Cloning and Expression of the Creatinase Gene from *Flavobacterium* sp. U-188 in *Escherichia coli*

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The gene coding for creatinase (creatine amidinohydrolase, EC 3.5.3.3) was isolated from *Flavobacterium* sp. U-188. The primary structure of creatinase deduced from the nucleotide sequence showed a protein (molecular weight, 42, 651) composed of 378 amino acids. The creatinase gene was over-expressed in *Escherichia coli* under the control of the *lac* promoter and the amount of this enzyme was over 20% of the soluble protein in the cell.

Creatinase (creatine amidinohydrolase, EC 3.5.3.3) catalyzes the hydrolysis of creatine to sarcosine and urea. In a previous paper¹⁾ we developed an enzymatic method for measurement of creatinine using creatinase in combination with creatininase (creatinine amidohydrolase, EC 3.5.2.10) and sarcosine oxidase (EC 1.5.3.1) from newly isolated bacteria. *Flavobacterium* sp. U-188 produces a large amount of creatinase when creatinine or creatine is added to the medium as an inducing substrate. However, the use of the expensive inducer is a disadvantage in industrial production.

In this paper we report the cloning and expression of the gene coding for creatinase from *Flavobacterium* sp. U-188 in *Escherichia coli*.

Materials and Methods

Bacterial strains and media. *Flavobacterium* sp. U-188²⁾ and *E. coli* were grown in LB medium³⁾ at 30°C and 37°C, respectively. *E. coli* HB101⁴⁾ was used as a cloning host for construction of a gene library and *E. coli* JM109⁵⁾ was used for sequencing and gene expression. For induction of the creatinase gene regulated by the *lac* promoter, JM109 harboring a recombinant plasmid was grown in LB medium containing 1 mM IPTG (isopropyl- β -D-thiogalactoside). Ampicillin and tetracycline were added at the concentration of 100 μ g/ml and 10 μ g/ml, respectively.

Chemicals. Restriction and modification enzymes were purchased from Takara Shuzo Co., Ltd. (Kyoto, Japan) or Boehringer Mannheim GmbH. Peroxidase was purchased from Toyobo Co., Ltd. (Osaka, Japan) and sarcosine oxidase was obtained from Seishin Pharmaceutical Co., Ltd. (Tokyo, Japan). IPTG and 4-aminoantipyrine (4-AA) were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and 2,4-dichlorophenolsulfonate (2,4-DCPS) was from Nacalai Tesque Co., Ltd. (Kyoto, Japan). Creatine was purchased from Sigma Chem. Co., Ltd. (St. Louis, U.S.A.). Other chemicals used were of reagent grade.

Preparation of chromosomal DNA and construction of gene library. Chromosomal DNA was prepared from *Flavobacterium* sp. U-188 basically by the method of Saito and Miura⁶⁾ except the addition of a 2-methoxyethanol extraction step introduced by Kirby⁷⁾ before phenol extraction as the DNA could not be digested with restriction enzymes without this step. The chromosomal DNA fragments in sizes of 4–9 kb generated by the partial digestion with *Sau*3AI were collected by agarose gel electrophoresis⁸⁾ and ligated to the dephosphorylated-*Bam*HI cut pBR322. *E. coli* HB101 was transformed with the ligated DNA and plated on a LB agar plate containing 100 μ g/ml of ampicillin.

Screening procedure. Each transformant was grown in 5 ml of LB medium containing 100 μ g/ml of ampicillin and 1% creatine at 37°C for 24 hr. Cultures were centrifuged and cells were resuspended in 1 ml of 10 mM phosphate buffer (pH 7.7) containing 1% creatine and 20 μ l of toluene. After incubation at 37°C for 1 hr, 0.1 ml each of 0.2% DCPS, 0.07% 4-AA, 70 u/ml of peroxidase and 40 u/ml of sarcosine oxidase were added to the mixture. If

creatinase is produced in a transformant and hydrolyzes creatine to sarcosine, the reaction mixture turns red.

DNA sequencing. Fragments of DNA were subcloned into pUC118 or pUC119⁹⁾ and their sequences were identified by a modified dideoxy chain termination method¹⁰⁾ using a 7-DEAZA Sequencing Kit (Takara Shuzo Co., Ltd.).

Assay of creatinase activity. All cells used for the enzyme assay were grown to early stationary phase, disrupted by ultrasonication, and centrifuged to obtain the crude extract. The activity of creatinase was measured by the amount of urea from creatine by the method of Watt and Chrisp.¹¹⁾ The reaction mixture consisting of 0.1 ml of the crude extract, 0.1 ml of 0.3 M potassium phosphate buffer (pH 7.7), and 0.8 ml of 0.1 M creatine was incubated at 37°C for 10 min and the reaction was stopped by adding 2 ml of a color reagent containing 50% ethanol, 1% *p*-dimethylaminobenzaldehyde, and 1 M HCl. The optical density was measured at 435 nm. One unit of creatinase was defined as the amount of enzyme which liberates 1 μ mol of urea under these conditions.

SDS-polyacrylamide gel electrophoresis and Western blot analysis. For SDS-polyacrylamide gel electrophoresis (SDS-PAGE)¹²⁾ the cell pellets from 10-ml culture were resuspended in 2 ml of water, disrupted by ultrasonication and centrifuged at 12,000 rpm for 10 min. The supernatant mixed with an equal volume of sample buffer was boiled for 5 min and 20 μ l of the mixture was put on a 10% gel. For measurement of the amount of creatinase produced in a recombinant strain, a gel was stained with Coomassie brilliant blue R-250 and analysed by a densitometer (model 800 fiber optic scanner, Kontes, Vineland, N.J.). Western blots¹³⁾ were reacted with creatinase-specific antiserum and stained with horseradish-peroxidase-conjugated second antibody using a Bio Rad Immun-Blot Assay Kit (Bio Rad Laboratories, Richmond, CA.). Creatinase specific antiserum prepared from rabbits immunized with the creatinase purified from *Flavobacterium* sp. U-188 was a gift from Y. Nakajima.

Results

Cloning of the creatinase gene

Out of 3200 transformants with ampicillin resistance and tetracycline sensitivity, three strains showed creatinase activity. The recombinant plasmids containing 1.9-kb, 5.2-kb or 4.3-kb inserts of DNA from *Flavobacterium* sp. U-188 were designated pKLS501, pKLS511, and pKLS521. The restriction maps of these insert DNA in the recombinant plasmids are shown in Fig. 1. The 1.9-kb fragment

Fig. 1. Restriction Maps of the Recombinant Plasmids Carrying Creatinase Gene.

Only the insert segments from *Flavobacterium* sp. U-188 are shown. Restriction sites: Bg, *Bgl*II; S, *Sal*I; Sm, *Sma*I; X, *Xho*I.

Fig. 2. Western Blotting Analysis of Extracts from *Flavobacterium* sp. U-188 (lanes 1 and 2), *E. coli* HB101 (lane 3), HB101 Harboring pKLS501 (lanes 4 and 5) and Creatinase Purified from *Flavobacterium* sp. U-188 (lane 6).

The samples in lanes 2, 3, and 5 were the extracts of cells grown in the medium containing creatine. The arrow indicates the position of creatinase.

of pKLS501 was common to the other recombinant plasmids.

The creatinase activity of HB101 harboring pKLS501 was 0.0084 u/ml culture and Western blot analysis showed that HB101 cells carrying the recombinant plasmid pKLS501 constitutively produced creatinase, while *Flavobacterium* sp. U-188 did not produce the enzyme at all in the absence of creatine (Fig. 2).

Sequencing of the creatinase gene

The nucleotide sequence of the 1.9-kb insert of DNA in pKLS501 was identified and found to contain a single open reading frame of

GATCGGGCGCATGGAGCCAACTCCAGGAGCCGATCGAGCTGGACGAGCTGGCGAGGTCATCCAGCTGTCGGCCGCTCACCTCGAGCGGCTGTTTCGC 100
 AAATACCTGGCTGCTCGCCCTCGCGCTACTACATGAGCTCGCTGCTGGCGCCGCCAGCTGTTGCGCCAGACCGAGTTCCCGGTGTCAGGTCGCG 200
 GCGAAGCTGGGGTTTCATTTCGGCGCAGACCTTCTCCAAAGAGTTACCGCGAACATTTTCGGCATGCCCGCAGTGGCGAGCGTCTCCAGCGATCCCTGCTGT 300
 GTCGCGCAGCGGTGCATCAGGCGCAGCTTCCCGTTGGCGCGCTCTCGTATGGCGGCTTTTTTTTGGCCGTTCTGTCAGCCCTCTCAGGCGCTGCAGCC 400
 CAGTGGCGAGCGGCTCGCCGCTCGCGTATGAGCTTATGACCCCGCCCGCGCGTTCGGCAGCCAGCGAATCTGACCAACTTGAGGTCGGATACGTG 500
 CAAAAGCGCCCGCGGGTTCCTGTTGCAATCTCTTCAACGGCAGCCAGTCCGGTGTGCCAGATCAGTGAATAACAGAGGGTTCCTGTCATGCAAAATGCC 600
SmaI MetGlnMetPr
 CAAAACACTCCGCGATCCGCAACGGTGAAGAGTCAAGTCCACCTTCTCCGCGCAGGAATACGCCAACCGCCATGCCAAGTGGCGCGCCAGCTGGCGGCC 700
 oLysThrLeuArgIleArgAsnGlyGluLysValLysSerThrPheSerAlaGlnGluTyrAlaAsnArgHisAlaLysLeuArgAlaHisLeuAlaAla
 GAGAACATCCATGCTGCGGTCTTCACTCGTACCACAACATCACTACTACTCCGACTTCTGCTACTGCTCTTCCGTGCTCCCTACCGCCCTGCTCGTGA 800
 GluAsnIleAspAlaAlaValPheThrSerTyrHisAsnIleAsnTyrTyrSerAspPheLeuTyrCysSerPheGlyArgProTyrAlaLeuValValT
 CGCAGCAGCAGTGCATCAGCATCAGCGCCAACTCGACGGTGGCCAGCGTGGCGCGCAGCGTGGGCACCGACAACATCGTCTACACCGACTGGCAGCG 900
 hrGlnAspAspValIleSerIleSerAlaAsnIleAspGlyGlyGlnProTrpArgArgThrValGlyThrAspAsnIleValTyrThrAspTrpGlnAr
 CGACAACACTACTTCGTGCGCATCCAGCAGCGCTGCCAGGGCGCGTGGCATCGGTATCGAGCAGCAGCCACTGAACCTGCAGAACCCGACAGAGTGGCC 1000
 gAspAsnTyrPheValAlaIleGlnGlnAlaLeuProArgAlaArgArgIleGlyIleGluHisAspHisLeuAsnLeuGlnAsnArgAspLysLeuAla
 GCGCGCTACCGCGATGCCAGCTGGTGGACGTCGCGCGCGCGCTGCATCGGCATCGCGATGATCAAGTCGGCTGAAGACGAGAGATGATCGCCATGGCG 1100
 AlaArgTyrProAspAlaGluLeuValAspValAlaAlaCysMetArgMetArgMetIleLysSerAlaGluLeuHisGluMetIleArgHisGlyAla
 CGCGCGTCGCGACATCGGTGGCGCGCGCATCGTCGAGCGCTCGGTGATCAGGTGCGCGACTACGAAGTGGCGCTGCACGCGACCGAGGCCATCGTGGC 1200
 laArgValAlaAspIleGlyGlyAlaAlaIleValGluAlaLeuArgAspGlnValProGluTyrGluValAlaLeuHisAlaThrGlnAlaMetValAr
 CGCCATTGCGGAGACCTTCGACAACGTCGAGCTGATGGACACCTGGACCTGGTTCCAGTGGCGCATCAACACCGACGGCGCGCACACCGCGTGAACACC 1300
 galAlaIleAlaGluThrPheAspAsnValGluLeuMetAspThrTrpThrPheGlnSerGlyIleAsnThrAspGlyAlaHisAsnProValThrThr
 CGCAAGGTCAACAAGCGGACATCTCAGCTGAACTGCTTCCCGATGATCGCGGTTACTACACCGCGCTGGAGCGTACCTGTCTTCCTCGACCACTGCT 1400
 ArgLysValAsnLysGlyAspIleLeuSerLeuAsnCysPheProMetIleAlaGlyTyrTyrThrAlaLeuGluArgThrLeuPheLeuAspHisCysS
 CGCAGCACCACCTGCGCATGTGGCAGGCCAACGTCGAAGTCAGCAGCGCGCGCTCAAGCTGATCAACCGGGCATCGCGTGCAGGACATCGCCAAAGGA 1500
 erAspAspHisLeuArgMetTrpGlnAlaAsnValGluValHisGluAlaGlyLeuLysLeuIleLysProGlyMetArgCysSerAspIleAlaLysGl
 GCTCAACGAGATCTTCTCAAGCAGATCTGCTGCACTACCGACCTTCGGTTACGGTCACTCCTTCGGGACCTCTAGCCACTACTACGCGCGCGAGCGG 1600
 uLeuAsnGluIlePheLeuLysHisAspLeuLeuGlnTyrArgThrPheGlyTyrGlyHisSerPheGlyThrLeuSerHisTyrTyrGlyArgAspGly
 CCTGGAGCTGCGCGAGGACATCGACACCGTTCTGGAGCGCGCATGGTGGTCTCCATGGAGCCGATGATCATGCTGCCCGAAGCTCGTCCGCGCGCGCGT 1700
 ProGlyAlaAlaArgGlyHisArgHisArgSerGlyAlaGlyHisGlyGlyLeuHisGlyAlaAspAspHisAlaAlaArgArgSerSerGlyArgArgT
 GGCGTACCGCGAGCAGCAGATCCTGATCGTCAACGAGAACGGTGGCGAGAACATCACCAGTCCCGTACGGTCCGGACAGAACATCATCCGCAATAA 1800
 rpArgThrAlaSerThrThrSer
 CCGGTGCGCGCGCAGGGTCAAGACCTGCGCGGCAACTGGCTGCAACACGCAAAACCGAACCTCCGTGCCACAGCGCTGGAAGCCCGCTTCCCGTGA 1900
 CTGCGGTTTCACTCCCGCTGCCGAACACAACAAGACCAACAGCGCATGATC

Fig. 3. The Nucleotide Sequence of the Insert DNA of pKLS501 and the Deduced Amino Acid Sequence of Creatinase.

The arrows indicate inverted repeats. The putative ribosome binding site is underlined.

1134 bp (Fig. 3). The protein deduced from the nucleotide sequence was composed of 378 amino acids with a molecular weight of 42,651. The deduced amino acid sequence was consistent with the sequence of 15 amino acids from the amino terminal of creatinase of *Flavobacterium* sp. U-188 found by a Protein Sequencer (Applied Biosystems Inc. Type 470A) (data not shown).

There was a typical ρ -independent terminator, a GC-rich dyad symmetry element followed by a run of T residues,¹⁴⁾ in the region between positions 338 and 366. As subcloning of the 1.9-kb fragment of pKLS501 downstream of a *lac* promoter or a *trp* promoter did not affect the creatinase productivity, this putative terminator was perhaps functional in *E. coli* (data not shown).

Subcloning and overexpression

The 864-bp *ClaI*–*SmaI* fragment of pKLS 501 was replaced by the 216-bp *PvuII*–*SmaI* fragment containing the *lac* promoter of pUC19 to construct pKLS508 (Fig. 4) in which the creatinase gene was expressed under the control of the *lac* promoter and operator. The creatinase activity of JM109 harboring pKLS508 was 1.80 units/ml culture with 1 mM IPTG, which is 50-fold higher than that without IPTG. SDS-PAGE analysis of the crude extract showed that the amount of creatinase produced by JM109 carrying pKLS508 in the presence of IPTG reached about 20% of the soluble protein in the cell (Fig. 5).

Fig. 4. Construction of pKLS508 for the Overexpression of Creatinase.

Abbreviations: *lacP*, *lac* promoter; B, *Bam*HI; Bg, *Bgl*II; C, *Cla*I; E, *Eco*RI; Pv, *Pvu*II; S, *Sma*I.

Fig. 5. SDS-PAGE Analysis of Crude Extracts from Recombinants.

lane 1, HB101 (pKLS501); lane 2, JM109 (pKLS508) not induced; lane 3, JM109 (pKLS508) induced by IPTG. The arrow indicates the position of creatinase.

Discussion

A large amount of creatinase is required for diagnostic use because of the low affinity of creatinase for creatine ($K_m = 40$ mM).²⁾ Furthermore the use of the expensive inducing

substrate is a high barrier for the commercial production of the enzyme.

In this paper we resolved these problems by cloning the creatinase gene and constructing an overproducing strain. High productivity of the creatinase by the recombinant strain led to simplification of the purification process and improvement of the quality of the enzyme. Moreover, the creatinase gene was subcloned into the Sleeper bacteriophage vector^{15,16)} and expressed by heat induction without addition of the inducer (data not shown).

The properties of the recombinant creatinase were not appreciably different from those of the *Flavobacterium* creatinase (data not shown). Therefore, we guess that the creatinase was produced as an active form in *E. coli* JM109 harboring pKLS508.

E. coli HB101 harboring pKLS501 expressed very low activity of creatinase although it was considered to contain the entire creatinase gene. This result suggests that a promoter of *Flavobacterium* was not functional in *E. coli*. The high production of the pKLS508 clone regulated under a *lac* promoter may result from the highly conservative sequence of the ribosome binding site and codon usage, minor tRNAs¹⁷⁾ of *E. coli* being almost never used.

The molecular weight of the creatinase predicted from the nucleotide sequence is 42,651 and this is consistent with that estimated by SDS-PAGE of the creatinase produced by JM109 carrying pKLS508. Recently, Hoeffken *et al.*¹⁸⁾ identified the primary and crystal structures of the creatinase from *Pseudomonas putida*, which has properties corresponded very closely to those of the creatinase from *Flavobacterium*. The amino acid sequence comparison between *Flavobacterium* creatinase and *Pseudomonas* creatinase showed about 80% homology (data not shown).

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References

- 1) M. Suzuki and M. Yoshida, *Clin. Chim. Acta*, **143**, 147 (1984).
 - 2) M. Suzuki and N. Saito, Japan Tokkyo Koho, 77-8395 (Mar. 9, 1977).
 - 3) R. W. Davis, D. Botstein and J. B. Roth, "Advanced Bacterial Genetics," Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1980, p. 201.
 - 4) H. W. Boyer and D. Roulland-Dussoix, *J. Mol. Biol.*, **41**, 459 (1969).
 - 5) J. Messing, *Gene*, **33**, 103 (1985).
 - 6) H. Saito and K. Miura, *Biochim. Biophys. Acta*, **72**, 619 (1963).
 - 7) K. S. Kirby, *Biochem. J.*, **64**, 405 (1956).
 - 8) R. C. A. Yang, J. Lis and R. Wu, in "Methods in Enzymology," Vol. 68, ed. by R. Wu, Academic Press, New York, 1979, pp. 176-182.
 - 9) J. Vieira and J. Messing, in "Methods in Enzymology," Vol. 153, ed. by R. Wu and L. Grossman, Academic Press, New York, 1987, pp. 3-11.
 - 10) S. Mizusawa, S. Nishimura and F. Seera, *Nucl. Acids Res.*, **14**, 1319 (1986).
 - 11) G. Watt and J. D. Chrisp, *Anal. Chem.*, **26**, 452 (1954).
 - 12) U. K. Laemmli, *Nature*, **227**, 680 (1970).
 - 13) W. H. Burnette, *Anal. Biochem.*, **112**, 195 (1981).
 - 14) W. H. Holmes, T. Platt and M. Rosenberg, *Cell*, **32**, 1029 (1983).
 - 15) E. Nakano and T. Masuda, *Agric. Biol. Chem.*, **46**, 313 (1982).
 - 16) Y. Koyama and E. Nakano, *Agric. Biol. Chem.*, **54**, 1315 (1990).
 - 17) T. Ikemura, *J. Mol. Biol.*, **151**, 389 (1981).
 - 18) H. W. Hoeffken, S. H. Knof, P. A. Bartlett, R. Huber, H. Moellering and G. Schumacher, *J. Mol. Biol.*, **204**, 417 (1988).
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