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Dipeptide synthesis by L-amino acid ligase from *Ralstonia solanacearum*

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ABSTRACT

Despite its utility, dipeptides have not been widely used due to the absence of an efficient manufacturing method. Recently, a novel method for effective production of dipeptides using L-amino acid α-ligase (Lal) is presented. Lal, which is only identified in *Bacillus subtilis*, catalyzes dipeptide synthesis from unprotected amino acids in an ATP-dependent manner. However, not all the dipeptide can be synthesized by Lal from *B. subtilis* (BsLal) due to its substrate specificity. Here, we attempted to find a novel Lal exhibiting different substrate specificity from BsLal. By *in silico* screening based on the amino acid sequence of BsLal, RSp1486a an unknown protein from *Ralstonia solanacearum* was found to show the Lal activity. RSp1486a exhibited different substrate specificity from BsLal, and preferably synthesized hetero-dipeptides where more bulky amino acid was placed at N terminus and less bulky amino acid was placed at C terminus in opposition to those synthesized by BsLal.

Dipeptide possesses useful and interesting properties. One is a physical property. For example, the solubility or the stability of L-tyrosine and L-glutamine in aqueous solution can be improved by the conversion to alanyl-tyrosine (Ala-Tyr) and alanyl-glutamine (Ala-Gln) [1]. The other is a unique physiological property. For example, valyl-tyrosine decreases blood pressure [2] or tyrosyl-arginine shows analgesic effect [3].

In spite of its utility, dipeptide has not been widely used due to the absence of an efficient manufacturing method. Several methods for producing dipeptide have been described; chemical or enzymatic condensation of protected amino acids [4–6]. However, these methods have not been satisfactory in regard to cost effectiveness and quality. Most methods need to introduce protecting groups into the amino acids and then remove them from the resultant dipeptides. The protecting reactions also trigger racemization of the substrate amino acids, which result in the formation of undesired by-products. For dipeptide to be more widely used, further cost-effective production method must be developed.

Recently, a new enzyme named L-amino acid α-ligase (Lal) has been identified in *Bacillus subtilis* by *in silico* screening [7]. This enzyme catalyzes dipeptide synthesis from unprotected amino acids in an ATP-dependent manner. The substrate specificity of Lal from *B. subtilis* (BsLal) was so wide that it could synthesize 44 different dipeptides. Moreover, the production of Ala-Gln was demonstrated through a fermentative process with BsLal in a genetically engineerd *Escherichia coli* strain [8].

However, not all dipeptides could be synthesized by BsLal. A substrate placed at N-terminal of dipeptide synthesized by BsLal is limited to Ala, Gly, Ser, Thr, and Met. In this report, we describe the finding of novel Lal exhibiting different substrate specificity from BsLal. We also present some of the characteristics of the enzyme.

Materials and methods

Materials

Escherichia coli JM109, BL21(DE3), and plasmid pUC19 were purchased from Nippon Gene (Tokyo, Japan). *Ralstonia solanacearum* JCM 10489 was purchased from RIKEN (Saitama, Japan). *R. solanacearum* MAFF 211270, 211272, 211282, 211396, 211402, 211403, 211544, 301520, 301522, 301523, 301526, and 301560 were purchased from National Institute of Agrobiological Sciences (Ibaraki, Japan). Plasmid pET-21a(+) was purchased from Merck (Tokyo, Japan). KOD-plus- DNA polymerase was purchased from Toyobo (Osaka, Japan). Restriction enzymes and DNA ligase were purchased from Takara Bio (Shiga, Japan). HisTrap HP was purchased from GE Healthcare (Buckinghamshire, England). Determiner L IP was purchased from Kyowa Medex (Tokyo, Japan). *N*-α-(5-Fluoro-2,4-dinitrophenyl)-L-alaninamide (FDAA) was purchased from Tokyo Chemical Industry (Tokyo, Japan). Dipeptides for standards were purchased from Cosmo Bio (Tokyo, Japan). All other chemicals used were commercially available and of chemically pure grade.

In silico screening of novel Lal

Amino acid homology search was performed using NCBI's BLAST service at http://www.ncbi.nlm.nih.gov/BLAST/.

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Genetic manipulation

DNA manipulations were basically performed according to Sambrook et al. [9]. Genomic DNA of *R. solanacearum* was prepared with DNeasy Tissue kit (Qiagen, Valencia, CA, USA). PCR product was purified with GFX PCR DNA and gel band purification kit (GE Healthcare). Plasmid DNA was prepared with a GFX micro plasmid prep kit (GE Healthcare). Plasmid pUC19 and pET21a(+) were used for sequence analysis and overexpression, respectively. Every primer for PCR was designed by nucleotide sequence of RSp1486 (DDBJ Accession No. AL646053) from *R. solanacearum* GMI1000. DNA sequencing was performed with an ABI PRISM 310 genetic analyzer (Applied Biosystems, Foster City, CA, USA). The nucleotide sequence was determined by complete sequencing of both strands, with multiple sequencing of some regions.

Construction of plasmid for sequence analysis

RSp1486a gene (DDBJ Accession No. AB428352) was amplified from the *R. solanacearum* JCM 10489 genome by PCR using the primer 5′-ATTAGGATCCGGTGCGTGAGCCAGGAGG-3′ to introduce a BamHI site and 5′-ATATAAGCTTCTGCACCGCGCTCGGCAC-3′ to introduce a HindIII site. The 1.4-kb PCR product was digested with BamHI and HindIII and inserted between the BamHI and HindIII sites of pUC19. The resulting plasmid was amplified in *E. coli* JM109, and the insert was sequenced.

Construction of plasmid for overexpression

RSp1486a gene was amplified from the *R. solanacearum* JCM 10489 genome by PCR using the primer 5′-GGGCCAACCATAT GAGCAAGAAGATTCT-3′ to introduce an NdeI site and 5′-ATA TCTCGAGGCTATGCAGCGGCAAAC-3′ to introduce a XhoI site. The 1.4-kb PCR product was digested with *Nde*I and *Xho*I and inserted between the NdeI and XhoI sites of pET-21a(+). The resulting plasmid, designed to express *RSp1486a* with a histidine (His) tag at its C terminal under the control of the T7 promoter and *lac* operator, was named pETRSp1486a. The pETRSp1486a was amplified in *E. coli* JM109 and introduced into *E. coli* BL21(DE3).

Overexpression of RSp1486a and protein purification

Escherichia coli BL21(DE3)/pETRSp1486a cultivated in Luria-Bertani medium containing 50 mg/l of ampicillin at 30 °C for 16 h with vigorous shaking. The culture broth was transferred to fresh Luria-Bertani medium and incubated at 37 °C with vigorous shaking. After 3 h, 0.1 mM of IPTG (isopropyl-β-D-thiogalactopyranoside; final concentration) was added, and cultivation was continued a further 16 h at 30 °C. Cells were harvested by centrifugation (5000*g*, 10 min, 4 °C). A cell pelette was resuspended with 50 mM of Tris–HCl (pH 8.0) and disrupted by sonication at 4 °C. Cellular debris was removed by centrifugation (10,000 *g* for 30 min, 4 °C), and the supernatant was collected as cell-free extract.

The cell-free extract was subjected to a HisTrap HP 1 ml column equilibrated with 50 mM Tris–HCl (pH 8.0), 500 mM NaCl, and 20 mM imidazole, then the column was washed with 10 column volumes of the same buffer. The His-tagged protein was eluted with 50 mM Tris–HCl (pH 8.0), 500 mM NaCl, and 500 mM imidazole and desalted through a HiTrap Desalting 5 ml column equilibrated with 50 mM Tris–HCl (pH 8.0). Protein concentration was determined using Coomassie Protein Assay Reagent (PIERCE) with bovine serum albumin as a standard.

Lal activity of RSp1486a

The reaction mixture (total volume was 500 μl) contained 50 mg/l of purified His-tagged RSp1486a protein, 12.5 mM ATP, 12.5 mM $MgSO_4$, and 12.5 mM substrate amino acid(s) in 50 mM Tris–HCl buffer (pH 8.0). As substrates, every combination of one or two amino acids selected from the following 19 kinds of L-amino acids was tested (190 combinations in total): glycine (Gly), L-alanine (Ala), L-serine (Ser), L-cysteine (Cys), L-aspartate (Asp), L-asparagine (Asn), L-threonine (Thr), L-methionine (Met), L-lysine (Lys), L-isoleucine (Ile), L-valine (Val), L-leucine (Leu), L-glutamate (Glu), L-glutamine (Gln), L-proline (Pro), L-arginine (Arg), L-histidine (His), L-tryptophan (Trp), L-phenylalanine (Phe). In addition, to test the reactivity with D-amino acid, a reaction with D-alanine (D-Ala) was conducted. As a blank sample, reaction mixture without amino acid was also prepared. The reaction was carried out at 30 °C. After overnight incubation, Lal activity was measured by determining released inorganic phosphate (Pi) using colorimetric assay with Determiner L IP. An aliquot of reaction mixture (1 μl) was mixed with 120 μl of solution I, and 40 μl of solution II in 96-well microplate. After a few minutes incubation, the absorbance at 595 nm was measured. A standard curve was prepared using KH_2PO_4 solution.

Determination of dipeptides

(i) *HPLC analysis.* HPLC analysis was performed as described previously [10,11]. Briefly, amino acids and dipeptides derivatized with FDAA were separated by reverse-phase HPLC and monitored by UV absorbance at 340 nm.

(ii) *NMR analysis.* The reaction mixture (total volume was 1.5 ml) contained 50 mg/l of purified His-tagged RSp1486a protein, 12.5 mM ATP, 12.5 mM MgSO4, and 12.5 mM substrate amino acid(s) in 50 mM phosphate buffer (pH 8.0). The reaction was carried out at 37 °C. After overnight incubation, reaction mixture was lyophilized and redissolved in 300 μl of deuterium oxide. Then, 3-trimethylsilyl-tetradeuterosodium propionate (TSP) was added as an internal concentration and chemical shift standard. In the case of the sample containing Cys as a substrate, a deuterated DTT was further added. 1H NMR spectra and 1H–13C heteronuclear multiple bond correlation (HMBC) NMR spectra were acquired at 30 °C with a DMX500 NMR spectrometer (Bruker biospin).

Characterization of RSp1486a

To classify the amino acids acceptable for RSp1486a as substrate, three types of reaction mixtures were prepared: (i) single amino acid was used as substrate (Xaa + Xaa); (ii) two amino acids of which one substrate was fixed as Gly were used (Gly + Xaa); (iii) two amino acids of which one substrate was fixed as Gln were used (Gln + Xaa). "Xaa" is 19 kinds of L-amino acids described above. As blank samples, reaction mixtures without Xaa were also prepared for each type. The reaction was performed at 30 °C for 16 h and the Lal activity was measured by determining released Pi.

For determining optimal pH, a 150 mM GTA buffer consisting of 50 mM 3,3-demethylglutaric acid, 50 mM Tris, and 50 mM 2-amino-2-methyl-1,3-propanediol was used as reaction buffer. The reaction mixture (total volume was 500 μl) contained 50 mg/liter of purified His-tagged RSp1486a protein, 10 mM ATP, 10 mM $MgSO_4$, 5 mM Phe, and 5 mM Ala in 150 mM GTA buffer (pH 6.0–10.0). The reaction was carried out at 37 °C for 1 h and stopped by adding 500 μl of 0.1 N HCl. After that, Phe-Ala was determined by HPLC.

The methods for determining optimal temperature were the same as those described in determining optimal pH except for the buffer (50 mM Tris–HCl [pH 9.0]) and the reaction temperature (in the range of 25–60 °C).

For determining the Michaelis constants (K_ms) for Ala, Phe, and ATP, the reactions were performed by varying concentration of Ala, Phe, or ATP. The standard reaction mixture (total volume was 500 μl) contained 100 mg/l of purified His-tagged RSp1486a protein, 60 mM ATP, 30 mM $MgSO_4$, 30 mM Phe, and 30 mM Ala in 50 mM Tris–HCl (pH 9.0). The reaction was carried out at 40 °C for 60 min (for determining K_ms for Ala and Phe) or 30 min (for determining K_m for ATP) and stopped by adding of 500 μl of 0.1 N HCl. After that, Phe-Ala was determined by HPLC.

Result

Searching for a novel Lal

To search for a novel Lal, BLAST search was performed using the amino acid sequence of BsLal as a query. RSp1486 protein encoded in the genome of *R. solanacearum*, showed the highest homology with BsLal (E-value = $3e^{-34}$, identities = 108/371 [29%], positives = 179/371 [48%]). RSp1486 was defined as "hypothetical protein" and its actual function was unknown. Furthermore, this protein possessed an ATP-grasp domain (PS50975) as well as various ligases including BsLal. From these results, we chose the RSp1486 as the most promising candidate of novel Lal. Since it was difficult to obtain the *R. solanacearum* strain GMI1000, the strain JCM 10489 was used as alternative strain. The homologous protein from the strain JCM10489 was named RSp1486a. Sequence analysis revealed that the RSp1486a was composed of 449 aa as well as RSp1486 and that the amino acid sequence was over 94% identical to RSp1486.

Lal activity of RSp1486a

The gene encoding RSp1486a was amplified with PCR, and expression plasmid pETRSp1486a was constructed. In this plasmid, the gene was driven by the T7 promoter to yield RSp1486a with a 6× His tag at its C terminus. Expression in the host, *E. coli* BL21(DE3), was confirmed by SDS–PAGE (Fig. 1). The molecular mass of the expressed protein was estimated at 50 kDa (Fig. 1, lane 3), which is fairly consistent with the value estimated from the amino acid sequence of the His-tagged RSp1486a (49.5 kDa). It was predicted that RSp1486a is a monomer protein by gel filtration chromatography (data not shown).

Purified RSp1486a was incubated with ATP and various combinations of amino acids (190 combinations in total). Each value of released Pi was obtained by subtracting the value of blank sample (2 mM) from actual value. A significant amount of Pi was detected in 96 combinations (Fig. 2). Especially, 4–10 mM of Pi was detected in the samples containing Ala, Ser, Cys, Met, His, and Phe. On the other hand, the same amount of Pi was detected in both the sample containing D-Ala and the blank sample. These results suggest that RSp1486a is a novel Lal with broad substrate specificity for L-amino acids as well as BsLal.

Structure determination of dipeptide products

Although it was revealed that the RSp1486a have a Lal activity, the structure of synthesized dipeptides could not be determined by the detection of released Pi. Therefore, some samples where relatively high concentration of Pi was detected were analyzed by NMR (Table 1). A ligase reaction using two kinds of amino acids as substrates could give four kinds of dipeptides. Interestingly, RSp1486a mainly synthesized only one hetero-dipeptide among all samples analyzed in this study. Furthermore, more bulky amino acid was mostly placed at N-terminus of the hetero-dipeptides.

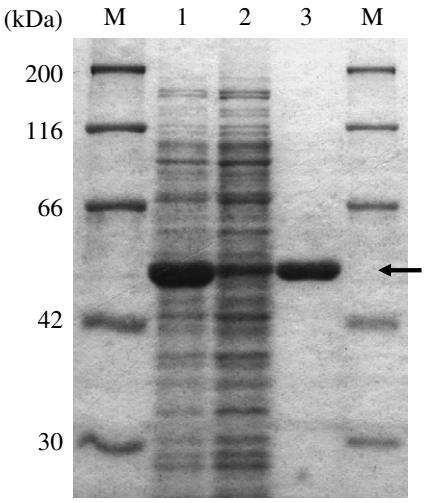

Fig. 1. SDS–PAGE analysis of cell free extract and purified enzyme. Lane M, molecular weight marker; lane 1, cell-free extract; lane 2, flow-through from the HisTrap HP column; lane3, purified fraction eluted with 500 mM imidazole buffer. The arrow indicates the position of RSp1486a at approximately 50 kDa.

Characterization of RSp1486a

To investigated which amino acid is acceptable as substrate for RSp1486a, we noted that Gln and Gly were acceptable for N terminus or C terminus of dipeptide and resultant Gln-Val or His-Gly, though Gln-Gln or Gly-Gly were not synthesized (Table 1). We estimated that the amino acid substrates could be classified as follows: substrate acceptable for both N and C terminus or for only N terminus, or for only C terminus. Then, to classify the amino acid substrate, three types of reaction mixtures were prepared described in Materials and methods. A total of 60 reaction mixtures were incubated for 16 h at 30 °C and the Lal activity was detected by determining released Pi. As a result, Ala, Cys, Phe, His, Met, and Ser were classified as substrates acceptable for both N and C terminus because significant activity was detected in type (i) reaction mixture containing single amino acid, respectively. Asn and Gln were classified as substrates acceptable for only N terminus because significant Lal activity was detected in only type (ii) reaction mixture containing Gly which is acceptable for only C terminus, respectively. Gly, Leu, Thr, and Val were classified as substrates acceptable for only C terminus because significant Lal activity was detected in only type (iii) reaction mixture containing Gln which is acceptable for only N terminus, respectively (Fig. 3).

Next, some of the characteristics of the RSp1486a were examined using Phe-Ala synthesizing reaction as a model. The activity was evaluated by determining Phe-Ala by HPLC. The optimal temperature and pH were around 9 and 40 °C, respectively (Fig. 4). GTP did not substitute for ATP. Mg was essential for the activities and Mn was not substitute for it. Apparent K_ms for Phe, Ala, and ATP were 10.5 (±0.80) mM, 5.1 (±0.38) mM, and 0.533 (±0.037) mM, respectively.

Lals from other strains of R. solanacearum

To investigate whether RSp1486-like gene is distributed in various other strains of *R. solanacearum*, we attempted to obtain the corresponding protein from following 12 strains: *R. solanacearum* MAFF 211270, 211272, 211282, 211396, 211402, 211403, 211544, 301520, 301522, 301523, 301526, and 301560 (DDBJ Accession No. AB428353, AB428354, AB428355, AB428356,

	Gly	Ala	Val	Leu	Ile	Ser	Thr	Cys	Met	Asn	Gln	Asp	Glu	Lys	Arg	His	Phe	Trp	Pro
Pro	−	+	−	−	−	+	−	++	+	−	−	−	−	−	−	+	++	−	−
Trp	−	+	−	−	−	−	−	+	−	−	−	−	−	−	−	−	+	−	
Phe	++	+++	+++	++	+++	+++	++	+++	+++	++	+++	++	++	++	++	+++	+++		
His	+++	+++	+++	+++	+	+++	+++	++	+++	+	+	+	+	+	+	++			
Arg	−	++	−	−	−	+	−	+	+	−	−	−	−	−	−				
Lys	−	++	−	−	−	+	−	+	+	−	−	−	−	−					
Glu	−	++	−	−	−	+	−	+	+	−	−	−	−						
Asp	−	++	−	−	−	−	−	+	+	−	−	−							
Gln	+	+++	++	−	−	+	+	+++	++	−	−								
Asn	−	++	+	−	−	−	−	++	+	−									
Met	++	+++	++	++	+	+++	+	+++	+++										
Cys	++	+++	−	++	++	+++	++	+++											
Thr	−	++	−	−	−	++	−												
Ser	++	+++	−	+	+	+++													
Ile	−	+	−	−	−														
Leu	−	+	−	−															
Val	−	++	−																
Ala	++	+++																	
Gly	−																		

Fig. 2. RSp1486a shows Lal activity to various amino acids. Lal activity was confirmed by the determination of Pi that is by-product of Lal reaction. Combinations of 19 kinds of L-amino acids were used as substrates. The amount of released Pi value was obtained by subtracting the value of blank sample (2 mM) from actual value and was indicated as follows. +++, >6 mM; ++, 4–6 mM; +, 2–4 mM; −, <2 mM.

Table 1
Structure and quantitative determination of diptptides by NMR analysis

Substrate 1	Substrate 2	Dipeptides produced (mM)			
		Major product		Other product(s)	
Ala	Ser	Ser-Ala	6.5	Ala-Ala	2.6
Ala	Met	Met-Ala	6	Ala-Ala	2.6
Ala	Gln	Gln-Ala	6.4	Ala-Ala	2.5
Ala	Phe	Phe-Ala	6.6	Ala-Ala	2
				Phe-Phe	1.2
Ala	His	His-Ala	9.7	Ala-Ala	1.2
His	Val	His-Val	8.6	His-His	0.1
His	Leu	His-Leu	7.5	His-His	0.5
His	Thr	His-Thr	5.3	His-His	1.5
His	Met	His-Met	8.5	His-His	0.2
His	Ser	His-Ser	6.2	His-His	0.8
His	Gly	His-Gly	8.5	His-His	0.5
Gln	Val	Gln-Val	5.7		
Phe	Val	Phe-Val	5.8	Phe-Phe	1.7
Cys	Ser	Ser-Cys	6.7		
Cys	Gln	Gln-Cys	6.8	Cys-Cys	0.6
Cys	Phe	Phe-Cys	10.7	Cys-Cys	1.4
				Phe-Phe	0.2

AB428357, AB428358, AB428359, AB428360, AB428361, AB428362, AB428363, and AB428364). From all strains, the corresponding proteins were obtained as His-tagged recombinant protein as well as RSp1486a. Then, to compare the substrate specificity with that of RSp1486a, each protein was incubated with various kinds of substrate combinations, and then the released Pi was determined. Among 13 proteins including RSp1486a, the significant difference of substrate specificity was not observed (data not shown). Sequence analysis also revealed that the amino acid sequence of each protein was at least 95% identical to one another.

Discussion

We reported here that the RSp1486a protein encoded in the *R. solanacearum* genome was found to exhibit L-amino acid ligase activity. This is the second report of Lal and the effectiveness of *in silico* screening for searching Lal was shown. In spite of its low amino acid sequence identity (28%) with firstly reported Lal from *B. subtilis* (BsLal), RSp1486a showed Lal activity equal to BsLal. Since there is a lot of unknown protein possessing ATP-grasp domain like RSp1486a, this finding raise the possibility of the existence of other Lals, whose amino acid sequence is not very similar to those of already known Lals.

It was previously reported that the BsLal is one of the enzymes concerned with the biosynthesis of bacilysin, which is di-peptidic antibiotic produced by *B. subtilis* [12]. The bacilysin contains an L-Ala at the N terminus and a non-proteinogenic amino acid, L-anticapsin, at the C terminus. The function of BsLal is the ligation of L-Ala and L-anticapsin. On the other hand, the physiological significance of RSp1486a is unclear. The gene encoding RSp1486 is included in 9 ORF clusters but their functions are almost unknown.

Ralstonia solanacearum is a plant pathogenic bacterium commonly found in the soils of tropical and subtropical countries where it devastates cultures of many crop plants [13]. The RSp1486a homolog proteins were found from all strains of *R. solanacearum* tested in this study. RSp1486a may be concerned with the biosynthesis of some di-peptidic secondary metabolite as well as BsLal.

The substrate specificity of RSp1486a was fairly broad as well as BsLal. But unlike Bslal, RSp1486a could synthesize dipeptides where bulky amino acid such as His or Phe is placed at the N terminus. The major product was also different from BsLal when the same combination of amino acids was used as substrates. For example, RSp1486a predominantly synthesized Gln-Ala but Ala-Gln when Ala and Gln were used as substrates. It seems that more bulky amino acid has higher affinity for N-terminal and less bulky amino acid has higher affinity for C-terminal. This difference is preferable for considering the utilization of Lal for production of various dipeptides. The finding of RSp1486a may expand the range of dipeptide produced by the effective dipeptide-manufacturing method using Lal.

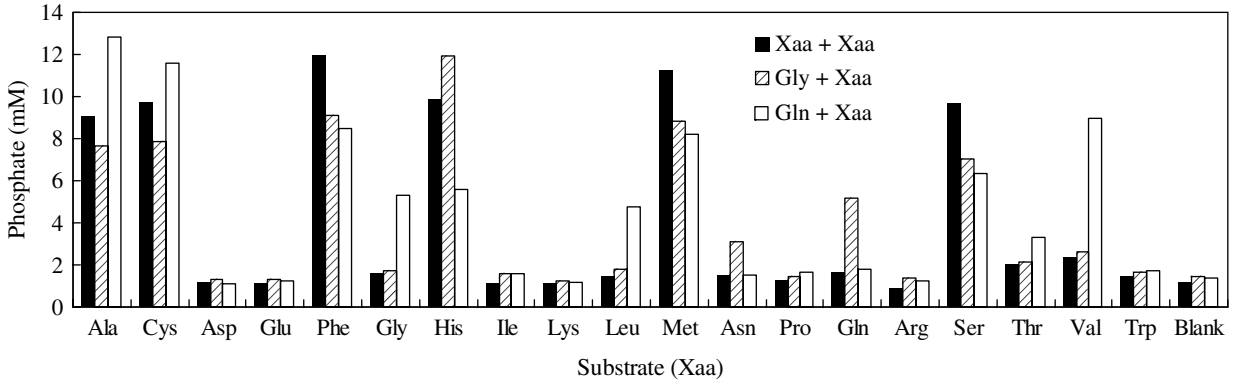

Fig. 3. Substrate specificity of RSp1486a. Puridied His-tagged RSp1486a (50 mg/l) was incubated with 20 mM ATP, 10 mM $MgSO_4$ and 40 mM Xaa (closed bar) or 20 mM each of Gly and Xaa (striped bar), or 20 mM each of Gln and Xaa (open bar) at 30 °C for 16 h and the amount of released Pi was determined.

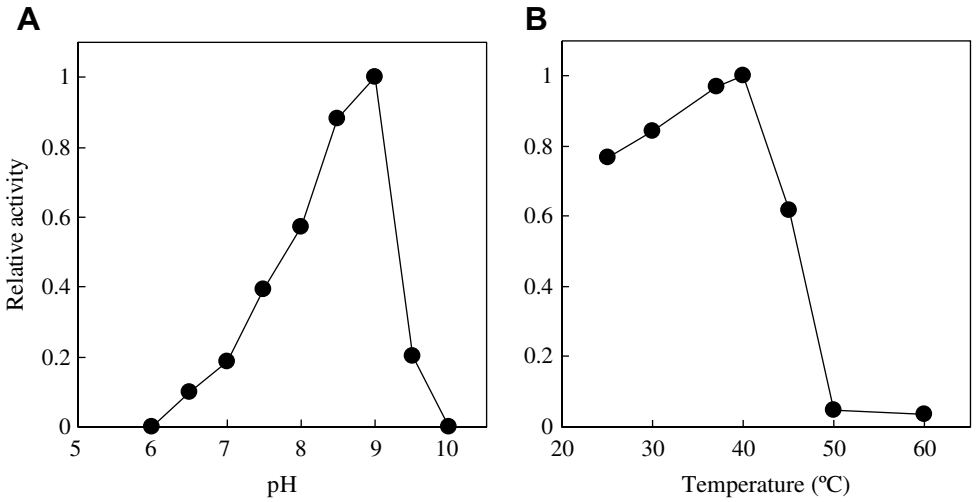

Fig. 4. Effect of pH (A) and temperature (B) on RSp1486a protein activity. Reaction conditions were described in Materials and methods. The amount of Phe-Ala relative to that formed at pH 9 (A) or 40 °C (B) was expressed.

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