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Isolation and Analysis of the *argG* Gene Encoding Argininosuccinate Synthetase from *Corynebacterium glutamicum*

KO, SOON-YOUNG[1], SEI-HYUN YIM[1], HEUNG-SHICK LEE[2], AND MYEONG-SOK LEE[1]*

[1]*Department of Biological Science, Sookmyung Women's University, Seoul 140-742, Korea*
[2]*Department of Biotechnology, Korea University, Jochiwon 339-700, Korea*

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Abstract The *argG* gene of *Corynebacterium glutamicum* encoding argininosuccinate synthetase (EC6345) was cloned and sequenced. The gene was cloned by heterologous complementation of an *Escherichia coli* arginine auxotrophic mutant (*argG*$^-$). The cloned DNA fragment also complements *E. coli argD*, *argF*, and *argH* mutants, suggesting a clustered organization of the genes in the chromosome. The coding region of the *argG* gene is 1,206 nucleotides long with a deduced molecular weight of about 44 kDa, comparable with the predicted size of the expressed protein on the SDS-PAGE. Computer analysis revealed that the amino acid sequence of the *argG* gene product had a high similarity to that of *Mycobacterium tuberculosis* and *Streptomyces clavuligerus*. Two conserved sequence motifs within the ArgG appear to be ATP-binding sites which correspond to 2 of the 3 conserved regions found in sequences of all known argininosuccinate synthetases.

Key words: *Corynebacterium glutamicum*, *argG*, argininosuccinate synthetase, heterologous complementation

Corynebacterium glutamicum, an aerobic Gram-positive organism, is widely used for the industrial production of amino acids and nucleic acids [1]. For industrial utilization, most genetic engineering has been conducted on this organism of the subgroup of corynebacteria. Advances have been made in the understanding of the biochemical pathways and genetic systems in this organism. Many genes involved in amino acid biosynthesis have been cloned and some of these genes have been used to design engineered strains with improved amino acid production [4, 5, 14, 18, 22, 36, 37]. For similar purpose, study on the genes for arginine biosynthesis in *C. glutamicum* has been started.

*Corresponding author
Phone: 82-2-710-9418; Fax: 82-2-6352-9418;
E-mail: mslee@sookmyung.ac.kr

In prokaryotes, arginine is synthesized from glutamate in eight enzymatic steps [7–9]. The pathway of arginine biosynthesis has two alternative patterns. In members of the families *Enterobacteriaceae* and *Bacillaceae*, acetylornithine is deacetylated via acetylornithine deacetylase (AODase) encoded by the *argE* gene [32, 34]. In *Neisseria gonorrhoeae*, members of the families *Pseudomonadaceae*, cyanobacteria, and photosynthetic bacteria, the acetyl group from acetylornithine is recycled by ornithine acetyltransferase (OATase) encoded by the *argJ* gene [2, 11, 32].

There are distinct organizations of the arginine biosynthetic gene clusters in different organisms. In *E. coli*, arginine is synthesized by enzymes encoded by the *argECBH* and *carAB* (encoding carbamolphosphate synthetase) gene clusters and other genes, *argF* and *argG*, scattered around the chromosome [6]. In *Bacillus subtilis*, *argABCDEF* or *I* and the genes *carAB* are located in a 12 kb DNA fragment [21, 23, 24]. In *Mycobacterium tuberculosis* and *Streptomyces clavuligerus*, the genes related to the pathway are clustered in the order of *argCJBDFRGH* and *argCJBDFGH* respectively [3, 35]. In *Pseudomonas aeruginosa* [10] and *Neisseria gonorrhoeae* [25], all arginine biosynthetic genes appear to be scattered in the chromosome with only *carA* and *carB* forming an operon. In *C. glutamicum*, the clustered organization of *argCJBDF* was reported [4, 5, 28, 29, 31].

The seventh step of the arginine biosynthetic pathway involves argininosuccinate synthetase (EC6345) which is encoded by the *argG* gene. Little is known about the structure of the gene and characteristics of the enzyme in *C. glutamicum*. The role of this enzyme in the arginine biosynthetic pathway is generally known to catalyze the ATP-mediated condensation of citrulline and aspartate to form argininosuccinate in the penultimate step of the arginine biosynthetic pathway.

Here, the cloning and analysis of the *argG* gene encoding argininosuccinate synthetase from *C. glutamicum* is described.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Growth Conditions

E. coli and *C. glutamicum* strains used in this study are listed in Table 1. *C. glutamicum* and *E. coli* strains were grown on a rotary shaker in Luria-Bertani (LB) medium at 30°C and 37°C, respectively. *E. coli* arginine auxotrophs were used for the complementation. M9 minimal medium was supplemented with all auxotrophic requirements other than arginine for the complementation analysis of *arg* mutants by *C. glutamicum* genomic library. Ampicillin was added at a final concentration of 50 μg ml^{-1}.

DNA Manipulation

Plasmid DNA from *E. coli* was isolated by the alkaline lysis method or with QIAGEN plasmid midi kit (QIAGEN, Germany). Agarose gel electrophoresis, DNA restriction, alkaline phosphatase treatment, and ligation were performed following classical protocols [30]. *E. coli* strains were transformed by electroporation with an electroporator (Invitrogen, U.S.A.) according to the manufacturer's recommendations.

Cloning of the *argG* Gene by Complementation

For cloning the gene, a *C. glutamicum* ASO19 genomic DNA library constructed into the *E. coli-Corynebacterium* shuttle vector pMT1 was utilized [20]. The *argG*-containing clones were screened by heterologous complementation. *E. coli* arginine auxotrophic mutant cells, as shown in Table 1, were transformed with the DNA library and plated onto the M9 minimal medium containing ampicillin, and appropriate supplements. Transformed colonies were isolated and screened for the plasmid content. To confine the *argG* region, the purified plasmids termed as pRG1 and pRG2 were digested by several restriction enzymes. The DNA was separated on Tris-acetate buffer by using agarose gels and analyzed.

Subcloning of the *argG* Gene

When pRG1 was introduced into an *E. coli argG* mutant, the cells grew in minimal agar plates. Thus, various deletion derivatives of pRG1 were constructed, and their ability to complement *argG* mutant was checked. Based on the complementing ability, the 3.0 kb *Kpn*I-*Kpn*I fragment, the 2.5 kb *Kpn*I-*Xba*I fragment, and the 3.8 kb *Eco*RI-*Xba*I fragment were isolated by a QIAquick gel extraction kit (QIAGEN, Germany) and were inserted into pBluescript II KS(+). These subfragments were termed as pRG11, pRG12, and pRG13 (See Table 1).

DNA Nucleotide Sequence Determination and Computer Analysis

According to the sequencing strategy shown in Fig. 5, subfragments were cloned into the pBluescript II KS(+).

Table 1. Bacterial strains and plasmids used in this study.

Strains or plasmids	Relevant characteristics	Reference
1. Strains		
C. glutamicum		
ASO19	Spontaneous rifampin-resistant mutant of ATCC 13059	17
E. coli		
DH5α	F^- ϕ80d*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *deo*R *end*Al *hsd*R17(r_k, m_k) phoA *sup*E44 *thi*-1 *rec*A1 *gyr*A96 *rel*A1 λ^-	BRL
BL21(DE3)	F^- *ompThsd*S_B($r_B^-m_B^-$) *gal dcm* (DE3)	Novagen
CGSC6176	F^- λ^- *argA81::Tn10 IN(rrnD-rrnE)1*	CGSC
CGSC5421	Hfr *lacZ43*(Fs) λ^- *relA1 argB62 thi-1*	CGSC
CGSC1184	F^- *galT23* λ^- *IN(rrnD-rrnE)1 argC24*	CGSC
CGSC4538	Hfr *thr-1 leuB6 proA30 lacZ4 glnV44* (AS) λ^- *rpsL8 argD37 thi-1 mu+*	CGSC
CGSC4896	Hfr *argF58 relA1 spoT1 metB1*	CGSC
CGSC5961	F^- *argG78 rpsL257*	CGSC
CGSC5359	F^- *galT23 LAM*$^-$*IN(rmD-rmE) argH56*	CGSC
2. Plasmids		
pMT1	Shuttle vector; Ap^r (*E. coli*), Km^r (*C. glutamicum*)	17
pBluescriptIIKS(+)	Ap^r *lacZ*	Stratagene
pET28a	Expression vector: Km^r	Novagen
pRG1	pMT1 with 5.4 kb insert carrying *argG*; Ap^r	This work
pRG2	pMT1 with 6.6 kb insert carrying *argG*; Ap^r	This work
pRG11	pBluescript II KS(+) with 3 kb *Kpn*I-*Kpn*I fragment; Ap^r	This work
pRG12	pBluescript II KS(+) with 2.6 kb *Kpn*I-*Xba*I fragment; Ap^r	This work
pRG13	pBluescript II KS(+) with 3.8 kb *Eco*RI-*Xba*I fragment; Ap^r	This work
pET-RG	pET28a with 1.2 kb *Eco*RI-*Hin*dIII fragment; Km^r	This work

Superscripts indicate resistance. Ap, ampicillin; Km, kanamycin; CGSC, *E. coli* Genetic Stock Center, Yale University, New Haven, Conn, U.S.A.

DNA was sequenced on a genetic analyzer (Perkin Elmer, U.S.A.), using the enzymatic method based on the DNA-sequencing procedure of Sanger. T3, T7, or M13 reverse primer was used to obtain the DNA sequence from one end of the subclones. The newly determined sequence was used to make synthetic 20-mer oligonucleotides (BRL, U.S.A.), and the cycle was repeated until the entire insert was covered. Analysis of nucleotide and amino acid sequences was performed using a DNAMAN computer program (Lynnon Biosoft, U.S.A.).

Expression of the ArgG Protein

For the identification of the ArgG protein, *E. coli* BL21(DE3) and pET28a vector (Novagen, U.S.A.), a bacteriophage T7 promoter expression plasmid, was used [12, 13, 16]. To

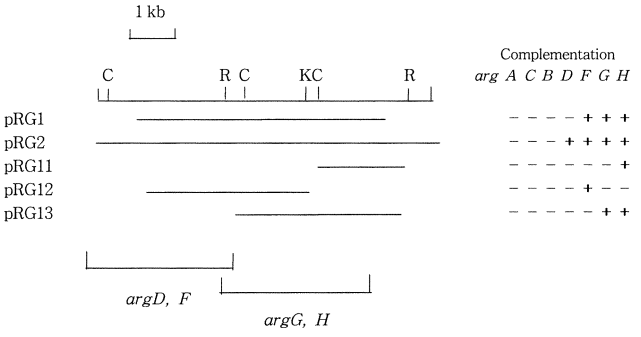

Fig. 1. Restriction map of the pRG1 and pRG2 inserts, and deletion analysis of the insert DNA. Abbreviation: C(*Cla*I), R(*Eco*RI), K(*Kpn*I).
Complementation of *E. coli* arginine auxotrophs by specific restriction-generated fragments subcloned into the plasmid, pBluescript II KS(+).

```
MT.....NRIV.LAYSGGLDTTVAIPYLKKMIDGEVIAVSLDLGQGGENM   44
-s.....e-vi.---------s---swig-etgr--v--ai-------h-   44
v-.....e-v-.---------s---gwiaeetga-----av-v-----dl   44
......mskvltslpage-vgiaf.------s--vawmrdKgavpctytadigqydepdi   53
-mhtlpmskvltslptge-vgiaf.------s--vawmrdKgavpctytadigqydepdi   59
--tilkh......lpvgq--giaf.------sa-llwmrqKgavpyaytanlgqpde-dy   53
-q.....ek-av---------scclkl-edkynyk-vs-av-v--peddl   45
-a.....kkva.---------s-c--i--ekygydevvtisvdvgqp-ee   44

DNVRQRALDAGAAESIVVDAKDEFAEEYCLPTIKANGMYMKQYPLVSAISRPLIVKH...  101
-vi------c--v-av----r-----g-----vln-al--dr----------...  101
-vi-k---ac--v-ae-a----------a----ar--dr------l---a----...  101
as-ps--say--eitrl--craalv--glaalacgafhirsggrpyfnttplgravtgtl  113
as-pd--kty--evarl--crrrwsrrgwgaltcgafhirsggrayfnttplgravtgtl  119
-aipr--mey--enarli-crkqlva-giaaiqcgafhnttggltyfnttplgravtgtm  113
kepeek-kkl-vlkhyti---e---vd-ifra----al-eg.---st-la----aik...  101
ikradakaekisnkhyti---e--vkd-vf-l-----s-eg.-vmgts-a----a-k...  100

LVEAGKQFNGTHVAHGCTGKGNDQVRFEVGFMDTDPNLEIIAPARDFAWTRDKAIAFAEE  161
--a-arehg-gi----------asla-d--vl--v--y----e--------  161
--a-a-khdasi----------a-iqalg-d-kc---v--y-m--------c-a  161
--r-mledgvqiwgd-s-f----ie--yryqllan-h-r-yk-wl-adfvtelggrkems  173
--r-mleddvqiwgd-s-f----ie--yryqllan-q-r-yk-wl-adfvtelggrkems  179
--a-m-edgvniwgd-s-y----ie--yrygll-nae-q-yk-wl-tdfidelggrhems  173
ia-la-eigadais----------f---svirtka-ei-----v--lnl--tee-qy-k-  161
v---arkegaval----------l---av-rq--mdv..---m-emnl--ewe-dy-k-  158

NNVPIEQSVKSPFSIDQNVWGRAIETGYLEDLWNAPTKDIYAYTEDPALGNA.PDEVIIS  220
-ai--nvtkr----------v---f--h----------tinwgv-----vg  221
kql--attk---y------f-------f---i----ie--fe--sn--eprea.---v--  220
ewllahglpyrdsteKaystdan-wgath-aktlehldtgietvdpimgvrfwdps-e-a  233
ewlvahdfpyrdsteKaystdan-wgath-aktlehldtgvetv-pimgvrfwdps-e-a  239
efmiacgfdykmsveKaystdsnmlgath-akdleylnssvKivnpnmgvKfwdes-k-p  233
kgi--pvdlek-----e-l---s--g-i--npmietp-ecf-w-v--kiakdeeey-e-e  221
hgi-v-vtksk-w-v-e-i-s-s--g-r---psfv-pee-few-ts-ekapeq-rv-d-g  218

FEGGKPVSIDGRPVSVLQAIEELNRRAGAQGVGRLDMVEDRLVGIKSREIYEAPGAIALI  280
--r-v---v--k---m-a----------v----------mv--  281
-as-v--a------t-----qq--e------i--i----------v----------  280
t-dvtvgfeq-----ingkefasavdlvmeanaiggrhglgmsdqienr-i--ksrgiye  293
p-dvtigfdq----tingkefasavdlvmeatpsaagtamgmsdqienr-i--ksrgiye  299
a-evtvrfeq-h--alngktfsddvemmleanriggrhglgmsdqienr-i--ksrgiye  293
-ka-v--a-n-qkfdainv-k-a-kl--rn----v-ii---vl-l----n--c----l--  281
--a-v---l--ekm-gyafvkkm-ei--en----t--i---vl-l-a--n--h-a-tv-r  278

KAHEALEDVTIERELAR.YKRGVDRRWAEEVYDGLWFGPLKRSLDAFIDSTQEHVTGDIR  339
t--te--h--l----g-.f--qt-q----l------ys---aa-e--vak-----s-ev-  340
t--qe--n--v------.f--q-eq--g-l-------s----a--g--eeanqq------  339
apgm--lhivy--lvnaihnedtlaayhn-grrlgrlmyeg-w--pqalmir-slqrwvg  353
apgm--lhaay--lvnaihnedtlaqyht-grplgrlmyeg-w--pqslmir-slqrwvg  359
apgm--lhiay--l-tgihnedtieqyhahgrqldrllyqg-wf-sqalmlrdslqrwva  353
t--k---qlvls--.elvf-em--sky-dli-k---he--rhd----v-k---rmn-iv-  340
qpnsrplrnssltrselkf-ki--dq-s-la-y--vde--yad-n----ks--r---ivk  338

MVLHAGSITINGRRSSHSLYDFNLATYDTGDTFDQTLAKGFVQLHGLSSKIANKRDREAG  399
l---g-h-av-----ae----------e--s---sa-r---yv------l-ar--lr  398
-t----tavvt----er-----d----------sk-q--idifa--a---a---lv  397
tavtgevtlrl.--gedysildttgpafsyhpdklsmertedsaf-pvdr-gqltm-nld  412
savtgevtlrl.--gedysildttgpafsyhpdklsmertedsaf-pvdr-gqltm-nld  418
sqitgevtlel.--gndysilntvsenl-ykperl-me--.dsvfspddr-gqltm-nld  411
ak-yk--lr-v--e-eca--qe-mvsfenk-mdqreivgmvkfhglqaaifesl-nk  397
vr-yk-al--la-s-pna--sed-vsf-sqtidqkgsegfakyhgfqarmyrkvm-kq  396

NN.....  401
.....
.....
.....
iadsrarleqyvglglvgtphptpigaaqaaatgligamdeggaeaiasrgeatdeetml  472
iadsrakleqyagsrfigtanp.pigaaqaaatgligampeggaqaiasrgevsaddell  477
itdtreklfgyaktgllsssaasgvpqvenlenkgq.....  447
.....
```

.....		*C. glutamicum*	
.....		*M. tuberculosis*	(71%)
.....		*S. clavuligerus*	(67%)
.....		*S. lavendulae*	(15%)
draamesgtd	482	*S. coelicolor*	(15%)
draamesgtd	482	*E. coli*	(21%)
.....		*M. vannielii*	(43%)
.....		*M. barkeri*	(38%)

Fig. 2. Comparison of the deduced amino acid sequence of *argG* with the amino acid sequences from different organisms. Some gaps (dot) were introduced to obtain the maximal alignment. Dashed line represents an identical amino acid among all organisms compared in this work. Conserved amino acid regions in argininosuccinate synthetase proteins are underlined. The percentages at the end of the sequences indicate degrees of identity to the *C. glutamicum* argininosuccinate synthetase.

clone the target gene in the bacteriophage T7 promoter expression plasmid, pET28a, the insert DNA was amplified by using PCR with two synthetic oligonucleotide primers, 5'-GGA- GCAGAATTCATGACTAACCGCATCGTT and 5'-CCTTAAGCTTTTCCGTGCTGTTCCATGTGG. These primers were constructed to carry two enzyme sites, *Eco*RI and *Hin*dIII, at both ends of the insert. pET28a vector was digested with *Eco*RI and *Hin*dIII, and extracted by using a QIAquick gel extraction kit (QIAGEN, Germany). The amplified 1.2 kb insert DNA was also digested with two endonucleases and ligated in the linearized pET28a to generate pET-RG. *E. coli* strain BL21 (DE3), a lysogen bearing the bacteriophage T7 polymerase gene under control of the *lac* UV5 promoter, was transformed with this recombinant plasmid termed pET-RG. The overnight culture of BL21 containing this plasmid in LB medium containing kanamycin (30 μg/ml) was grown at 37°C to OD_{600} of approximately 0.5. The gene was induced with 0.1 mM IPTG (isopropyl-β-D-thiogalactoside) for 2.5 h. Total cell protein samples were run on a SDS-polyacrylamide gel electrophoresis followed by staining with Coomassie Brilliant Blue. A suspension of cells containing the vector alone was used as a control.

RESULTS AND DISCUSSION

Cloning of the *argG* Gene from *C. glutamicum* by Complementation

Recombinant plasmids complementing *argG* gene defects in *E. coli* were selected from the *C. glutamicum* genomic library by heterologous complementation. A *C. glutamicum* ASO19 genomic library was previously constructed in *Corynebacterium-E. coli* shuttle vector pMT1 [20]. This DNA library was used for the transformation of *E. coli argG* auxotrophic mutant cell.

Ampicillin-resistant transformants were selected by plating them on LB medium containing ampicillin and screened for complementation of the arginine auxotrophy on M9 minimal medium in the absence of arginine. All of the transformed colonies showed the arg^+ phenotype, i.e. they grew fluently on M9 minimal medium when retransformed into the the *E. coli argG* auxotroph. The plasmid content was analyzed by several restriction endonuclease digestions. As a result, two clones were obtained which contained plasmids, termed as pRG1 and pRG2. Recombinant plasmid DNA pRG1 containing the 5.4 kb insert was able to complement other *E. coli* arginine auxotrophs, *argD*, *argF*, and *argH* (Fig. 1). This suggests a clustered organization of the three genes in the chromosome. Previously, the clustered organization of *argJBD* was reported in *C. glutamicum* [29].

From these results and restriction analysis of the pRG1, the clustered organization of *argCJBDFGH* is inferred. This organization of arginine biosynthetic genes is similar to those of different organisms. The *argCJBDF* of *Bacillus subtilis* and the *argCJBDFRGH* of *Mycobacterium tuberculosis* are known to be clustered on the chromosomal DNA [3, 15, 23].

Sequence Analysis of the *C. glutamicum argG* Gene

Three subfragments were used for the nucleotide sequencing based on the sequencing strategy shown in Fig. 1. Analysis of the cloned DNA sequence showed a complete ORF (nt145 - nt1347). The sequence of this ORF shows strong homology to that of the *argG* gene from different organisms, confirming that this corresponds to the complete coding region of *argG* gene with a total G+C content of 55%. This analysis also reveals that the most probable translation start codon (ATG) of the ORF is preceded by the consensus Shine-Dalgarno box, AAGGAG (data not shown).

The degree of identity of the *C. glutamicum argG* gene to the corresponding sequences of the *M. tubeculosis*, *S. clavuligerus*, *M. vannielii*, *E. coli*, and *S. coelicolor* were 71, 67, 43, 21, and 15%, respectively (Fig. 2). Comparison of the amino acid sequences of argininosuccinate synthetase from other organisms indicates that the protein (401 amino acids) encoded by the *argG* (44,152 Da) gene has two motifs, AHGCTGKGN and RAGAQGVGR (amino acid

	16
E. coli	IAFSGGLDTSAAL
S. cerevisiae	LAYSGGLDTSVIL
M. vanielii	LAYSGGLDTSCCL
M. barkri	LAYSGGLDTSVCI
S. clavuligerus	LAYSGGLDTSVAI
C. glutamicum	LAYSGGLDTTVAI
	127
E. coli	GDGSTYKGNDIERFY
S. cerevisiae	SHGCTGKGNDQIRFE
M. vanielii	SHGATGKGNDQFRFE
M. barkri	AHGCTGKGNDQLRFE
S. clavuligerus	AHGCTGKGNDQVRFE
C. glutamicum	AHGCTGKGNDQVRFE
	264
E. coli	NRIGGR-HGLGMSDQIENRIIEAKSRGIYE
S. cerevisiae	ASNLARANGVGRIDIVEDRYINLKSRGCYE
M. vanielii	NKLAGR-NGVGRVDIIEDRVLGLKSRENYE
M. barkri	NEIAGE-NGVGRTDMIEDRVLGLKARENYE
S. clavuligerus	NERAGA-QGIGRIDMVEDRLVGIKSREVYE
C. glutamicum	NRRAGA-QGVGRLDMVEDRLVGIKSREIYE

Fig. 3. Conserved amino acid regions in argininosuccinate synthetase proteins. Numbers refer to the position of the corresponding amino acid residue in the *E. coli* sequence. The underlined sequences are the possible nucleotide-binding sites. The amino acid sequences are aligned by introducing gaps indicated by dashes.

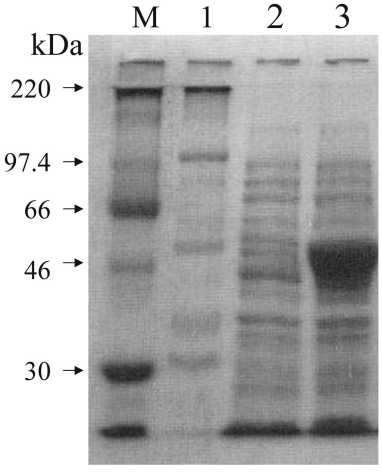

Fig. 4. Identification of the *argG* product by SDS-PAGE. The proteins were detected by staining with Coomasie brilliant blue. M, size marker; lane 1, uninduced cells of pET28a; lane 2, uninduced cells of pET-RG; lane 3, 4 h-induced cells of pET-RG with IPTG.

363–371 and 494–502), which appear to be ATP-binding sites (Fig. 2) [19, 27, 33, 35]. These sequences correspond to 2 of the 3 conserved regions found in all known argininosuccinate synthetase sequences (Fig. 2; Fig. 3). This observation of the putative ATP-binding region of the enzyme is consistent with the fact that the enzyme catalyzes the ATP-mediated condensation of citrulline and aspartate to form argininosuccinate in the arginine biosynthetic pathway. In addition, there was another conserved region, LAYSGGLDTTVAI, within the amino terminus of the protein, whose function is unknown (Fig. 3).

Identification of ArgG Protein

The *argG* gene fragment was amplified by PCR with two synthetic oligonucleotide primers. The expression plasmid pET-RG was constructed by ligating the amplified *argG* fragment with the predigested pET28a vector. *E. coli* was transformed with pET-RG. The expression of the ArgG protein was identified by SDS-PAGE. It is clear from Fig. 4 that a 48 kDa protein, which included 4 kDa of a pET vector region, was detectable in the clone containing pET-RG. This is consistent with the predicted size, 44 kDa, of the argininosuccinate synthetase deduced from the sequences of the *hisG* gene. Taken together, the *argG* gene encoding argininosuccinate synthetase from *C. glutamicum* has been cloned and analyzed, and further analysis on the properties of the gene and enzyme is in progress.

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