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論 文 内 容 要 旨

Introduction

Polyamines are essential low polycations molecules for cell growth and proliferation in all of living organism. The key intermediate in polyamine biosynthesis is putrescine ($H_2N(CH_2)_4NH_2$; Put), which is a polyamine and is further converted to higher polyamines like spermidine and spermine. Putrescine and spermidine are major polyamine in bacteria whereas spermidine is mainly found in plant and animals.

In the principle, two different biosynthetic pathways lead to the formation of putrescine: (i) the ornithine decarboxylase (ODC) catalyze direct formation of putrescine from ornithine and (ii) the arginine decarboxylase (ADC) pathway. In ADC pathway, arginine is first decarboxylased by ADC to form agmatine which is subsequently converted to putrescine. ODC and ADC were found mainly in animal and plant, respectively. However both pathways occur simultaneously in many bacteria.

Polyamines play a unique role in *Selenomonas ruminantiu* which is gram negative bacterium isolated from sheep rumen. Cadaverine and putrescine covalently link to the α-carboxyl group of D-glutamic acid residue of peptidoglycan and play important role in maintenance of the integrity of cell envelope. Both of them are formed from L-lysine or L-ornithine by lysine decarboxylase (LDC) which shows decarboxylation activity against both of L-lysine and L-ornithine. On the other, L-ornithine specific decarboxylase (ODC) is not found in *S. ruminantium*. Hence LDC is an important enzyme which supplies cadaverine and putrescine to form a unique peptidoglycan.

The production of LDC in *S. ruminantium* is higher regulated and is strictly linked to the growth phase of the bacterium, i.e. a drastic decrease of LDC activity occurred on late log phase of cell growth, which was due to the rapid degradation of LDC. Yabuki in our laboratory found that intracellular free cadaverine contact also decreased after late log phase. However, at the stationary phase, spermidine and spermine content were kept almost same level as those in late log phase. These facts suggested that another putrescine synthesis pathway which is active even at stationary phase such as ADC pathway should be

present in *S. ruminantium*. In order to clarify this hypothesis, DFML or DFMO, the specific inhibitor for LDC and ODC were used. Takatsuka and Kamio (2004) observed that *S. ruminantium* could grow normally even in the presence of 5 mM DFML or DFMO when 10 mM L-arginine was supplied. This case, the peptidoglycan contains putrescine instead of cadaverine. This result indicates *S. ruminantium* can form putrescine not only directly from ornithine but also from arginine. Recently, Liao in our laboratory found ADC in *S. ruminantium*. Purification and characterization of ADC and cloning of ADC gene from *S. ruminantium* chromosomal DNA was accomplished.

In general, putrescine was converts from agmatine in different 3 pathways. (Fig. 1) The first pathway, agmatinase (agmatine ureohydrolase, AUH, EC 3.5.3.11) encoded by *spe*B hydrolyzes agmatine and forms putrescine. This pathway was found in many bacterias such as *E.coli*, *Samonella typhi*, *Salmonella typhimurium*, *Klebsilla aerogenes*, *Bacillus subtilis*, *Bacillus anthracis* and also in human. The second pathway was found in lactic acid bacteria, *Enterococcus faecalis* (previously refered to as *Streptococcus faecalis*) and *Lathyrus sativus* seedling. Agmatine deiminase (agmatine iminohydrolase, EC 3.5.3.12) encoded by *agu*A converts agmatine to *N*-carbamoylputrescine (NCP). Then putrescine carbamoyltransferase (EC 2.1.3.6) catalyzes the phosphorolysis of *N*-carbamoylputrescine to carbamoylphosphate and putrescine. The last pathway consists of two enzymes; AguA and NCP-carbamoylputrescine. However, agmatine utilization pathways involved in putrescine synthesis in *S. ruminantium* have not been reported before.

Lioa *et.al.* found two open reading frames which showed high homology with those of agmatine deiminase and *N*-carbamoylputrescine amidohydrolase at downstream of ADC gene. In my doctor thesis, I investigated agmatine conversion to putrescine *in vivo* and *in vitro* to conform the existence of active of ADC pathway in *S. ruminantium*.

RESULTS AND DISCUSSION

S. ruminantium has ADC pathway.

S. ruminantium were cultured in CD medium in presence of 5 mM DFMO and ^{14}C-arginine. Cells were harvested and disrupted in the presence of 5%TCA, and radioisotope products in supernatant were analyzed by TLC. The result clearly showed that ^{14}C-arginine was incorporated into cell and converted to agmatine and putrescine. (Fig. 2) Therefore, it is strongly suggested that *S. ruminantium* has ADC pathway.

From the nucleotide sequence of *adc* and its the flanking regions, *orf6* and *orf7* which showed homology with proteins in agmatine utilization enzymes were found. The *orf6* gene of 1116 bp long encodes 372 amino acids protein (ORF6). ORF6 showed 54% and 43% identity with agmatine deiminase of *Pseudomonas aeruginosa* and *Streptococcus mutans*, respectively, and 48% identity with Peptidyl-arginine deiminase-like protein of *Listeria monocytogenes.* Predicted protein of ORF7 which composed of 292 amino acids has homology to *N*-carbamoylputrescine (NCP) amidohydrolase of *Pseudomonas aeruginosa* (57% identity) and showed high homology with carbon-nitrogen hydrolase family protein form *Streptococcus pneumoniae* (71% identity), *Erwinia carotovora* (58% identity), *Caulobacter crescentus* (57% identity) and *Burkholderia pseudomallei* (55% identity). These results suggested *S. ruminantium* has ADC pathway which comprised of ADC, agmatine deiminase and NCP-amidohydrolase.

Analysis activity of recombinant AguA and AguB

The *orf6* and *orf7* genes were amplified by PCR and cloned to pET and recombinant ORF6 and ORF7 proteins were expressed as a C-terminal His_6-tag fusion protein in *E.coli* Rosetta (DE3) cell. Recombinant proteins were purified using Ni^{2+} chelating affinity, DEAE-5PW and phenyl-5PW column chromatographies. Enzyme activities of recombinant ORF6 and ORF7 were investigated using agmatine and NCP as substrates, respectively.

ORF6 reaction product was analyzed by API 2000 mass spectrophotometer, and its molecular weight of 131.8 was same as chemical

synthetic NCP (Fig. 3). Both NCPs were used as substrates for ORF7 reaction, and converted to putrescine. From these results, ORF6 and 7 were confirmed as agmatine deiminase and *N*-carbamoylputrescine amidohydrolase, respectively. Therefore, I designated *orf6* and *7* as *aguA* and *aguB*, respectively.

AguA and AguB in *S. ruminantium*.

Expression of AguA and AguB proteins in *S. ruminantium* cell were determined by a western blotting. (Fig. 9) *S. ruminantium* produced both proteins in TYG medium. The possible polyamine synthesis pathway in *S. ruminantium* was depicted in Figure 4.

Properties of AguA and AguB

The molecular mass of AguA was calculated as 41,391.0 Da and determined as 45 kDa on SDS-PAGE and gel filtration column. Therefore AguA was estimated to be monomeric enzyme. On the other hand, AguB was calculated as 32,531.7 Da and determined to be 37 kDa on SDS-PAGE while 80 kDa on gel filtration column. Hence AguB was suggested to be a homodimer. (Fig. 5)

A very few information has been available about AguA and AguB from bacteria whileas both enzymes were mostly found in plant. Only purification of AguA and AguB from *P. aeruginosa* was report. AguA of *P aeruginosa* is homodimer of 43 kDa subunits (Table1) Most of AguA are homodimers such as from *Arabidopsis thaliana* (43 kDa subunit), *Zea mays* (44 kDa subunit) rice (95 kDa subunit) except from soy bean showed a monomer of 70 kDa. For AguB, mostly C-N hydrolases studied so far are forming homomultimers. AguB from *P. aeruginosa* and *A. thaliana* showed same as homohexamer of 33 kDa, and 35 respectively. (Table 1)

Figure 6 showed the effect of pH and temperature on AguA. The optimum pH and temperature were 6.5 and 45 °C, respectively. Optimum pH of AguA was same as enzyme from corn and soy bean. AguA from *P. aeruginosa* showed higher optimum pH at 8.0. Optimum temperature of AguA from *S. ruminantium* and *P. aeruginosa* were same. While AguA from corn showed high optimum temperature at 60 °C. The enzyme was stable at pH range from 6.0 to 9.0 and at the temperature below 40 °C for 30 min. The effects of pH and temperature on

AguB were shown in figure 7. Optimum pH is about 7.0 and the enzyme is stable from pH 6.0 to 8.0. More than 80% activity remained at this pH range. In the optimum pH range of AguB were same as found in other sources except AguB from *A. thaliana* showed high optimum pH at 8-9. The optimum temperature of AguB was 45 °C. AguB activity was stable until 70 °C. Optimum temperature of AguB from *S. ruminantium* was almost same as AguB from other sources.

The *Km* and *Vmax* valve obtained from a Lineweaver-Burk plot were 6.67 mM and 1.22 nmol/min/μg protein of AguA towards agmatine and 0.22 mM and 0.33 nmol/min/μg protein of AguB towards *N*-carbamoylputrescine, respectively. (Fig. 8) *Km* values of AguA and AguB from other sources were showed Table 1. AguA from rice seedling show high *Km*, 15 mM. While *Km* value of AguA from *P. aeruginosa, A. thaliana,* corn, maize shoot, soybean and cucumber were lower than 1 mM. *Km* value of AguB from *S. ruminantium* was almost same as that of AguB from *P. aeruginosa, A. thaliana* and corn.

The effects of metal ions on enzyme activities were shown in Table 2. Metal ions such as Al^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+}, Fe^{3+}, Ni^{2+}+ and Zn^{2+} inhibited the AguA activity at 1 mM except Mn^{2+} , which was the same as found in AguA from Soy bean, rice seedling and corn. On the other hand, AguB was strongly inhibited by Co^{2+}, Fe^{2+}, Mn^{2+} and Zn^{2+}. Iodoacetamide and Hg^{2+} were the strong inhibitors with AguA and AguB activity. These results suggested that cysteine involved in the catalysis of both enzymes.

Effect of site-directed mutagenesis for the activities of AguA and AguB.

Alignment of AguA among several homologues obtained from GenBank revealed that C 361 was strong conserved. On the other hand, triad conserved regions contain glutamate, lysine and cysteine among nitrilase family and K147, C153 and E 160, are also found in AguB from *S. ruminantium*. Therefore, role of these conserved cysteines was determined by site-directed mutagenesis. As the result, C361A mutant of AguA showed 20% of the activity of the wild type enzyme. C153A and E 160A mutated enzymes of AguB completely lost its activities. These results confirm the effect of thiol-modifying agent, iodoacetamide and Hg^{2+} to cysteine of the AguA and AguB.

Conclusions

1. Agmatine utilization pathway in *S. ruminantium* consists of 2 enzymes agmatine deiminase encoded by *aguA* and *N*-carbamolyputrescine amidohydrolase encoded by *aguB*.
2. Properties of AguA and AguB were characterized by using recombinant enzymes. It is suggested that AguA and AguB convert agmatine to putrescine in ADC pathway of *S. ruminantium*.
3. The conserved residues C 361 for AguA, C153 and E160 for AguB were important residues for enzyme activities.

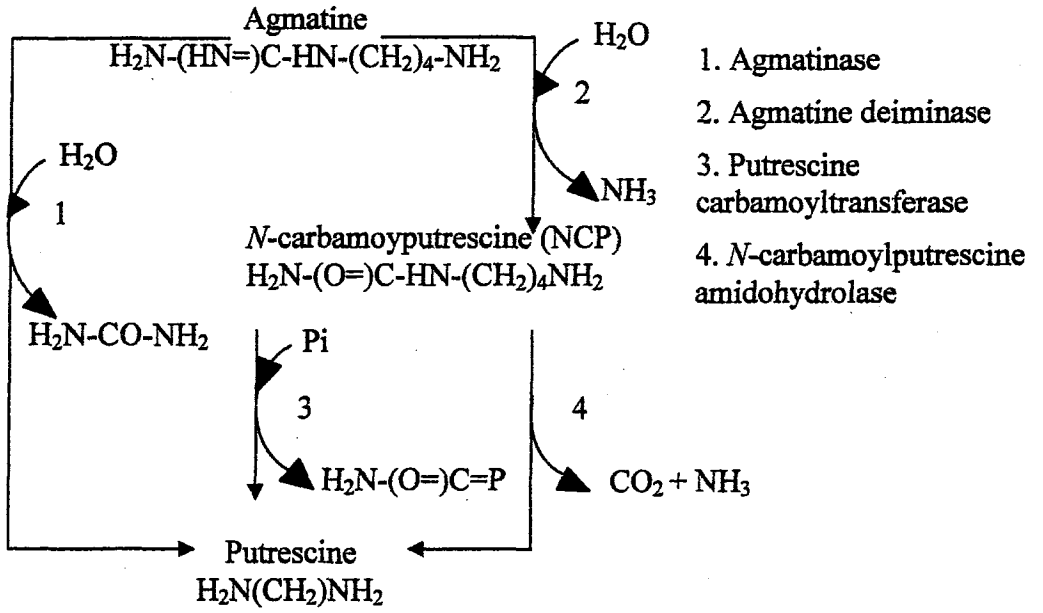


Fig. 1. Three distinct enzymatic systems for the biosynthetic conversion of agmatine to putrescine

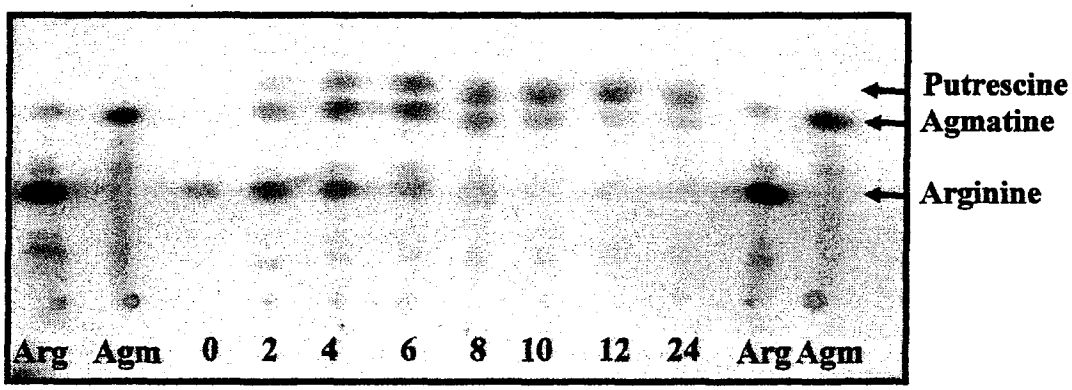


Fig. 2. Conversion of ^{14}C-arginine in *S. ruminantium* cell. ^{14}C-arginine incoorperated into cell and converted to agmatine and putrescine, respectively.

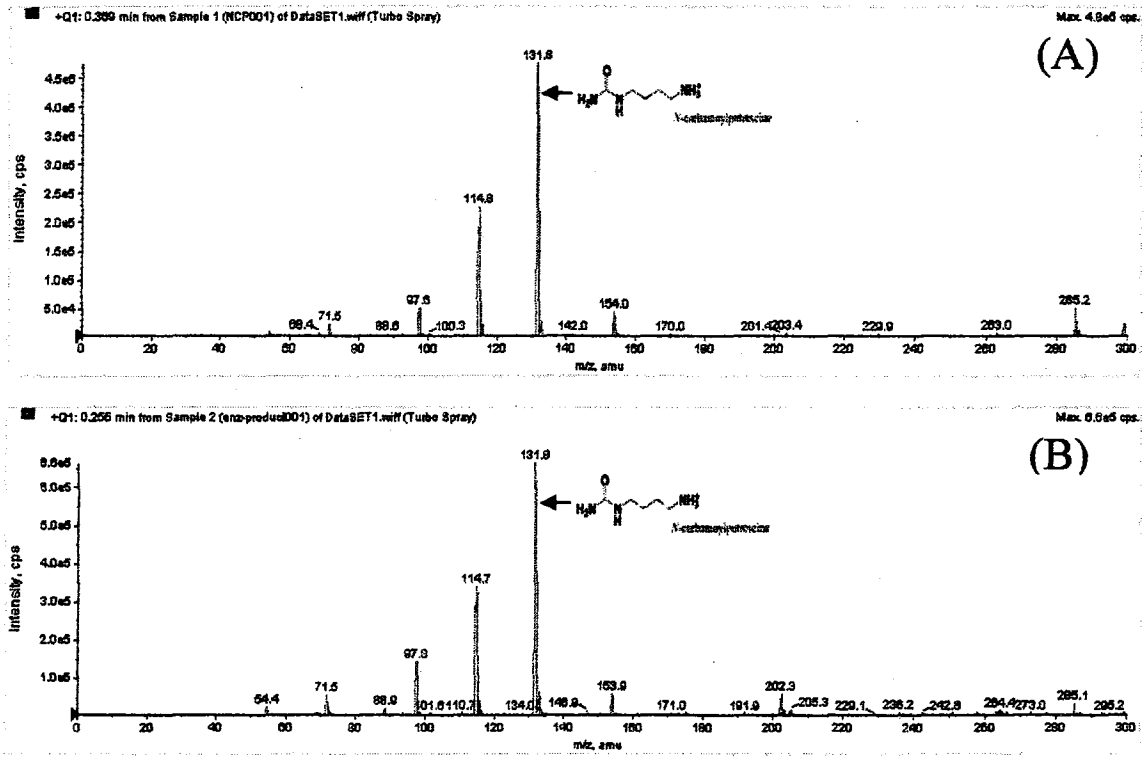


Fig. 3. Determination of *N*-carbamoylputrescine by Mass spectrophotometer (A) *N*-carbamoylputrescine from chemical synthesis (B) from AguA reaction.

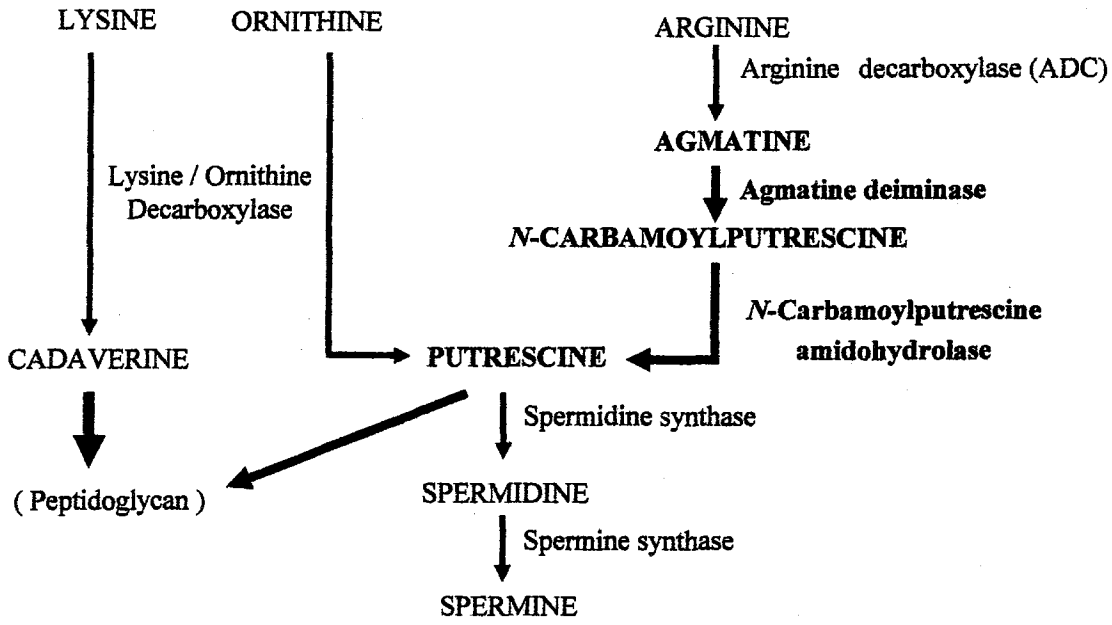


Fig. 4. Polyamine synthesis pathway in *S. ruminantium.*

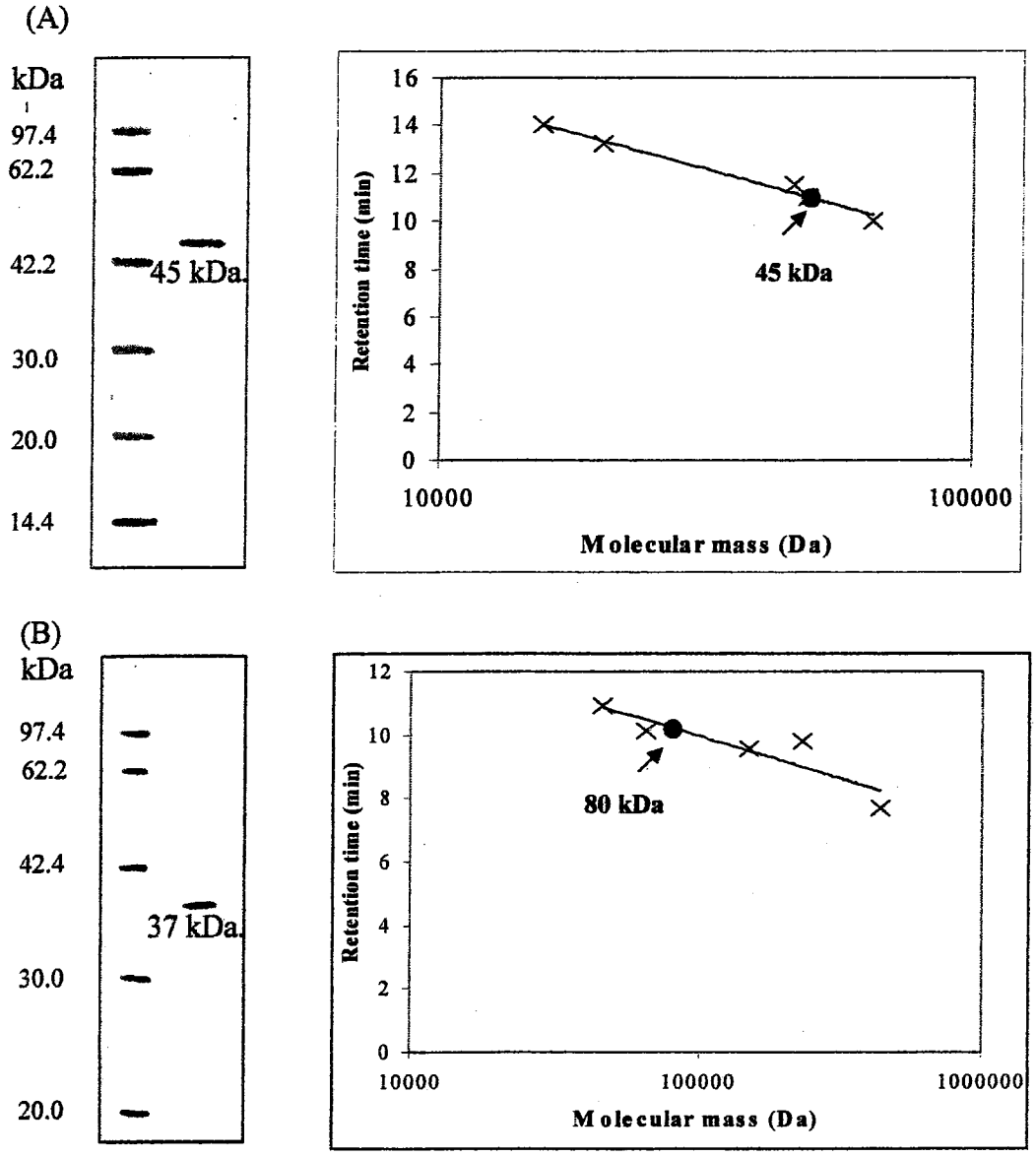
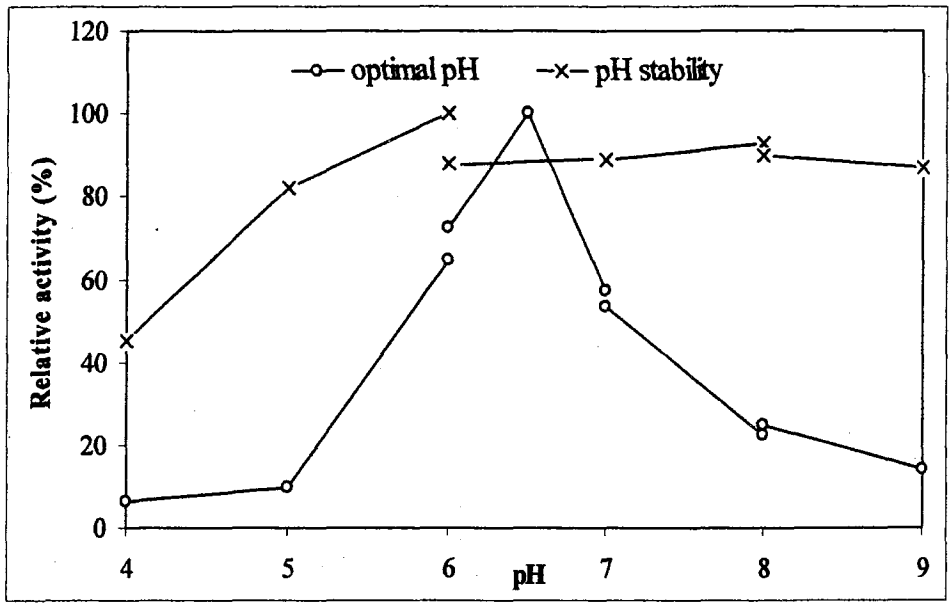


Fig.5. Molecular mass of purified AguA and AguB. AguA showed molecular mass of about 45 kDa on SDS-PAGE and gel filtration column (A). AguB was determined to be 37 kDa on SDS-PAGE and native structure 80 kDa on gel filtration column (B).

(A)



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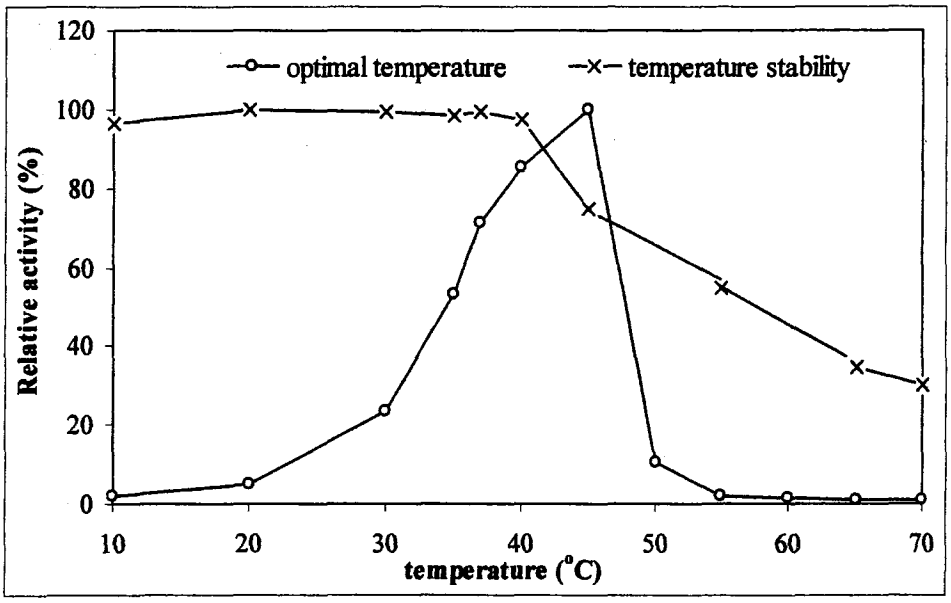
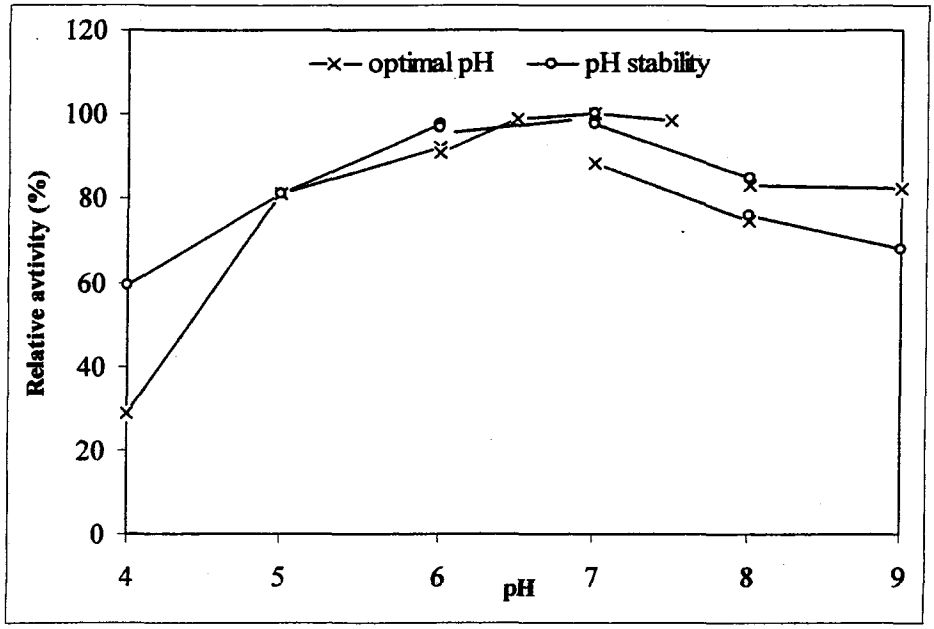


Fig. 6. Effect of pH (A) and temperature (B) on AguA activities. (A) AguA was incubated at 37 °C in 50 mM buffer of each pH for 30 min and the activity was measured. A maximum activity was taken as 100%. In case of pH stability, the enzyme was treated at each pH in 50 mM buffer at 45 °C for 30 min and remaining activity was measured under the standard condition. (B) AguB For pH stability, the enzyme was treated at each pH in 50 mM buffer at 37 °C for 30 min, followed by assaying the remaining activity under standard condition.

(A)



(B)

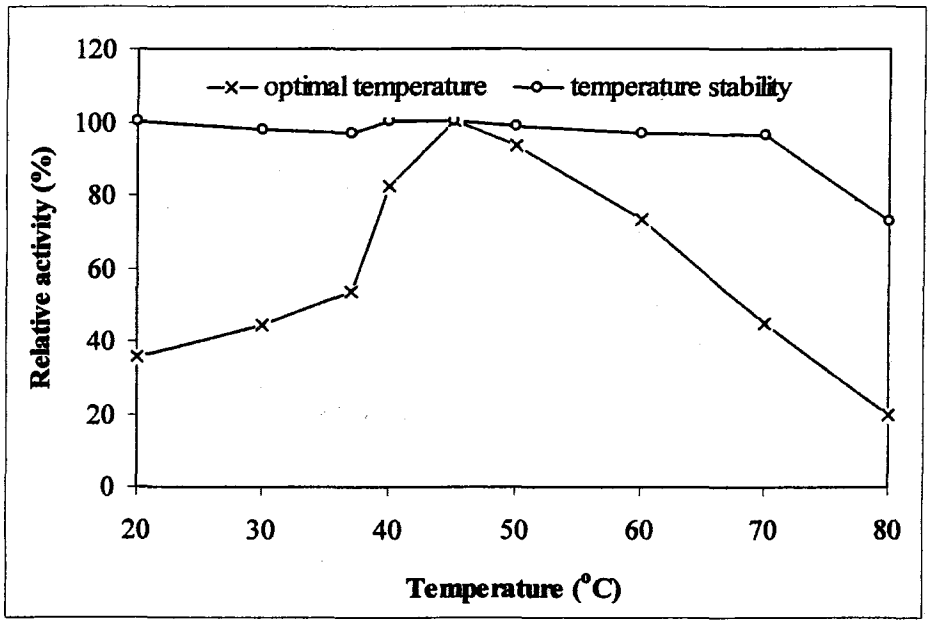
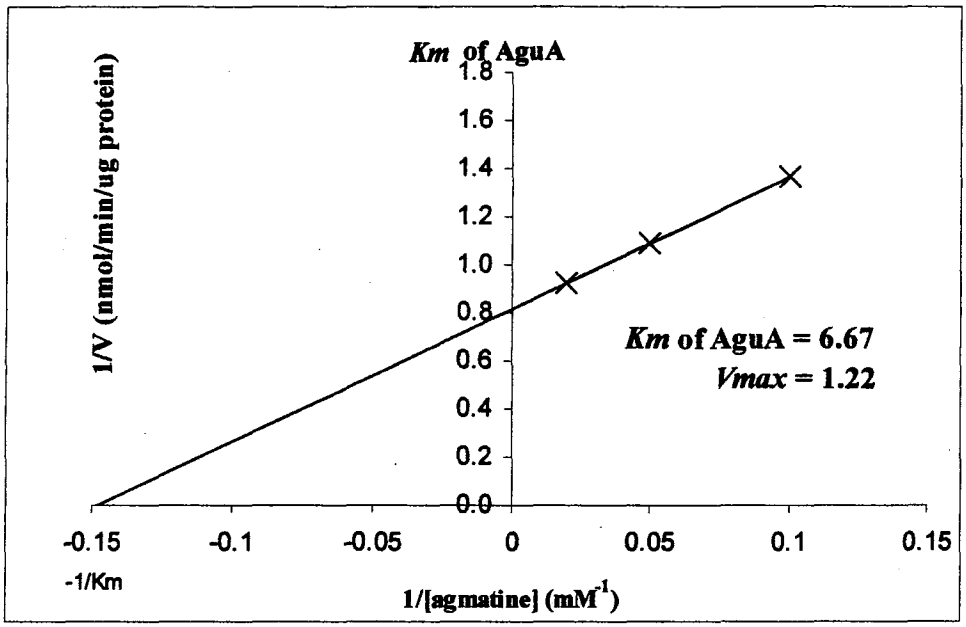


Fig.7. Effect of pH (A) and temperature (B) on AguB activities. AguA was incubated at 37 °C in 50 mM buffer of each pH for 30 min. A maximum activity was taken as 100%. In case of stability, the enzyme was treated at each pH in 50 mM buffer at optimum temperature for 30 min, followed by assaying the remaining activity under the standard condition. For pH stability, the enzyme was treated at each pH in 50 mM buffer at 37 °C for 30 min, followed by assaying the remaining activity under standard condition.

(A)



(B)

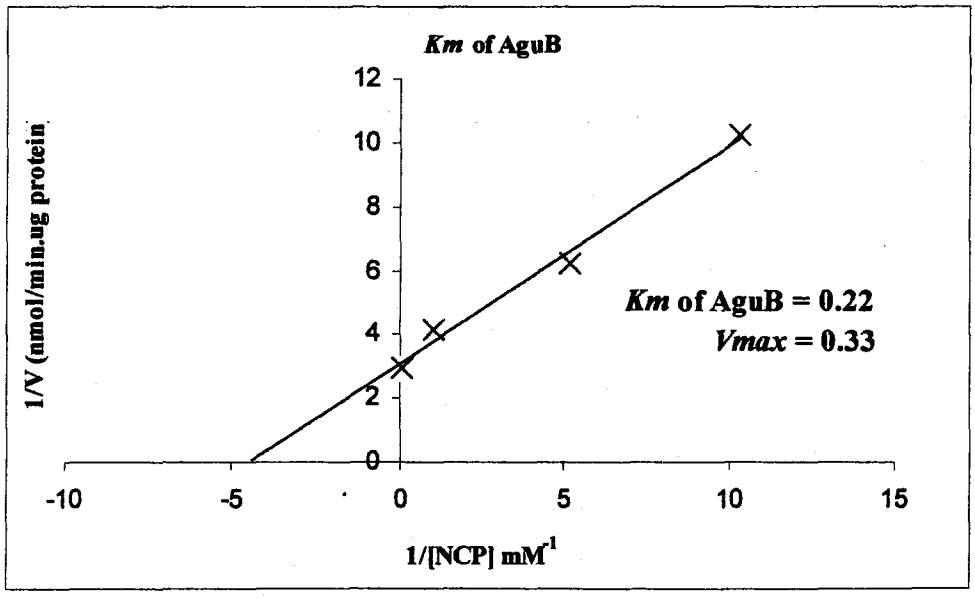


Fig. 8 Kinetic studies of AguA (A) and AguB (B)

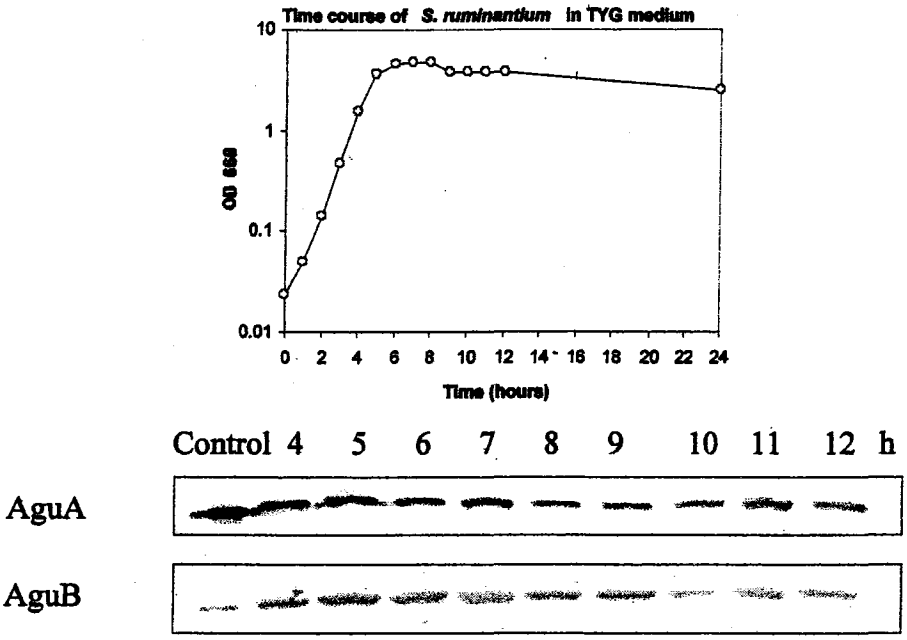


Fig.9. Cross reaction of anti-AguA and anti-AguB with crude extracts of *S. ruminantium* after culture in TYG medium for 4-12 h.

Table 1. Molecular mass and *Km* of agmatine deiminase and *N*-carbamoylputrescine amidohydrolase with other sources.

source	AguA		AguB	
	Molecular mass (kDa)	*Km* (mM)	Molecular mass (kDa)	*Km* (mM)
S. ruminantium	45 monomer	6.67	37 homodimer	0.22
P. aeruginosa	43 homodimer	0.60	33 homohexamer	0.50
A. thaliana	43 homodimer	0.11	35 homohexamer	0.13
Zea mays	43 homodimer	0.19		
Rice seedling	95 homodimer	15.00		
Soybean	70 monomer	2.50		
Cucumber	36 and 47 dimer	0.02		

Table 2. Effects of metal ions and some chemical reagents on agmatine deiminase and *N*-carbamoylputrescine amidohydrolase.

Conc. 1 mM	Relative activity (%)	
	Agmatine deiminase	*N*-carbamoylputrescine amidohydrolase
None	50	100
EDTA	100	94
Iodoacetamide	10	0
Hg^{2+}	0	0
Al^{3+}	6	68
Ca^{2+}	68	118
Co^{2+}	10	11
Cu^{2+}	5	110
Fe^{2+}	2	0
Fe^{3+}	2	118
Mg^{2+}	62	124
Mn^{2+}	120	37
Ni^{2+}	0	64
Zn^{2+}	1	40

論文審査結果要旨

大腸菌等の一般に知られているグラム陰性菌のペプチドグリカン層には，ムレインリポタンパク質が共有結合して存在し，外膜を細胞壁につなぎ止めて安定化する重要な働きを演じている。しかし，グラム陰性で嫌気性菌である *Selenomonas ruminantium* においては，本ムレインリポタンパク質が欠失して，まるでこれに取って代わるがごとくカダベリン若しくはプトレシンがペプチドグリカンのD-グルタミン酸残基のα-カルボキシル基すべてに共有結合して存在し，細胞分裂に必須の構成成分となっている。*S. ruminantium* において，カダベリン及びプトレシンはそれぞれL-リジンおよびL-オルニチンから構成的に細胞質内で合成され，続いて細胞質膜上でリピド中間体：ポリアミントランスフェラーゼによりペプチドグリカンリピド中間体に転移される。本菌においてリジン脱炭酸酵素は，L-リジンだけでなくL-オルニチンも基質として認識し，反応産物としてプトレシンを生成するオルニチン脱炭酸酵素活性をも持っていることが明らかになっている。

Difluoromethylornichine（DFMO）は上記酵素を特異的に阻害し，合成培地における本菌の生育を完全に阻害するが，天然培地及びアルギニン含有合成培地においては本阻害剤の阻害効果は見られない。このような背景から本研究者は，本菌のポリアミン合成系においてオルニチン経路の他に未知の経路でプトレシンが合成されていると考え，この未知回路の検索に取り組んだ。

最初に本研究者は，(1) DFMOならびに標識アルギニン含有合成培地で生育させた *S. ruminantium* において細胞質画分に標識アグマチン並びにプトレシンを検出した。本発見は *S. ruminantium* におけるプトレシンはアグマチンを経路して生合成されていることを示唆する。従って，本研究者は，本研究室の寥等により決定された *S. ruminanitum* のDNA塩基配列データを参考にアグマチン回路に関わるagmatine deiminaseおよびN-carbamoylputrescineamidohydrolaseの2種類の酵素遺伝子（*aguA* 及び *aguB* と命名）を *S. ruminanitum* 染色体DNAからクリーニングし，さらに大腸菌における組換え酵素の発現並びに単離・精製に成功した。(2) つぎに本研究者は，本2種類の酵素の基質特異性，pH安定性，熱安定性，*Km* 値，分子質量，ならびにサブユニット等の基本的諸性質を決定した。また，遺伝子操作により本2種類の活性部位を決定した。

今日，動物細胞におけるプトレシン合成においてアグマチン経路とガン細胞の誘発に関して注目されている。*S. ruminantium* におけるプトレシン合成に係るアグマチン経路の発見及び生化学的証明は，本菌におけるポリアミン合成制御の理解ばかりでなく動物細胞におけるアグマチン経路によるプトレシン構成制御機構を理解する上でのモデルと成り得るものである。

従って，審査員一同は，本研究者に博士（農学）の学位を授与するに値するものと認定した。