-Glutamine DL-Theanine Glutathione γ-L-Glutamyl-*p*-nitroanilide		[45]
Extracellular	113		9.0	45	L-Glutamine (0.096 mM) D-Glutamine DL-Theanine Glutathione γ-L-Glutamyl-*p*-nitroanilide		[75]
Aspergillus oryzae AJ11728	82		9.0	37–45	L-Glutamine (1.2 mM) D-Glutamine		[22]

Table 3
Effects of NaCl on glutaminase activities

Bacterial species	Residual activity (%)	NaCl concentration (%)	References
Bacillus subtilis	90	16	[76]
Escherichia coli	65	18	[77]
Micrococcus luteus			
I	130	16	[6]
II	100	16	[6]
Pseudomonas fluorescens	75	18	[77]
Cryptococcus albidus	65	18	[77]
Aspergillus oryzae	20	18	[21]
Aspergillus sojae	6	18	[77]

that are inactivated in the absence of salt [6]. We have cloned and overexpressed gene encoding salt-tolerant *Micrococcus* glutaminase in *E. coli* [27]. *Micrococcus* glutaminase may play potentially significant roles in industrial processes that require high-salt environments. *R. etli* establishes nitrogen-fixing symbiosis with *Phaseolus vulgaris* (bean). *R. etli glsA* gene encoding glutaminase A was cloned in order to study the regulation of the glutaminase which plays a catabolic role in maintaining the balance between glutamic acid and glutamine, the nitrogen donors of the cell [28]. Periplasmic glutaminase–asparaginase gene of *P. fluorescens* was cloned and expressed in *E. coli*. Studies on the genomic sequence of glutaminase–asparaginase of *P. fluorescens* showed that it depends on the sigma factor σ^{54} for expression and it shares considerable similarity with other reported glutaminase.

Recent progress in genomics has provided a wealth of genome sequences, and several sensitive homology search programs have discovered glutaminase homologues in many microorganisms. Comparison of the amino acid sequences of glutaminase homologues from various microorganisms reveal that glutaminase homologues can be classified into at least two major subgroups. The first class includes glutaminase which conserves amino acid residues that locate in the active site of glutaminase from *Pseudomonas* 7A (Thr20, Tyr34, Thr100, Asp101, Lys173, and Glu294). Glutaminases from *P. aeruginosa*, *Acinetobacter glutaminasificans*, and *Mycobacterium leprae*, and glutaminase–asparaginase of *P. fluorescens* belong to this class (Fig. 3). The second class contains the enzyme that does not have the amino acids conserved in the active site of glutaminase from *Pseudomonas* 7A, and exhibit more than 30% sequence identity with *Micrococcus* glutaminase. The second group include glutaminases of *Corynebacterium glutamicum*, *B. halodurans*, *B. subtilis*, and *E. coli* (Fig. 4).

6. Structure of glutaminase

The three-dimensional structures of glutaminase–asparaginases from two bacteria, *Pseudomonas* 7A [29] and *A. glutaminasificans* [30] were determined. Crystals of *Pseudomonas* 7A glutaminase–asparaginase (PGA) were reacted with 6-diazo-5-oxo-L-norleucine (DON), and 5-diazo-4-oxo-L-norvaline (DONV) that are analogues of glutamic acid and aspartic acid, respectively (Fig. 5). The derivative crystals remained isomorphous to native PGA crystals, and their structures were refined. The structures of PGA–DON and PGA–DONV complexes suggested that Thr20 was the primary catalytic nucleophile and Tyr34, which was polarized by Glu294, activate Thr20 [31] (Fig. 6). Ortlund et al. [31] proposed that initial nucleophilic attack on the amide carbon of glutamine or asparagine by Thr20 leads to acyl–enzyme intermediate and generates an ammonia molecule as a byproduct, and hydrolysis of acyl–enzyme intermediate yields the free enzyme. They also proposed that water is activated by the Thr100–Asp101–Lys173 catalytic triad. However, amino acid residues that locate in the active site of glutaminase from *Pseudomonas* 7A are not conserved among all glutaminase–asparaginases. This fact suggests that the proposed reaction mechanism of PGA is not applied to all glutaminases or glutaminase–asparaginases. *Micrococcus* glutaminase and its major fragment containing about 80% of the protein were crystallized [32]. *Micrococcus* glutaminase shows only 11.9 and 12.4% sequence identity with glutaminase–asparaginases from *Pseudomonas* 7A and *A. glutaminasificans*, respectively. The low sequence identities between *Micrococcus* glutaminase and glutaminase–asparaginases with known structures could be indicative of a unique structure of *Micrococcus* glutaminase.

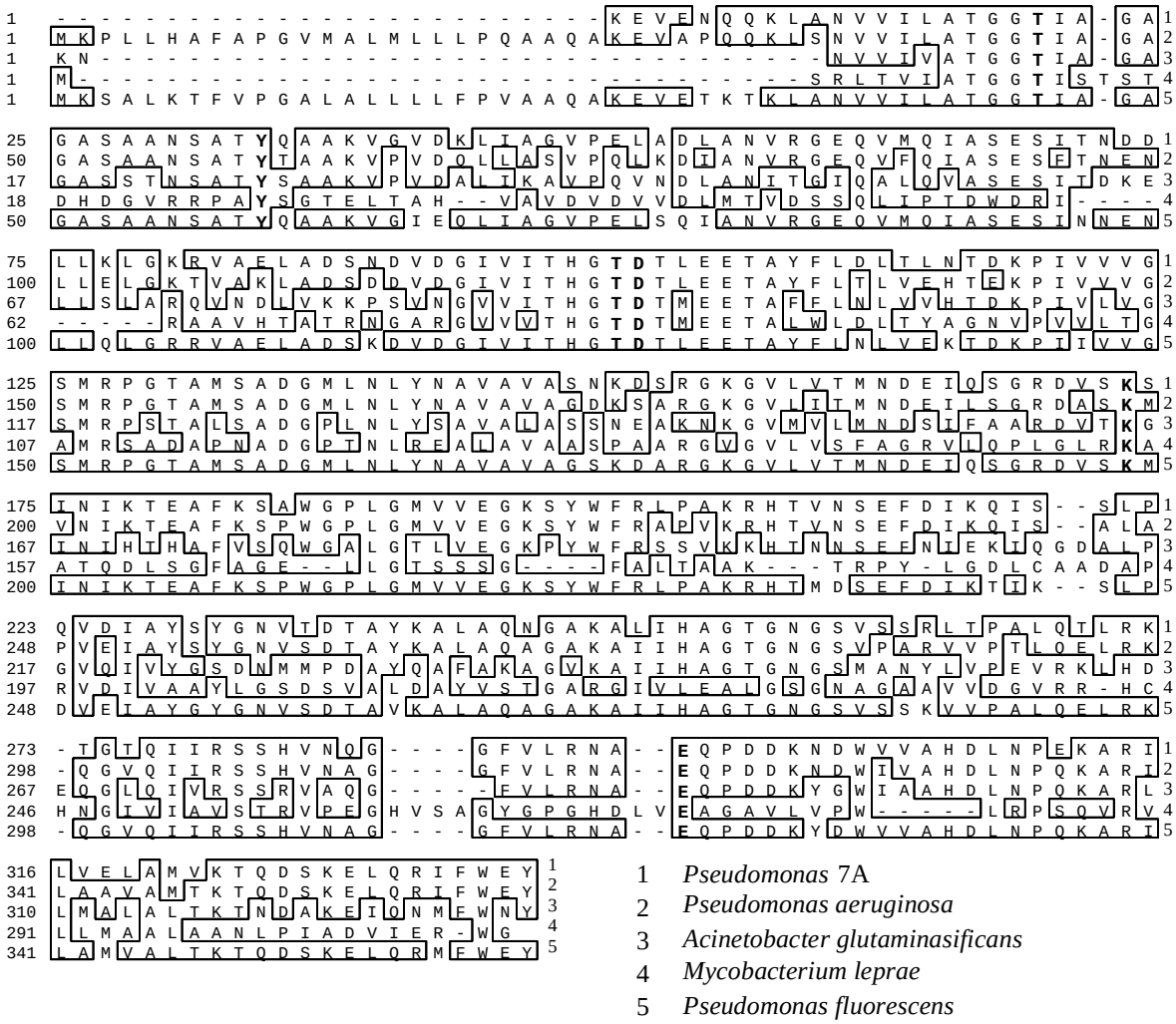
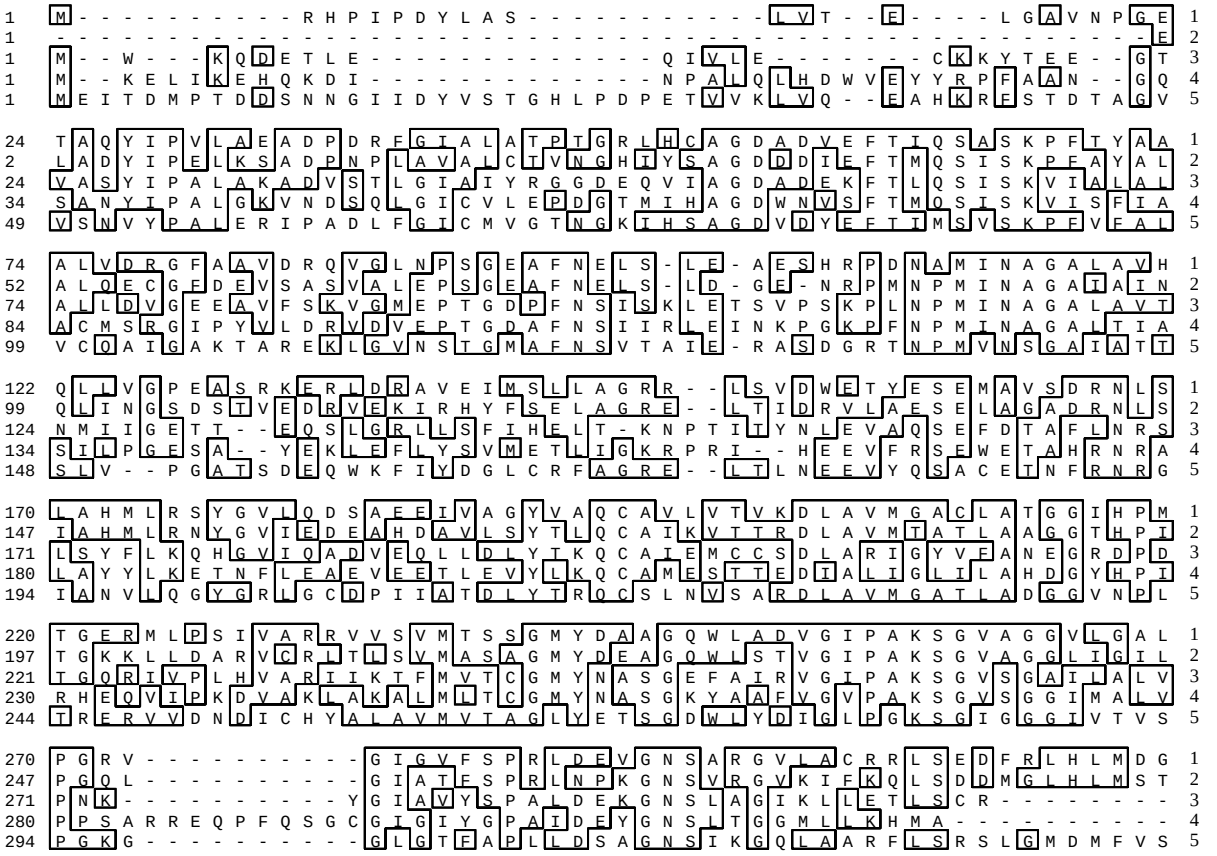


Fig. 3. Comparison of the amino acid sequence of glutaminase of *Pseudomonas* 7A with those of *Pseudomonas aeruginosa*, *Acinetobacter glutaminasificans*, and *Mycobacterium leprae*, and that of glutaminase–asparaginase from *P. fluorescens*. The conserved amino acid residues are boxed. The amino acid residues that locate in the active site of glutaminase from *Pseudomonas* 7A are in bold type.

7. Salt tolerance of glutaminase

Several independent studies suggested that halophilic enzymes present a higher proportion of glutamic and aspartic acid residues than their nonhalophilic homologues [24,25]. Fukuchi et al. [33] showed that the differences in the amino acid compositions between proteins of halophilic and mesophilic or thermophilic bacteria are much greater on the protein surface than in the interior. They concluded that the average glutamic acid composition for halophilic enzymes is equivalent to that for nonhalophilic enzymes, because of the increased glutamic acid content in nonhalophilic enzymes. The high content of aspartic acid but not glutamic acid in halophilic enzymes is significant and assumed to reflect the mechanisms of molecular adaptation to high-salt concentrations. Amino acid composition of salt-tolerant *Micrococcus* glutaminase was compared with those of halophiles and mesophiles. *Micrococcus* glutaminase showed similar amino acid compositions with those of mesophiles rather than halophiles. This result suggests that an increase of as-



1 *Micrococcus luteus* 456 A.A. (1-309)
2 *Corynebacterium glutamicum* 287 A.A. (1-286)
3 *Bacillus halodulans* 308 A.A. (1-302)
4 *Bacillus subtilis* 327 A.A. (1-319)
5 *Escherichia coli* 340 A.A. (1-333)

Fig. 4. Comparison of the amino acid sequence of glutaminase of *Micrococcus luteus* K-3 with those of glutaminases of *Corynebacterium glutamicum* [54], *Bacillus halodurans* [55], and *B. subtilis*. The conserved amino acid residues are boxed.

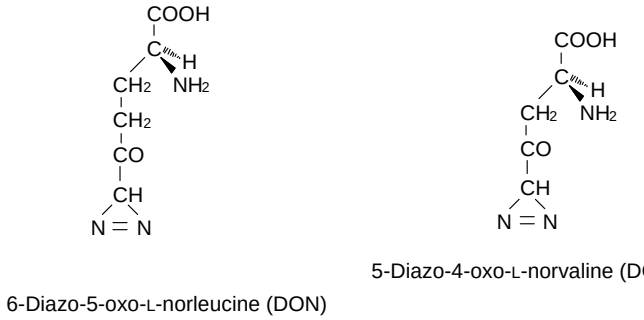


Fig. 5. Structural analogs of L-glutamine and L-asparagine.

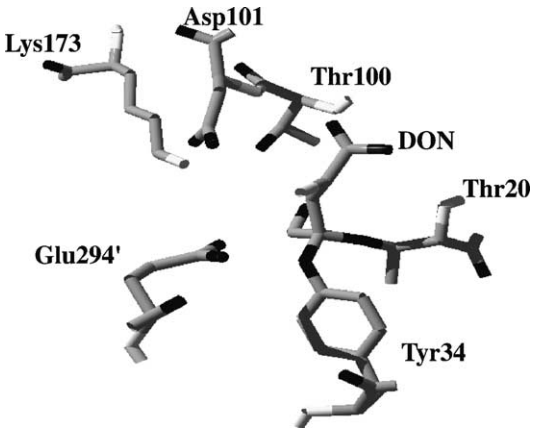


Fig. 6. Standard view of the active site with the bound inhibitor modeled in. DON is proposed to form an a-keto ether linkage with an active-site Thr OH yielding a tetrahedral acyl–enzyme intermediate. Thr20 is proposed to act as the primary nucleophile assisted by the Tyr34 and proximal Glu294′ (from subunit B).

partic acid residues on protein surface would not be the only possible way to adapt to high-salt concentrations, and salt-tolerant proteins have different haloadaptation mechanisms from those of halophilic enzymes.

8. Applications of glutaminase in pharmaceutical and food industries

E. coli asparaginase under the brand name Elspar has been used for the treatment of acute lymphocytic leukemia for more than 20 years [34]. A parallel interest in L-glutaminase has arisen from demonstrations that microbial glutaminase also exhibits antitumor activity [1,2,35]. A glutaminase–asparaginase enzyme from *Achromobacter* has shown antileukemic effects in patients with acute lymphoblastic leukemia and acute myelois leukemia in a preliminary clinical trial [36]. Glutaminase–asparaginase from *Pseudomonas* 7A has shown significant antitumor activity in mice [37], especially when combined with glutamine antimetabolites [38], and furthermore has shown promise as a possible therapeutic agent for the treatment of retroviral diseases [39]. *A. glutaminasificans* glutaminase–asparaginase is chemically modified by succinylation, and the succinylated enzyme has broader antitumor activity than *E. coli* asparaginase [40].

Theanine (γ-L-glutamylethylamide) is one of the major components of amino acids in Japanese green

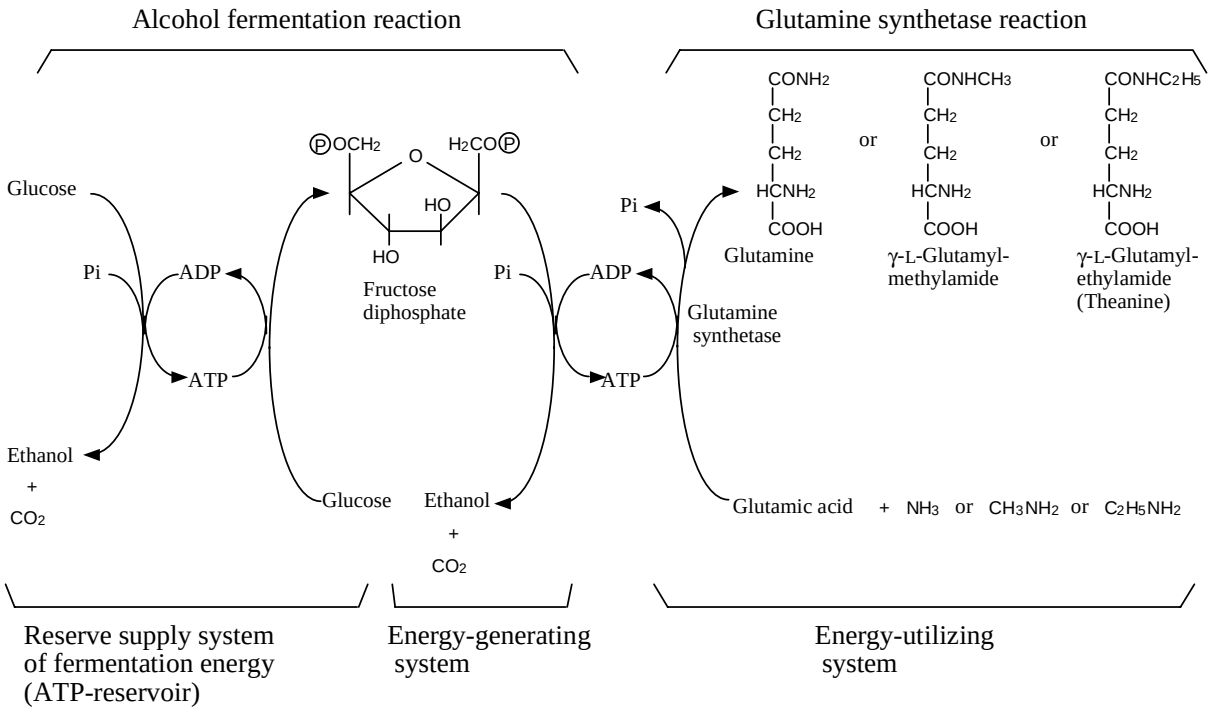


Fig. 7. Theanine production by coupling a sugar fermentation reaction of baker's yeast as an ATP-generating system.

tea, and unique as a taste-enhancing amino acid of infused green tea. Recently, increasing attention has been drawn towards the physiological roles of theanine, especially in a clinical point of view because of their ability to suppress stimulation by caffeine, to improve the effects of antitumor agents, and their role as antihypertensive agents. Theanine is synthesized by theanine synthetase (EC 6.3.1.6) in plants. Tachiki et al. [41] have developed a method of producing theanine from glutamate and ethylamine using a combination reaction of bacterial glutamine synthetase with a sugar fermentation reaction of baker's yeast as an ATP-regenerating system (Fig. 7).

On the other hand, the glutaminase from *P. nitroreducens* IFO 12694 has been found to catalyze simultaneously a γ-glutamyl transfer reaction and hydrolysis of the donor in a mixture containing γ-glutamyl donor and γ-glutamyl acceptor [42]. A new enzymatic method for producing theanine has been industrially developed by using glutaminase from *P. nitroreducens* [42] (Fig. 8). In fact, in a mixture containing high concentrations of substrates (0.7 M glutamine and 1.5 M ethylamine) and 0.5 units/ml glutaminase (borate buffer, pH 11), 270 mM (47 g/l) theanine was formed in a 7-h reaction at 30 °C.

In spite of its demonstrated potential as an antileukemic agent, glutaminase is generally regarded as a key enzyme that controls the taste of fermented foods such as soy sauce, especially in Asian countries. Soy sauce is a traditional seasoning of the Orient, which has gained worldwide acceptance today. Glutamic acid and aspartic acid have been well known as important amino acids contributing not only fine taste, "*Umami*" and sharp taste, "Sour" but also nutritional effects to food [43]. Aspartic acid, a product of asparaginase–glutaminase catalyzed reaction is converted by aspartic acid decarboxylase into alanine, a decisive amino acid component of soy sauce. The consumption of soy products and soy has been increasing in Western countries due to purported health benefits of soy (cancer protective, estrogenic effects). In addition to providing soy proteins and isoflavones, soy sauce also functions as a flavor enhancer. The current fermented soy sauce in Japan is manufactured from a mixture of defatted soybean grits and wheat kernels of almost equal amounts [44]. Its production involves solid state fermentation

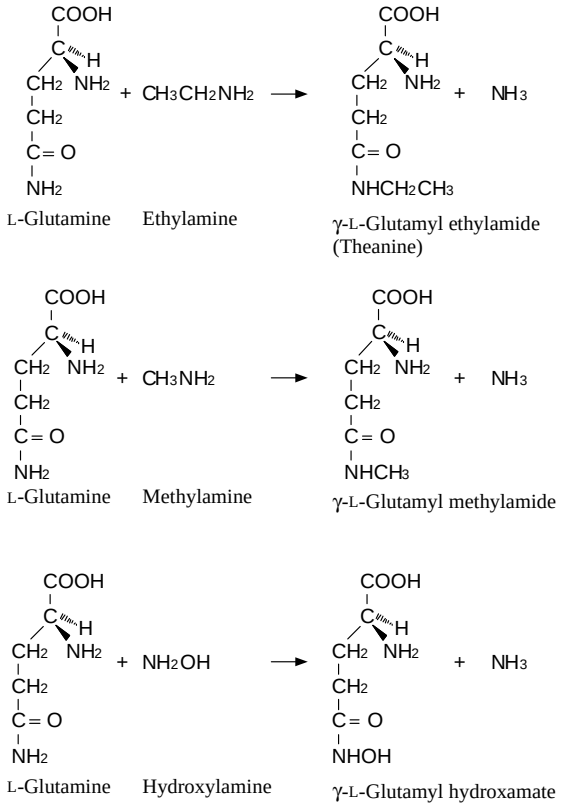


Fig. 8. Synthesis of useful compounds (theanine, γ-L-glutamyl methylamide) by glutaminase.

using *A. oryzae* in industrial and traditional *koji* fermentations since this mold secretes a large variety of proteases and peptidases during the fermentation. During soy sauce mash fermentation, glutamic acid and glutamine are formed from peptides by the action of peptidases and the glutamine is then converted into glutamic acid by the action of glutaminase. In the absence of glutaminase most of the glutamine liberated from the peptides is chemically and irreversibly converted to pyroglutamic acid, which has no taste [45] (Fig. 9).

Glutaminase from *A. oryzae*, which is commonly used for soy sauce fermentation, is shown to be inhibited by 17–18% salt concentration in the soy sauce fermentation process [21]. Therefore, salt-tolerant glutaminase may play potentially significant roles in fermentation process that require high-salt environments. The two isozymes of *Micrococcus* glutaminase

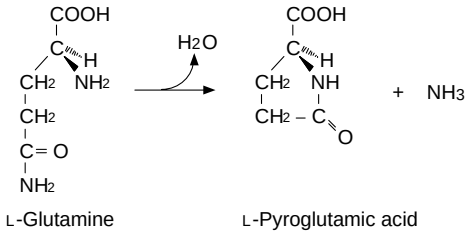


Fig. 9. Conversion of glutamine into pyroglutamic acid.

(I and II) were found to be highly salt tolerant. Glutaminase I was stable and exhibited about 1.3-folds higher activity in the presence of 8–16% NaCl than in the absence of NaCl, while glutaminase II was not stabilized and activated under the same condition. These properties of glutaminase I except for its optimal pH (pH 8) render it suitable for use in soy sauce fermentation [6]. Attempts to increase the glutamate content of soy sauce by salt-tolerant glutaminase form yeasts such as *Candida*, *Cryptococcus*, and *Torulopsis* were also done in the past [46].

Strain improvements of *koji* molds for overproduction of glutaminase enzyme have also been attempted employing mutation, protoplast fusion and haploidization [47–49]. Since glutamine is also converted nonenzymatically to a flavorless compound, pyroglutamic acid [44,50], increased expression of glutaminase could result in an increased amount of glutamic acid [51,52] and an improved quality of soy sauce [45].

Even though, glutamic acid is the primary amino acid responsible to impart a delicious taste in fermented foods [42], practically few attempts have been made to produce glutamate by immobilizing glutaminase or glutaminase-producing microorganisms. Yeast cells with a salt-tolerant and thermostable glutaminase are immobilized in silica gel and alginate–silica complex gel (AS gel). Glutamic acid (10 mg/ml) is continuously produced by passing a filtrate of wheat gluten hydrolyzed by proteolytic enzymes through the column containing the cells immobilized in AS gel [53].

9. Conclusion and future prospects

Microbial glutaminases hold a very important place among commercially important enzymes due to their proven role as antileukaemic and as flavor-enhancing agents. In spite of this, glutaminase or glutaminase–asparaginase enzymes have received scant attention from investigators when compared to other established industrial enzymes or even their counterpart asparaginase. A major share of recent research on glutaminase has focused on mammalian glutaminase, their biochemistry, regulation and genetic make-up, justifiably due to the role of glutaminase in mammalian metabolism. However, this scenario is changing as a number of recent studies are attempting being dedicated to attain an indepth knowledge of regulatory, structural and biochemical aspects as well as the gene expression of glutaminases from various microbial sources. Still, there is plenty of room for research on glutaminases including the isolation of salt-and thermo-tolerant enzymes, which would significantly enhance their applications in the food industry. Furthermore, a detailed understanding of the regulation of gene expression based on molecular approaches and other means would contribute immensely towards developing successful strategies for strain improvement which is a prerequisite for any industrially important enzyme.

References

[1] J. Roberts, J.S. Holcenberg, W.C. Dolowy, Nature 227 (1970) 1136.
[2] J. Roberts, J.S. Holcenberg, W.C. Dolowy, Life Sci. 10 (1971) 251.
[3] A.A. Pekhov, V.A. Zanin, N.V. Margretova, T.T. Berezov, Bull. EKSP Biol. Med. 99 (1985) 557.
[4] S.C. Hartman, in: P.D. Boyer (Ed.), The Enzymes, vol. IV, Academic Press, New York, 1971, p. 79.
[5] J.S. Holcenberg, J. Roberts, W.C. Dolowy, in: S. Prusiner, E.R. Stadtman (Eds.), The Enzymes of Glutamine Metabolism, Academic Press, New York, 1973, p. 277.
[6] M. Moriguchi, K. Sakai, R. Tateyama, Y. Furuta, M. Wakayama, J. Ferment. Bioeng. 77 (1994) 621.
[7] S. Prusiner, E.R. Stadtman, J. Biol. Chem. 251 (1976) 4363.
[8] K. Soda, M. Ohshima, T. Yamamoto, Biochem. Biophys. Res. Commun. 46 (1972) 1278.
[9] T. Tachiki, T. Yamada, M. Ueda, Y. Naemura, N. Imamura, Y. Hamada, J. Shoide, Biosci. Biotechnol. Biochem. 60 (1996) 1160.
[10] M. Ohshima, T. Yamamoto, K. Soda, Agric. Biol. Chem. 40 (1976) 2251.
[11] H.E. Wade, H.K. Robinson, B.W. Philips, J. Gen. Microbiol. 69 (1971) 299.

[12] G.E.N. Nelson, R.E. Petrson, A. Ciegler, Dev. Ind. Microbiol. 169 (1974) 385.
[13] A. Imada, S. Igarasi, K. Nakahama, M. Isono, J. Gen. Microbiol. 76 (1973) 85.
[14] K. Dharmaraj, N. Selvakumar, D. Chandramohan, R. Natarajan, Indian J. Mar. Sci. 6 (1977) 168.
[15] A. Sabu, T.R. Keerthi, S. Rajeev Kumar, M. Chandrasekaran, Process Biochem. 35 (2000) 705.
[16] S. Renu, M. Chandrasekaran, Biotechnol. Lett. 14 (1992) 471.
[17] W.R. Cook, J.H. Hoffman, R.W. Berlohr, J. Bacteriol. 148 (1981) 365.
[18] M.A. Dura, M. Flores, F. Toldra, Int. J. Food Microbiol. 76 (2002) 117.
[19] S. Prusiner, J.N. Davis, E.R. Stadtman, J. Biol. Chem. 251 (1976) 3447.
[20] M. Soberon, A. Gonzalez, J. Gen. Microbiol. 133 (1987) 1.
[21] T. Yano, M. Ito, K. Tomita, H. Kumagai, T. Tochikura, J. Ferment. Technol. 66 (1988) 137.
[22] M. Klein, H. Kaltwasser, T. Jahns, FEMS Microbiol. Lett. 206 (2002) 63.
[23] K. Koibuchi, H. Nagasaki, A. Yuasa, J. Kataoka, K. Kitamoto, Appl. Microbiol. Biotechnol. 54 (2000) 59.
[24] S.P. Kennedy, W.V. Ng, S.L. Salzberg, L. Hood, S. DasSarma, Genome Res. 11 (2001) 1641.
[25] D. Madern, C. Ebel, G. Zaccai, Extremophiles 4 (2000) 91.
[26] C. Thammarongtham, G. Turner, A. Moir, M. Tanticharoen, S. Cheevadhanarak, J. Mol. Microbiol. Biotechnol. 3 (2001) 611.
[27] R. Nandakumar, M. Wakayama, Y. Nagano, T. Kawamura, K. Sakai, M. Moriguchi, Protein Express. Purif. 15 (1999) 155.
[28] A. Huerta-Saquero, J. Carderon, R. Arreguin, A. Calderon-Flores, S. Duran, Protein Express. Purif. 21 (2001) 432.
[29] J. Lubkowski, A. Wlodawer, H.L. Ammon, T.D. Copeland, A.L. Swain, Biochemistry 33 (1994) 10257.
[30] H.L. Ammon, I.T. Weber, A. Wlodawer, R.W. Harrison, G.L. Gilliland, K.C. Murphy, L. Sjolin, J. Roberts, J. Biol. Chem. 263 (1988) 150.
[31] E. Ortlund, M.W. Lacount, K. Lewinski, L. Lebioda, Biochemistry 39 (2000) 1199.
[32] P. Chantawannakul, K. Yoshimune, Y. Shirakihara, A. Shiratori, M. Wakayama, M. Moriguchi, Acta Cryst. D59 (2003) 566.
[33] S. Fukuchi, K. Yoshimune, M. Wakayama, M. Moriguchi, K. Nishikawa, J. Mol. Biol. 327 (2003) 347.
[34] J.G. Kidd, Cancer Res. 33 (1970) 3.
[35] T. Oki, M. Shirai, M. Oshima, T. Yamamoto, K. Soda, FEBS Lett. 33 (1973) 286.
[36] A.S.D. Spiers, H.E. Wade, Cancer Treat. Rep. 63 (1979) 1019.
[37] J. Roberts, F.A. Schmid, H.J. Rosenfeld, Cancer Treat. Rep. 63 (1979) 1045.
[38] W.G. McGregor, J. Roberts, Proc. Am. Assoc. Cancer Res. 30 (1989) 2302.
[39] J. Roberts, W.G. McGregor, J. Gen. Virol. 72 (1991) 299.
[40] R.P. Warrell, T.C. Chou, C. Gordon, C. Tan, J. Roberts, S.S. Sternberg, F.S. Philips, C.W. Young, Cancer Res. 40 (1980) 4546.
[41] S. Wakisaka, Y. Ohshima, M. Ogawa, T. Tochikura, T. Tachiki, Appl. Environ. Microbiol. 64 (1998) 2952.
[42] T. Tachiki, T. Yamada, K. Mizuno, M. Ueda, J. Shiode, H. Fukami, Biosci. Biotechnol. Biochem. 62 (1998) 1279.
[43] M.O. Mahony, R. Ishii, Br. J. Psychol. 77 (1986) 161.
[44] T. Yokotsuka, in: C.O. Cheichester (Ed.), Advances in Food Research, vol. 130, Academic Press, New York, 1986, p.195.
[45] J.B. Gilbert, V.E. Price, J.P. Greenstein, J. Biol. Chem. 180 (1949) 209.
[46] T. Nakadai, S. Nasuno, J. Ferment. Bioeng. 67 (1989) 158.
[47] S. Ushijima, T. Nakadai, Agric. Biol. Chem. 51 (1987) 1051.
[48] S. Ushijima, T. Nakadai, K. Uchida, Agric. Biol. Chem. 51 (1987) 2781.
[49] S. Ushijima, T. Nakadai, K. Uchida, Agric. Biol. Chem. 54 (1990) 2393.
[50] S. Sugiyama, Food Microbiol. 1 (1984) 339.
[51] S. Yamamoto, H. Hirooka, J. Ferment. Technol. 52 (1974) 564.
[52] S. Yamamoto, H. Hirooka, J. Ferment. Technol. 52 (1974) 570.
[53] Y. Fukushima, H. Motai, J. Ferment. Bioeng. 69 (1990) 189.
[54] M. Pompejus, B. Kroeger, H. Schroeder, O. Zelder, G. Haberhauer, Pat. WO 0100843-A 101, 2001.
[55] H. Takami, K. Nakasone, Y. Takaki, G. Maeno, R. Sasaki, N. Masui, F. Fuji, C. Hirama, Y. Nakamura, N. Ogasawara, S. Kuhara, K. Horikoshi, Nucleic Acids Res. 28 (2000) 4317.
[56] J. Roberts, J.S. Holcenberg, W.C. Dolowy, J. Biol. Chem. 247 (1972) 84.
[57] J.S. Holcenberg, D.C. Teller, J. Roberts, W.C. Dolowy, J. Biol. Chem. 247 (1972) 7750.
[58] E.K. Novak, A.W. Philips, J. Bacteriol. 117 (1974) 593.
[59] M. Kikuchi, H. Hayashida, E. Nakano, K. Sakaguchi, Biochemistry 10 (1971) 1222.
[60] E.A. Kozlov, N.A. Kovalenko, S.R. Mardeshev, Biokhimiya 37 (1972) 56.
[61] S.C. Hartman, J. Biol. Chem. 243 (1968) 853.
[62] S. Prusiner, E.R. Stadtman, J. Biol. Chem. 251 (1976) 3457.
[63] H. McIlwain, J. Gen. Microbiol. 2 (1948) 187.
[64] M. Ohshima, T. Yamamoto, K. Soda, Bull. Inst. Chem. Res. Kyoto Univ. 54 (1976) 170.
[65] E.A. Kabanova, Z.I. Lebedeva, T.T. Berezov, Biokhimiya 51 (1986) 432.
[66] S.M. Badr El-Dn, M.S. Foda, Zentralbl Bakteriol. Parasitenkd Infektionskr Hyg. 131 (1976) 489.
[67] J. Roberts, J. Biol. Chem. 251 (1976) 2119.
[68] S. Duran, L. Sanchez-Linares, A. Huerta-Saquero, G. Dupont, A. Huerta-Zepeda, J. Calderon, Biochem. Genet. 34 (1996) 453.
[69] I. Sato, H. Kobayashi, Y. Hanya, K. Abe, S. Murakami, G. Scorzetti, J.W. Fell, J. Ind. Microbiol. Biotechnol. 22 (1999) 127.
[70] J.S. Holcenberg, in: S.P. Colowick, O.N. Kaplan (Eds.), Methods in Enzymology, vol. 113, Academic Press, New York, 1985, p. 257.
[71] S.C. Hartman, T.F. McGrath, J. Biol. Chem. 248 (1973) 8506.

[72] S.C. Hartman, E.M. Stochaj, J. Biol. Chem. 248 (1973) 8511.
[73] A. Huerta-Saqero, J. Calderon, R. Arregiun, A. Calderon-Flores, S. Duran, Protein Express. Purif. 21 (2000) 432.
[74] K. Tomita, T. Yano, H. Kumagai, T. Tochikura, J. Ferment. Technol. 66 (1988) 299.
[75] Y. Ogasawara, T. Hamada, S. Mori, H. Hashida, Nippon Shoyu Kenkyusho Zasshi 22 (1996) 167.
[76] T. Yokotsuka, T. Iwasa, S. Fujii, Nippon Shoyu Kenkyusho Zasshi 13 (1987) 18.
[77] Y. Shimizu, A. Ueyama, K. Goto, J. Brew. Soc. Jpn. 86 (1991) 441.