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Generating oxidation-resistant variants of *Bacillus kaustophilus* leucine aminopeptidase by substitution of the critical methionine residues with leucine

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Abstract

Bacillus kaustophilus leucine aminopeptidase (bkLAP) was sensitive to oxidative damage by hydrogen peroxide. To improve its oxidative stability, the oxidation-sensitive methionine residues in the enzyme were replaced with leucine by site-directed mutagenesis. The variants, each with an apparent molecular mass of approximately 54 kDa, were overexpressed in recombinant *Escherichia coli* M15 cells and purified to homogeneity by nickel-chelate chromatography. The specific activity for M282L, M285L, M289L and M321L decreased by more than 43%, while M400L, M426L, M445L, and M485L showed 191, 79, 313, and 103%, respectively, higher activity than the wild-type enzyme. Although the mutations did not cause significant changes in the K_m value, more than 67.8% increase in the value of k_{cat}/K_m was observed in the M400L, M426L, M445L and M485L. In the presence of 50 mM H_2O_2, most variants were more stable with respect to the wild-type enzyme, indicating that the oxidative stability of the enzyme can be improved by engineering the methionine residues.

Introduction

Leucine aminopeptidases (LAPs) are widely distributed cytosolic exopeptidases that selectively remove N-terminal amino acid residues from polypeptides and proteins. LAPs play an important role in protein degradation and in the metabolism of biologically active peptides (Terenius et al. 2000; Goldberg et al. 2002; Lowther and Mattews 2002). They have also been shown to be valuable for the preparation of debittered protein hydrolysates (Rao et al. 1998), to improve flavor development (Toldrá et al. 2000) and to convert L-homophenylalanyl amide into L-homophenylalanine, the versatile intermediate for a class of angiotension 1-converting enzyme inhibitors (Kamphuis et al. 1990). Due to their great potential in industrial applications, the enzyme from a variety of microorganisms has been extensively studied (Gonzales and Robert-Baudouy 1996).

Proteins are well known to be sensitive to oxidative damage and the oxidized proteins have been found to increase in aged organisms, leading to the proposal that protein oxidation contributes to the aging process (Berlett and Stadtman 1997). Oxidative modification of residues within proteins may be conducted by physiological and non-physiological systems (Stadtman 1986, 1993). Methionine residues are remarkable for their high susceptibility to chemical

oxidation with methionine sulfoxide as the general product (Vogt 1995). Methionine sulfoxidation has been reported to affect the function of a number of biologically active proteins (Stauffer and Etson 1969; Hayes et al. 1998; Hatakeyama et al. 2000; Wang et al. 2001). Recently, a thermophilic *Bacillus kaustophilus* leucine aminopeptidase (bkLAP) gene encoding a protein of 497 amino acids with a calculated molecular mass of 53.7 kDa was cloned and expressed in *Escherichia coli* (Lin et al. 2004). In this investigation, we replaced each of the twelve methionine residues at positions 68, 224, 229, 282, 285, 289, 299, 321, 400, 426, 445, and 485 in the primary amino acid sequence of bkLAP by Leu in an attempt to improve its resistance to chemical oxidation. The results showed that most of the engineered enzymes have an increased resistance toward H_2O_2 inactivation.

Materials and methods

Materials, strains, plasmid, and growth conditions

Growth medium components were obtained from Difco Laboratories (Detroit, MI, USA). Restriction and DNA modifying enzymes were acquired from Promega Co. (Madison, WI, USA) and used in accordance with the supplier's instructions. Oligonucleotides for sequencing and mutagenesis were synthesized by Mission Biotechnology Inc. (Taipei, Taiwan). Nickel nitrilotriacetate (Ni^{2+}-NTA) resin was from Qiagen Inc. (Valencia, CA, USA). Reagents for polyacrylamide electrophoresis including acrylamide, bisacrylamide, ammonium persulfate, and TEMED were obtained from Bio-Rad Laboratories (Hercules, CA, USA). All other chemicals were commercial products of analytical or molecular biological grade.

Escherichia coli Novablue (Novagen Inc., Maison, Wis., USA) was used for the preparation and construction of recombinant plasmids. *E. coli* M15 from Qiagen Inc. was used for T5 RNA polymerase-mediated overproduction of wild-type and mutant proteins. Plasmid used was pQE-LAP (Lin et al. 2004). The *E. coli* cells containing plasmids were grown aerobically at either 28 °C or 37 °C in Luria-Bertani (LB) medium supplemented with 100 μg ampicillin/ml for Novablue strain or 100 μg ampicillin/ml and 25 μg kanamycin/ml for M15 strain.

Table 1. Oligonucleotides used for the site-directed mutagenesis

Mutants	Primer sequence[a]
M68L	5′-CCATTTTTGCCGCTGGGGGCGAAGCGG
M224L	5′-GAGAAAGAAGACCTGGAGCGGCTCGGC
M229L	5′-GAGCGGCTCGGCCTGGGAGCGCTTTTG
M282L	5′-CCGCGCGACAGCCTGGTCGACATGAAA
M285L	5′-AGCATGGTCGACCTGAAAACCGATATG
M289L	5′-ATGAAAACCGATCTGGCCGGCGCGGCG
M299L	5′-GTGCTCGGGGCGCTGGAAGCGATCGGC
M321L	5′-GCGACCGACAATCTGATCAGCGGCGAG
M400L	5′-AAAACGGGCGCGCTGACGAACAATGAG
M426L	5′-CTTGAAGCGTCGCTGGAAACCGGGGAG
M445L	5′-GGACACGCCATTCTGGGCGGGGCGTTC
M485L	5′-GCGACAGGCGTGCTGGTGCGCACGCTC

[a]Only the sense primer sequences are shown.

DNA methods

Conventional techniques for DNA manipulation were performed as described by Sambrook et al. (1989). Mutagenesis was done by the Quick Change method (Stratagene Co., La Jolla, CA, USA) according to the manufacture's protocol with a pair of complementary mutagenic primers (Table 1). DNA sequencing then confirmed the presence of the desired mutation in the selected transformants.

Expression and purification of wild-type and mutant enzymes

The recombinant *E. coli* M15 cells were grown at 37 °C in 250 ml of LB medium containing the above-mentioned antibiotics to an optical density at 600 nm of approximately 0.8. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM and the cultivation proceeded at 28 °C for 12 h. The cells were pelleted by centrifugation at 12,000 × *g* for 20 min at 4 °C, resuspended in 3 ml of binding buffer (5 mM imidazole, 0.5 M NaCl, and 20 mM Tris-HCl; pH 7.9), and disrupted by sonication. The extracts were clarified by centrifugation at 12,000 × *g* for 20 min at 4 °C and the resulting supernatants were mixed with Ni^{2+}-NTA resin pre-equilibrated with the binding buffer (5 mM imidazole, 0.5 mM NaCl, and 20 mM Tris-Hcl; pH 7.9). The His_6-tagged enzymes were eluted from the resin with a buffer containing 0.5 M imidazole, 0.5 M NaCl, and 20 mM Tris-HCl (pH 7.9).

Enzyme assay and kinetic characterization

LAP activity was assayed by monitoring the hydrolysis of leucine-*p*-nitroanilide (Leu-*p*-NA) (Lin et al. 2004). One unit of LAP activity is defined as the amount of enzyme that releases 1 μmol of *p*-NA per min at 55 °C.

The K_m and k_{cat} values were estimated by monitoring the hydrolysis of Leu-*p*-NA in the 0.5 ml reaction mixtures containing various concentrations of the substrate (0.2 to 6 mM) in 50 mM Tris-HCl buffer (pH 8.0) and a suitable amount of enzyme. The reaction mixtures were incubated at 55 °C for 10 min. Values of K_m and k_{cat} were calculated by fitting the initial rates as a function of soluble starch concentration to the Michaelis-Menten equation.

Chemical oxidation

To evaluate the oxidative inactivation of bkLAP, the wild-type enzyme (270 μg/ml) was incubated with up to 100 mM H_2O_2 in a 50 mM Tris-HCl buffer (pH 8.0) at 4 °C and 28 °C, respectively, for 30 min. Catalase (2,390 U/ml) was added to the sample to a final concentration of 1,330 U/ml to quench the remaining H_2O_2. Then, the solution (500 μl) was used for measurement of the residual activity under the standard conditions.

To determine the oxidative stability of mutant enzymes, protein concentrations were adjusted to 300 μg/ml with 50 mM Tris-HCl buffer (pH 8.0). The enzyme solutions were incubated with 50 mM H_2O_2 and kept at 28 °C for designated time periods. After quenching, 500 μl of the enzyme solution was withdrawn to determine the residual activity under the standard assay conditions.

Raman measurement

FT-Raman spectra of the enzymes were obtained by a Bruker RFS-100 FT-spectrophotometer (Bruker Optik GmbH, Lubeck, Germany). Continuous wave near infrared excitation at 1064 nm was provided by a diode laser pumped Nd:YAG laser (Coherent Lubeck GmbH, Lubeck, Germany), and the laser power focused on the sample position was about 50 mW. The scattered light from the sample was collected at 180° with an ellipsoidal mirror and was passed through the Michelson interferometer. A liquid nitrogen cooled GaAs detector was employed to provide superior signal-to-noise performance. Raman spectra were produced over the Raman shift 0–3500 cm^{-1}. Typically, 100 interferograms were coadded at 4 cm^{-1} resolution with a sampling time of about 3 minutes.

Electrophoresis and determination of protein concentrations

Polyacrylamide gel electrophoresis (PAGE) was performed in a vertical mini-gel system (Mini-Protean III system; Bio-Rad Laboratories, Richmond, CA, USA) with a 10% non-denaturing polyacrylamide gel. Electrophoresis was done at 4 °C and a constant voltage of 100 V for 4 h.

Sodium dodecylsulfate-PAGE (SDS/PAGE) with 4% polyacrylamide stacking and 10% polyacrylamide separating gels was performed using the Laemmli buffer system (Laemmli 1970). Before electrophoresis, the purified enzymes were mixed with 2 × SDS-sample buffer, heated at 100 °C for 5 min, and centrifuged at 12,000 × *g* for 10 min. Protein bands were stained with 0.25% Coomassie Brilliant Blue dissolved in 50% methanol-10% acetic acid, and destained in a 30% methanol-10% acetic acid solution.

Results and discussion

Oxidation of bkLAP by hydrogen peroxide

The purified bkLAP was evaluated for resistance to oxidative inactivation by hydrogen peroxide. As shown in Figure 1, the enzyme was barely affected by H_2O_2 at 4 °C. However, less than 12% of the LAP activity remained in the presence of 100 mM H_2O_2 at 28 °C, indicating that the performed temperature had a profound effect on the oxidative inactivation. Interestingly, bkLAP was still active when it was incubated with 100 mM H_2O_2 at 4 °C for 1 h (data not shown). It is well known that most of the biologically active proteins are sensitive to chemical oxidation, but the α-amylase of alkaliphilic *Bacillus* sp. KSM-K38 has been shown to be highly resistant to oxidative inactivation even at 1.8 M H_2O_2 (Hagihara et al. 2001). A mutant subtilisin of *Bacillus amyloliquefaciens* is also resistant to inactivation by 1 M H_2O_2 (Estell et al. 1985). These unusual oxidatively stable characteristics suggest these enzymes have great potential for commercial applications.

Both native and chemically modified bkLAP undergo analogous mobility on non-denaturing gel

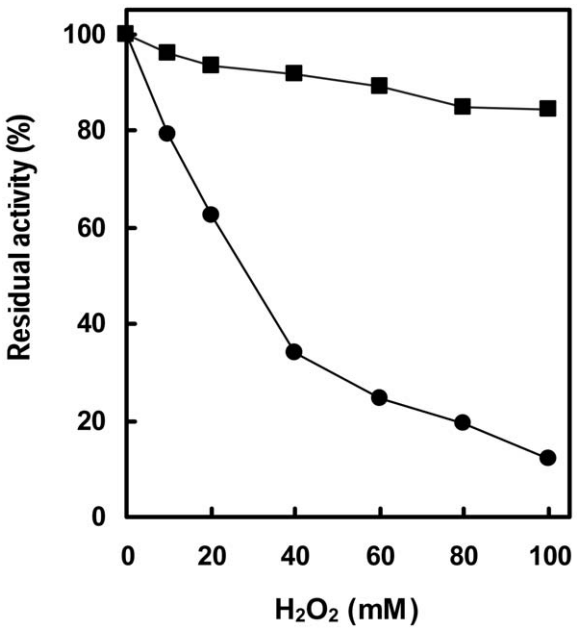

Figure 1. Inactivation of the purified bkLAP by H_2O_2. The enzyme was incubated for 30 min in the presence of fresh H_2O_2 and 50 mM Hris-HCl buffer (pH 8.0) at 4 °C (■), and 28°C (●), respectively. Residual activity is expressed as a percent of a nontreated enzyme control. The data is representative of three measurements.

(data not shown). As reported by Gonzales and Robert-Baudouy (1996), group II LAP is a hexameric enzyme consisting of six identical subunits. Accordingly, it seems that the chemical treatment does not lead to the disassociation of the hexameric structure of bkLAP.

Raman spectroscopy has been widely used as a noninvasive and powerful technique in biological study, particularly for protein structural characterization. It offers invaluable information on the secondary structure and on the microenvironment of protein side groups (Thomas 1999; Pelton and Mclean 2000). Near IR FT-Raman was therefore employed to detect the structural change of bkLAP as affected by the chemical oxidation. The Raman spectra clearly indicated that the secondary structure of the enzyme showed little change and remained dominantly in a disordered arrangement as evidenced by the vibrational stretch of amide I at 1664 cm^{-1} and amide III around 1250 cm^{-1} (Figure 2). Based on eletrophoretic mobility and Raman spectroscopy, it is evident that the chemical oxidation does not cause a serious change on the protein structure of the enzyme.

Purification and kinetic characterization of wild-type and mutant enzymes

In order to obtain an oxidatively stable enzyme, we replaced each of the twelve methionine residues in the primary sequence of bkLAP with leucine by directed mutagenesis. After verification of the gene sequence, the mutated plasmids were transformed into *E. coli* M15 for IPTG-induced gene expression. SDS/PAGE analysis of the total cell proteins revealed that the mutant enzymes had an apparent molecular mass of approximately 54 kDa (data not shown), in agreement with that of His_6-tagged bkLAP (Lin et al. 2004). To determine the specific activity of each variant, the enzymes were purified to homogeneity by metal chelate column chromatography (data not shown). M68L, M224L, and M229L exhibited a comparable catalytic activity relative to the wild-type enzyme. A significant increase in enzymatic activity was also found in M400L, M426L, M445L, and M485L. Notably a dramatically reduced LAP activity was observed in M285L, M289L, and M321L (Table 2). To understand further the basis for variation in specific activity, the kinetic constants, k_{cat} and K_m, were determined for wild-type and mutant enzymes. As shown in Table 2, M285L, M289L, and M321L were severely compromised catalytically with more than 81% decrease in k_{cat}, indicating that these three substations significantly affect catalytic function of the enzyme. M400L, M426L, M445L, and M485L exhibited a similar K_m value coupled with an increased catalytic efficiency (k_{cat}/K_m) relative to the wild-type enzyme. The remaining variants had a catalytic efficiency comparable to that of the wild-type bkLAP. In the hexameric bovine lens LAP (blLAP), its catalytic domain has a triple-layered structure consisting of a central eight-stranded β-sheet sandwich between five α-helices on each side and two zinc ions are located near the edge of the eight-stranded, saddled-shaped β-sheet with the six active sites situating in the interior of the hexamer (Burley et al. 1992). On the basis of X-ray structure and kinetic studies, a detailed reaction mechanism for LAP has been proposed and specific residues have been implicated in metal coordination and in catalysis (Kim and Lipscomb 1993; Sträter and Lipscomb 1995; Sträter et al. 1999). It is interesting that the amino acid residues essential for zinc coordination and catalysis are conserved in bkLAP (Lin et al. 2004). Comparison of thirteen M17 family proteins revealed that Met-285 and Met-289 of bkLAP are conserved in the aligned enzymes and

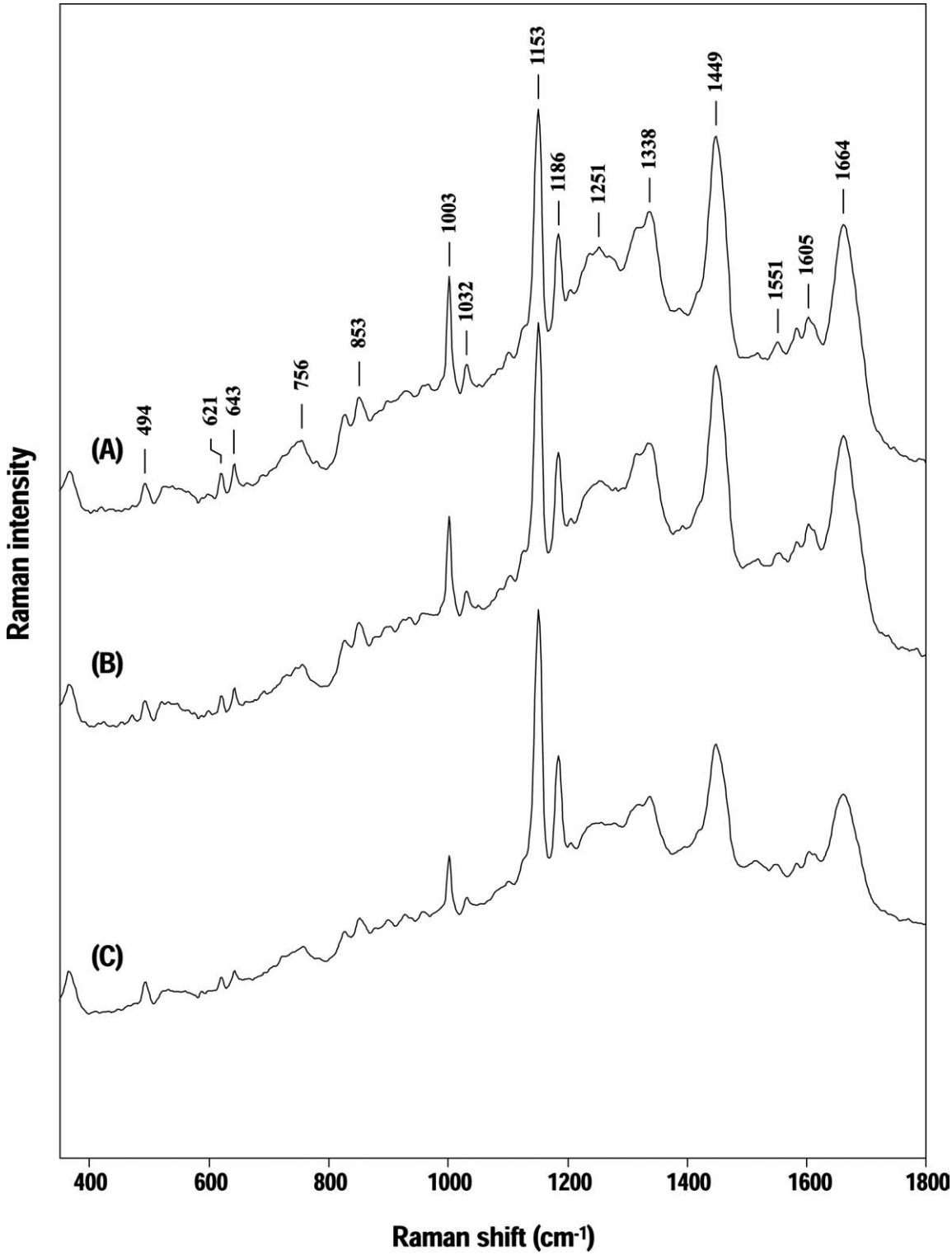

Figure 2. FT-Raman spectra of native and oxidized forms of bkLAP. A, native bkLAP; B, bkLAP treated with 50 mM H_2O_2; C, bkLAP treated with 100 mM H_2O_2.

Table 2. Specific activities and kinetic parameters of wild-type and mutant bkLAPs[a]

Enzyme	Specific activity (U mg^{-1})	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m (mM^{-1} min^{-1})
Wild-type	91.6 ± 3.7	2.1 ± 0.2	378.5 ± 5.9	180.2
M68L	109.8 ± 8.2	2.5 ± 0.4	342.3 ± 5.1	136.9
M224L	76.3 ± 5.2	1.7 ± 0.1	300.5 ± 3.8	176.8
M229L	116.8 ± 6.1	2.2 ± 0.3	372.4 ± 7.4	169.2
M282L	51.4 ± 2.9	2.6 ± 0.5	287.2 ± 5.8	110.5
M285L	7.3 ± 1.6	3.1 ± 0.2	34.6 ± 1.3	11.2
M289L	13.8 ± 3.1	2.6 ± 0.3	54.2 ± 2.5	20.8
M299L	64.2 ± 7.3	2.1 ± 0.4	210.1 ± 9.3	100.1
M321L	27.1 ± 2.8	1.9 ± 0.1	71.7 ± 5.1	37.7
M400L	267.1 ± 12.2	1.7 ± 0.1	514.1 ± 9.7	302.4
M426L	163.6 ± 10.1	2.2 ± 0.2	723.1 ± 8.5	328.7
M445L	378.2 ± 17.1	1.8 ± 0.1	655.3 ± 7.9	364.1
M485L	185.9 ± 10.6	1.7 ± 0.1	666.5 ± 8.6	392.1

[a]The data represent the average values of three measurements.

situate nearby the catalytically important residues respective to blLAP Lys-262 and Asp-273 (Figure 3). To our knowledge, there is only one enzyme in which methionine residue has been shown to function in the catalytic cycle (Kachurin et al. 1995). Furthermore, substitutions of Met-285 and Met-289 in the enzyme with leucine did not abolish the LAP activity (Table 2). These results suggest that these two residues may be not essential for the catalytic function, but they could play a role in the local structural rearrangements of more critical residues in the active site. However, we are currently unable to explain why M321L retains only 30% activity relative to that of the wild-type enzyme. Structural determination of *B. kaustophilus* LAP should help to clarify the role of this residue.

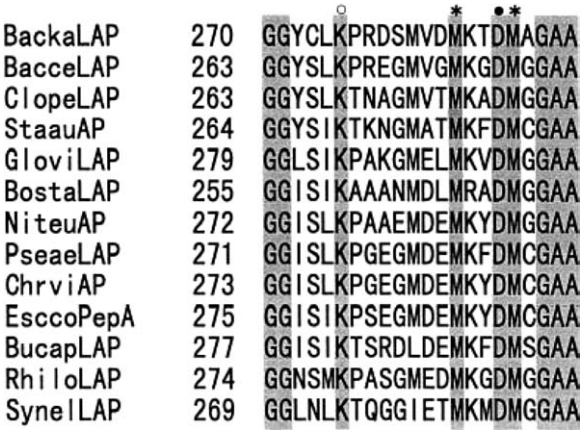

Figure 3. Sequence comparison of M17 family enzymes surrounding the conserved methionine residues. Amino acid residues, expressed in one-letter codes, are numbered from the translational methionine of each enzyme. Strictly conserved residues are in gray boxes and the conserved Met-285 and Met-289 residues of bkLAP are indicated by an asterisk. The residues essential for zinc-binding and catalytic activity are marked by closed and open circles, respectively. BackaLAP, *Bacillus kaustophilus* LAP (TrEMBL Q7X2C8; BacceLAP, *Bacillus cereus* LAP (TrEMBL Q816E3); ClopeLAP, *Clostridium perfringes* LAP (Swiss-prot O32106); GloviLAP, *Gloeobacter violaceus* LAP (TrEMBL Q7NHC6); BostaLAP, *Bos taurus* LAP (Swiss-prot P00727); NiteuAP, *Nitromonas europaea* AP (TrEMBL Q82X54); PseaeLAP, *Pseudomonas aeruginosa* LAP (Swiss-prot O68822); ChrviAP, *Chromobacterium violaceum* AP (TrEMBL Q7NTY9); EsccoPepA, *Escherichia coli* PepA (TrEMBL Q7X4V5); BucapLAP, *Buchnera aphidicola* LAP (TrEMBL Q89AG2); RhiloLAP, *Rhizobium loti* LAP (Swiss-prot Q984S1); SynelLAP, *Synechococcus elongates* (Swiss-prot Q8DI46).

Oxidative stability of wild-type and mutant enzymes

The sensitivities of wild-type and three selected mutant enzymes toward hydrogen peroxide were evaluated. As shown in Figure 4, the inactivation of these enzymes was time-dependent. The wild-type enzyme was stable after a 5 min treatment, while less than 30% of the original activity retained when the incubation prolonged to 30 min. M321L also exhibited a lesser activity under the same condition, indicating that subtle change in the size and functional group of the amino acid side chain at this position has little effect with respect to inactivation of the enzyme by hydrogen peroxide. However, a significant increase in the oxidative stability was observed in M224L and M299L (Figure 4). The remaining variants, except M285L and M289L, also exhibited an increased oxidative stability (data not shown). Although the roles of methionine residues in proteins have not been well defined, a review of available studies leads to the

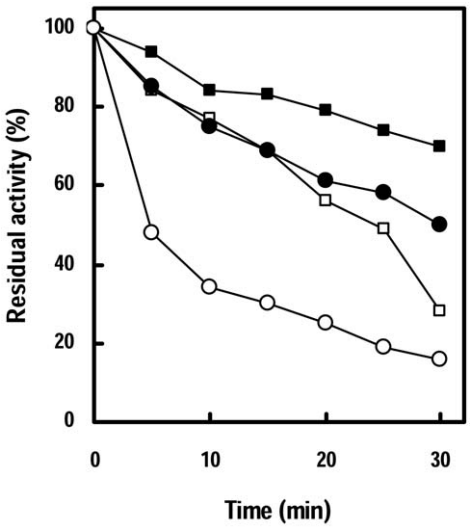

Figure 4. Oxidative stability of the purified wild-type and three selected mutant enzymes. For the determination of oxidative stability, the wild-type bkLAP (□), M224L (●), M299L (■), and M321L (○) were treated with fresh H_2O_2 as described in Materials and methods. Residual activity is expressed as a percent of a nontreated enzyme control. The data is representative of three measurements.

conclusion that methionine may function as an antioxidant and play a key role in the regulation of cellular metabolism (Levine et al. 2000). A potential strategy for protecting a protein against methionine oxidation is to replace those residues with amino acids with oxidatively resistant side chains. This has been achieved with subtilisin (Estell et al. 1985), D-amino acid oxidase (Ju et al. 2000), *N*-carbamoyl D-amino acid amidohydrolase (Chien et al. 2002), and α-amylase (Lin et al. 2003). Generally, replacement of the critical methionine residues in bkLAP with oxidation-resistant leucine also increased the oxidative stability of the enzyme. In *E. coli* glutamine synthetase, the eight oxidizable methionine residues are arrayed along the border of the active site to quench reactive oxygen radicals (Levine et al. 1996). With regard to this finding, it is possible that Met-68, Met-224, Met-229, Met-282, Met-299, Met-400, Met-426, Met-445, and Met-485 of bkLAP might play a role in the protection of other functionally important residues from oxidative damage.

In summary, this work demonstrates that the oxidative stability of bkLAP can be improved by replacement of the critical methionine residues with Leu. The experimental data could have significant benefit in the establishment of stable enzymes used for industrial purposes.

Acknowledgements

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