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ORIGINAL PAPER

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Digestion by serine proteases enhances salt tolerance of glutaminase in the marine bacterium *Micrococcus luteus* K-3

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Abstract Salt-tolerant glutaminase (*Micrococcus* glutaminase, with an apparent molecular mass of 48.3 kDa, intact glutaminase) from the marine bacterium *Micrococcus luteus* K-3 was digested using protease derived from *M. luteus* K-3. The digestion products were a large fragment (apparent molecular mass of 38.5 kDa, the glutaminase fragment) and small fragments (apparent molecular mass of 8 kDa). The digestion was inhibited by phenylmethanesulfonyl fluoride (PMSF). Digestion of intact glutaminase by serine proteases including trypsin, elastase, lysyl endopeptidase, and arginylendopeptidase also produced the glutaminase fragment. The N-terminus of the glutaminase fragment was the same as that of intact glutaminase. The N-termini of two small fragments were Ala394 and Ala396, respectively. The enzymological and kinetic properties of the glutaminase fragment were almost the same as those of intact glutaminase except for salt-tolerant behavior. The glutaminase fragment was a higher salt-tolerant enzyme than the intact glutaminase, suggesting that *Micrococcus* glutaminase is digested in the C-terminal region by serine protease from *M. luteus* K-3 to confer salt tolerance on glutaminase.

Keywords Glutaminase · Marine bacterium · *Micrococcus luteus* · Salt tolerance · Serine protease

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Introduction

The mechanisms of the adaptation of proteins to high salt concentrations remain an unsolved problem because of the lack of information with regard to salt-tolerant or halophilic enzymes. Halophilic enzymes are found in halophilic microorganisms that require at least a 2.5 M NaCl medium for optimum growth, and the enzymes require a high salt concentration for activity or stability (Madern et al. 2000). Several independent studies suggested that halophilic enzymes possess a higher proportion of acidic residues than nonhalophilic homologues (Lanyi 1974; Rao and Argos 1981; Baldacci et al. 1990; Benachenhou and Baldacci 1991). A relationship between acidic residues and salt binding was suggested by a stabilization model proposed for tetrameric malate dehydrogenase from *Haloarcula marismortui* (Madern et al. 1995). Amino acid residues located on the protein surface are proposed to bind to a network of hydrated salt ions that cooperatively contribute to protein stabilization. Fukuchi and Nishikawa (2001) suggested that the amino acid composition of protein surfaces differs between thermophilic and mesophilic bacteria. Recently, we have reported that an abundance of aspartic acid is found on the surface of proteins in halophilic microorganisms compared to those of mesophilic microorganisms (Fukuchi et al. 2003). The high content of aspartic acid, but not glutamic acid, in halophilic enzymes is significant and assumed to reflect mechanisms of molecular adaptation to high salt concentrations.

Salt-tolerant proteins are distinguished from halophilic proteins by their high stability and activity even at low-solvent salt concentrations. *Micrococcus* glutaminase is included in salt-tolerant proteins that are distinguished from halophilic proteins by their high stability and activity even at low-solvent salt concentrations (Moriguchi et al. 1994). The amino acid composition of *Micrococcus* glutaminase is similar to those of nonhalophilic enzymes (Fukuchi et al. 2003). The mechanisms

of salt-tolerant proteins seem to differ from those of halophilic proteins. Biochemical and biophysical data have been accumulated for several salt-tolerant enzymes, including glutaminase from *Lactobacillus rhamnosus* (Weingand-Ziade et al. 2003), α-amylase from *Bacillus dipsosauri* (Deutch 2002), protease from *Aspergillus* sp. FC-10 (Su and Lee 2001), α-type carbonic anhydrase from *Dunaliella salina* (Premkumar et al. 2003), and thermolysin from *B. thermoproteolyticus* (Inouye et al. 1998). However, the mechanisms of salt tolerance have not been well studied.

Glutaminase (EC 3.5.1.2), which catalyzes the hydrolytic degradation of L-glutamine to L-glutamic acid, plays an important role in the food processing industry, especially for Japanese soy sauce fermentation since it increases the amount of flavoring savory, L-glutamic acid (Nandakumar et al. 2003). However, glutaminases from *koji* mold (*Aspergillus oryzae* and *A. sojae*), which is commonly used for soy sauce fermentation, are markedly inhibited by the high salt concentration found in the soy sauce fermentation process (about 3 M sodium chloride, Yano et al. 1988). Since L-glutamine is nonenzymatically converted into the flavorless compound, L-pyroglutamic acid (Gilbert et al. 1949; Kuroshima et al. 1969; Schneider et al. 2003), the increased activity of glutaminase could result in an increased amount of L-glutamic acid, with decreasing production of L-pyroglutamic acid. Therefore, salt-tolerant glutaminase may play significant roles in industrial processes that require high-salt environments.

In a previous paper, we homogeneously purified *Micrococcus* glutaminase. The molecular mass of the native enzyme and the subunit were found to be 86 and 43 kDa, respectively, using gel filtration and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The enzyme is a salt-tolerant protein active in the absence and presence of 3 M sodium chloride (Moriguchi et al. 1994). Therefore, the enzyme is considered to be useful for application in Japanese soy sauce fermentation.

The gene encoding *Micrococcus* glutaminase was cloned in *Escherichia coli*. The molecular weight of the deduced amino acid sequence of glutaminase was determined to be 48,247 kDa. These results suggest the requirement of the processing enzyme for the formation of the 43-kDa mature enzyme from the 48,247-kDa sequence (Wakayama et al. 1996). In this study, we found that *Micrococcus* glutaminase is digested by proteases in the cell or during the course of purification, and that protease is the processing enzyme. Interestingly, the glutaminase fragment (38.5 kDa) exhibits a higher salt tolerance than that of the intact glutaminase (48.3 kDa) (Chantawannakul et al. 2003). We describe the formation mechanism and kinetics of the glutaminase fragment and discuss the role of the C-terminal region (amino acids 369–456) truncated from the intact glutaminase in the regulation of salt.

Materials and methods

Materials

Plasmid pKSGHE3-1 encoding *Micrococcus* glutaminase was prepared as previously described (Nandakumar et al. 1999). L-Glutamate dehydrogenase (beef liver) was acquired from Oriental Yeast (Tokyo, Japan) and DEAE-Toyopearl from Tosoh (Tokyo, Japan). Arginylendopeptidase was obtained from Takara Shuzo (Kyoto, Japan), and elastase was obtained from Sigma (St. Louis, Mo., USA). Protein calibration standard II was acquired from Bruker Daltonik (Leipzig, Germany). Lysyl endopeptidase and all other chemicals were obtained from Wako Pure Chemicals (Osaka, Japan).

Purification of recombinant glutaminase

Purification of *Micrococcus* glutaminase was carried out as previously described (Nandakumar et al. 1999). *Escherichia coli* JM 109 cells transformed with pKSGHE 3-1, a high-expression plasmid containing the *Micrococcus* glutaminase gene (Nandakumar et al. 1999), were cultured in Luria-Bertani medium at 30°C for 18 h. Isopropyl 1-thio-β-D-galactoside was added to the culture medium at a final concentration of 0.1 mM after 16 h of cultivation. The buffer (buffer I) used was composed of 10 mM Tris-HCl buffer (pH 7.2) containing 10 mM $MgCl_2$ and 10% glycerol. Cells in the buffer I (0.1 g/ml) were disrupted by sonication, and cell debris was removed by centrifugation. The supernatant obtained was dialyzed against buffer I and then applied to a DEAE-Toyopearl column equilibrated with buffer I. The column was washed with buffer I containing 130 mM NaCl, and the enzyme was then eluted using buffer I containing 150 mM NaCl. The homogeneous enzyme, examined using SDS-PAGE, was pooled and dialyzed against buffer I. The recombinant protein was purified to homogeneity with a similar specific activity (1,246 U/mg) to that of purified enzyme from *Micrococcus luteus* K-3 strain (1,230 U/mg) (Nandakumar et al. 1999).

Enzyme and protein assays

Glutaminase activity was assayed by determining the formation of L-glutamic acid by L-glutamate dehydrogenase as previously described. The standard reaction conditions were as follows. The reaction mixture (0.5 ml) contained 0.1 M Tris-HCl buffer (pH 7.5) and 30 mM L-glutamine. After 10 min at 30°C, the reaction was stopped by boiling for 3 min, and it was then centrifuged. The supernatant (0.05 ml) was added to the reaction mixture for glutamic acid determination, which contained 50 mM Tris-hydrazine buffer (pH 9.0), 1.5 mM NAD^+, 0.5 mM ADP, and 5 U/ml of glutamate dehydrogenase in a total volume of 1 ml. The

absorbance at 340 nm was measured after incubating the mixture for 60 min at 30°C. One unit of glutaminase was defined as the amount of enzyme that catalyzed the formation of 1 μmol L-glutamic acid per minute. The protein concentration was determined by the method of Lowry et al. (1951) or its absorbance at 280 nm (Kuramitsu et al. 1990). The molar extinction coefficients of tryptophan and tyrosine were assumed to be 5,800 and 1,450 M^{-1} cm^{-1}, respectively.

Determination of N-terminal amino acid sequence

The intact glutaminase and the glutaminase fragment were subjected to SDS-PAGE, and then electroblotted onto polyvinylidene difluoride (PVDF) membrane. A portion of the PVDF membrane containing the protein band was cut out, and the protein was subjected to automatic Edman degradation with a protein sequencer.

Analytical determinations

For MALDI-TOF mass spectrometry, the glutaminase fragment was dialyzed against water, and the same volume of matrix solution consisting of saturated α-cyano-4-hydroxycinnamic acid in 50% acetonitrile and 0.05% trifluoroacetic acid was added. One microliter of this solution was applied to the target. MALDI-TOF mass spectra were recorded using a Bruker Autoflex spectrometer. Protein calibration standard II was used for calibration.

Protease digestion

The reaction mixture contained 100 mM potassium phosphate buffer, pH 8.0, 0.44 mg/ml of the intact glutaminase, and 0.5 μg/ml trypsin, 7 μg/ml elastase, 0.02 U/ml lysyl endopeptidase or 0.5 μg/ml arginylendopeptidase in a total volume of 50 μl. The reaction mixture was incubated at 30°C for 30 min, and the digestion was stopped by the addition of 5 μl of 10 mM PMSF.

Results

Digestion of *Micrococcus* glutaminase by proteases from *Micrococcus luteus* K-3

The intact glutaminase was purified from the cell-free extract of *Escherichia coli* JM 109 cells transformed with pKSGHE 3-1, the expression plasmid for *Micrococcus* glutaminase, according to the procedure described in the text. When the purified intact glutaminase (48.3 kDa, 170 μg/ml) was digested by the incubation with 300 μg protein/ml of the cell-free extract from *M. luteus* K-3 at 30°C for 24 h, the glutaminase fragment (38.5 kDa), and the small fragment (8 kDa) were produced (Fig. 1). The digestion was inhibited in the presence of 1 mM PMSF, indicating that *Micrococcus* glutaminase may be digested by certain serine proteases in *M. luteus* K-3.

Properties of the intact glutaminase and the glutaminase fragment

The effect of NaCl concentration on the activity of the intact glutaminase was different from that of the glutaminase fragment (Fig. 2). The glutaminase fragment showed a higher activity than that of the intact glutaminase at NaCl concentrations of more than 1.7 M. Figure 3 shows the stabilities of the intact glutaminase and the glutaminase fragment at various NaCl concentrations. The glutaminase fragment was more stable than the intact glutaminase at more than 1.7 M NaCl, and a very small increase was observed on its stability as the concentration of NaCl increased. Table 1 shows the effects of salt on the kinetic constants for the intact glutaminase and the glutaminase fragment. K_m and k_{cat} values of the intact glutaminase were affected by NaCl, while salt concentration had little effect on those of the

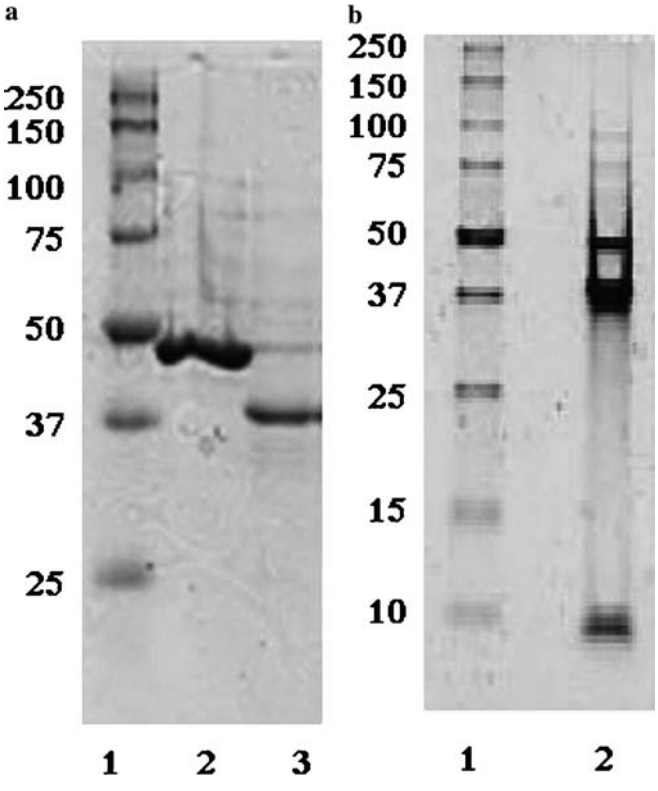

Fig. 1a, b SDS-PAGE analysis of the intact glutaminase and the glutaminase fragment. **a** The intact glutaminase and the glutaminase fragment were analyzed by 10% SDS-PAGE gel. *Lane 1* Molecular weight markers as indicated in kiloDaltons, *lane 2* intact glutaminase, *lane 3* glutaminase fragment. **b** The glutaminase fragment was analyzed by 15% SDS-PAGE gel. *Lane 1* Molecular weight markers, *lane 2* mixture of the intact glutaminase and the glutaminase fragment

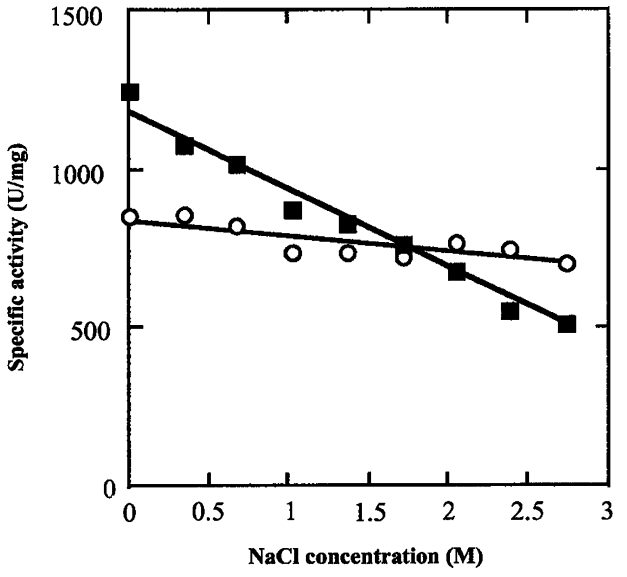

Fig. 2 Effect of salt concentration on the activity of the intact glutaminase and the glutaminase fragment. The activities of the intact glutaminase (*filled square*), and the glutaminase fragment (*open circle*) were assayed at various NaCl concentrations

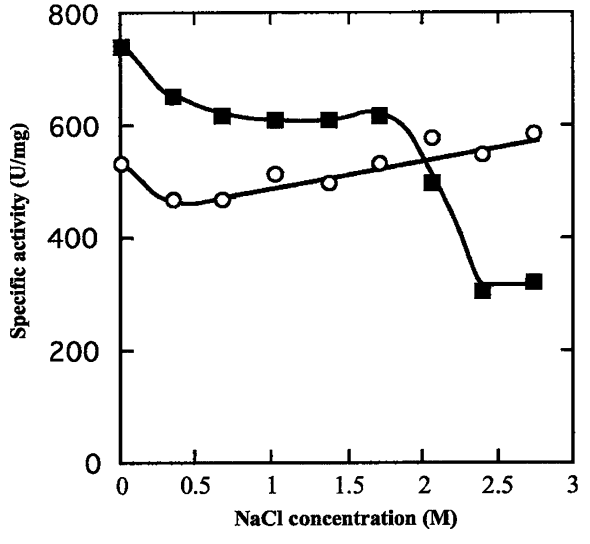

Fig. 3 Effects of salt concentration on the stabilities of the intact glutaminase and the glutaminase fragment. After the intact glutaminase (*filled square*) and the glutaminase fragment (*open circle*) were treated in at various NaCl concentrations in 0.1 M Tris-HCl buffer (pH 7.5) at 40°C for 30 min, residual activity was measured

glutaminase fragment. These results suggest that the glutaminase fragment is a more salt-tolerant enzyme than the intact glutaminase.

Digestion of *Micrococcus* purified glutaminase by commercial serine proteases

The digestion of purified intact glutaminase, using commercial serine protease, trypsin, elastase, lysyl endopeptidase, or arginylendopeptidase, also produced the glutaminase fragment, which had molecular mass of about 38.5 kDa (data not shown). The glutaminase fragments produced by the commercial proteases showed salt tolerance approximately equal to that of the glutaminase fragment produced by proteases from *M. luteus* (data not shown).

N-terminal sequence

The N-terminal amino acid sequence of the glutaminase fragment, determined to be MRHPIPDYLA, was the same as that of the intact glutaminase and the amino acid sequence deduced from the gene of the intact glutaminase. Thus, the glutaminase fragment was C-terminally truncated. The two small fragments produced by C-terminal proteolysis and separated by SDS-PAGE were transferred onto a PVDF membrane. Automatic Edman degradation revealed the N-terminal amino acid sequences of two 8-kDa fragments. The two peptides were started with the amino acid sequence AARDENDGPI and RDENDGPIRT. These sequences were identical to internal regions of the intact glutaminase following Ala394 and Ala396, respectively.

Determination of molecular mass

The glutaminase fragment was analyzed by MALDI-TOF mass spectrometry. The experimental value of 38,972.2 Da matched a region of the intact glutaminase, which encompasses residues 1–368 with a molecular mass of 38,983.6 Da.

Discussion

Our results showed that the glutaminase fragment has a greater activity and stability than that of the intact

Table 1 Effects of salt concentration on kinetic constants for the intact glutaminase and the glutaminase fragment

NaCl (M)	Intact glutaminase			Glutaminase fragment		
	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m [1/(mM·min)]	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m [1/(mM·min)]
0	6.1	8.24×10^4	1.35×10^4	3.8	5.24×10^4	1.38×10^4
1.7	3.9	4.78×10^4	1.23×10^4	3.1	3.94×10^4	1.27×10^4
2.6	2.3	2.42×10^4	1.05×10^4	3.7	4.93×10^4	1.33×10^4

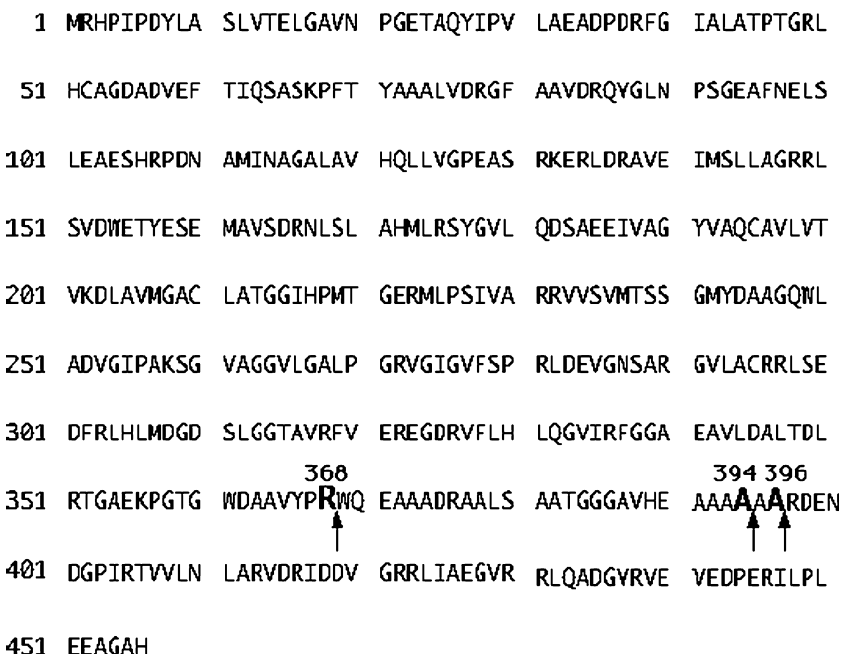

Fig. 4 The region susceptible to hydrolysis by *Micrococcus* proteases. The *arrows* indicate the positions at which the peptides were digested by proteases

glutaminase at NaCl concentrations greater than 1.7 M. On the other hand, the specific activity of the glutaminase fragment was lower than that of the intact glutaminase at NaCl concentrations less than 1.7 M NaCl concentrations. The C-terminal region of the intact glutaminase might be responsible for the function to increase its specific activity at low NaCl concentrations. At NaCl concentrations greater than 1.7 M NaCl, the C-terminal region might decrease the activity and stability of the intact glutaminase.

Since proteases in *Micrococcus luteus* K-3 digested the intact glutaminase and produced the glutaminase fragment, the glutaminase fragment may exist in vivo. However, *M. luteus* K-3 cannot survive in a medium containing more than a 1.7 M NaCl concentration (Moriguchi et al. 1994). The physiological meaning of the digestion of the intact glutaminase is unknown.

The molecular mass of the glutaminase fragment indicates that the peptide bond between Arg368 and Trp369 was hydrolyzed. This leads us to consider that the intact glutaminase was digested by a serine protease, which recognizes an arginine residue such as trypsin and arginylendopeptidase. The N-terminal amino acid of the C-terminal 8-kDa peptides derived from the intact glutaminase were Ala395 and Arg397. These results also suggest that the intact glutaminase possesses a region that is susceptible to hydrolysis by proteases, and that it encompasses residues 369–396 (Fig. 4).

The C-terminal additional peptide might be responsible for the conformational change. We hypothesize that the conformation change in the C-terminal domain of *Micrococcus* glutaminase concomitant with increasing NaCl concentration might bring about a subtle conformational change in the entire glutaminase structure. This subtle change may affect the interaction between glutaminase and NaCl. There is great interest in the changes in activity and stability of the enzyme in the presence of NaCl due to the additional peptide chain. There are no reports concerning the regulation of salt tolerance of the enzyme by the additional peptide chain as described in this paper, and this mechanism might be used as a new control procedure to affect the activity and stability of halophilic or salt-tolerant proteins under a high salt concentration environment. The concentration of NaCl used in this study might be extreme for normal biological process. Not many studies regarding the relationship between NaCl effects on structure and catalysis of enzymes that work under such extreme conditions have been carried out. In addition to pure biological interest in NaCl effects on enzyme structure and function, from the standpoint of the application of salt-tolerant enzymes in the food industry, such as Japanese soy sauce fermentation, the structure and function of salt-tolerant enzymes acting under extreme conditions such as high salinity should be investigated.

For further understanding of the mechanisms of the salt tolerance of *Micrococcus* glutaminase, crystals of the intact glutaminase and the glutaminase fragment have been prepared (Chantawannakul et al. 2003) and structural analyses of two crystals are currently being attempted.

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