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Racemization study on different *N*-acetylamino acids by a recombinant *N*-succinylamino acid racemase from *Geobacillus kaustophilus* CECT4264

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ABSTRACT

N-Succinylamino acid racemase (NSAAR) with *N*-acylamino acid racemase (NAAAR) activity together with a D- or L-aminoacylase allows the total transformation of *N*-acetylamino acid racemic mixtures into optically pure D- or L-amino acids, respectively. In this work we have cloned and expressed the *N*-succinylamino acid racemase gene from the thermophilic *Bacillus*-related species *Geobacillus kaustophilus* CECT4264 in *Escherichia coli* BL21 (DE3). *G. kaustophilus* NSAAR (GkNSAAR) was purified in a one-step procedure by immobilized cobalt affinity chromatography and showed an apparent molecular mass of 43 kDa in SDS-gel electrophoresis. Size exclusion chromatography analysis determined a molecular mass of about 150 kDa, suggesting that the native enzyme is a homotetramer. Optimum reaction conditions for the purified enzyme were 55 °C and pH 8.0, using *N*-acetyl-D-methionine as substrate. GkNSAAR showed a gradual loss of activity at preincubation temperatures over 60 °C, suggesting that it is thermostable. As activity was greatly enhanced by Co^{2+}, Mn^{2+} and Ni^{2+} but inhibited by metal-chelating agents, it is considered a metalloenzyme. The Co^{2+}-dependent activity profile of the enzyme was studied with no detectable inhibition at higher metal ion concentrations. GkNSAAR showed activity towards both aliphatic and aromatic *N*-acetylamino acids such as *N*-acetyl-methionine and *N*-acetyl-phenylalanine, respectively, with k_{cat}/K_m values ranging from 1×10^3 to 9×10^3 s^{-1} M^{-1}. Kinetic parameters were better for *N*-acetyl-D-amino acids than for *N*-acetyl-L-specific ones.

1. Introduction

Optically pure amino acids are of considerable economic importance because of the broad spectrum of their industrial applications. Proteinogenic amino acids are used in human nutrition and health or as important additives in animal feed, and as flavour enhancers in foodstuffs and sweeteners [1]. Additionally, non-natural amino acids are valuable intermediates for the preparation of semisynthetic antibiotics, pesticides, and other products of interest for the pharmaceutical, food, and agrochemical industries [2]. Several methods have been described for enzymatic production of amino acids, such as the hydantoinase [3] or amidase [4,5] processes. An alternative method uses *N*-acylase to produce optically pure amino acids from *N*-acylamino acids. L-Acylases catalyze the transformation of *N*-acetyl-D,L-amino acids to L-amino acids and *N*-acetyl-D-amino acids. After separation of the products, the *N*-acetyl-D-amino acid is racemized under drastic thermal conditions and returned to the enzyme reaction [6]. D-Amino acids can also be obtained using D-selective acylase following the same procedure. Enzymatic racemization of the remaining non-hydrolyzed D- or L-acetylamino acid could improve the "acylase process" by eliminating costly racemization and separation steps. *N*-Acylamino acid racemase together with a D- or L-aminoacylase can produce a final yield of 99% optically pure amino acid in one-step from racemic mixtures of *N*-acetylamino acids [7].

N-Acylamino acid racemase (NAAAR) allows the racemization of *N*-acetylamino acid under physiological conditions. The Tokuyama group was the first to detect NAAAR activity in some actinomycetes strains [8]. Subsequently, NAAARs from *Streptomyces atratus* and *Amycolatopsis* sp. TS-1-60 were purified [9,10]. The recombinant genes encoding NAAARs from the latter, *Amycolatopsis orientalis* subsp. *lurida* and *A. azurea* were cloned and expressed in *Escherichia coli* [7,11,12]. The atomic structure of NAAAR of the ancient bacterium, *Deinococcus radiodurans* has recently been solved [13]. Biochemical characterization of native and recombinant NAAARs has shown their activity on a broad range of *N*-acylamino acids but not on the corresponding amino acids [14]. NAAAR required a high

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concentration (>10 mM) of divalent metal ion for enzyme activity [15]. Substrate inhibition has also been detected in NAAAR from *Amycolaptosis* sp. [15], restricting the use of this enzyme in a practical industrial process [16].

A previous study re-assigned the "correct" function of NAAAR from *Amycolatopsis* as o-succinylbenzoate synthase (OSBS), showing an example of catalytic promiscuity (OSBS/NAAAR) [15]. Further studies discovered that alternative substrates such as *N*-succinyl-methionine or *N*-succinyl-phenylglycine, structural analogues of the substrate for the OSBS reaction, were better substrates for the NAAAR reaction than *N*-acetyl-methionine [17]. Recently, this enzyme has been characterized in *Geobacillus kaustophilus* together with two other proteins encoded in the same operon. These three enzymes would constitute a novel pathway for the conversion of D- to L-amino acids, and OSBS/NAAAR was denominated *N*-succinylamino acid racemase with a new physiological function in the muconate lactonizing enzyme (MLE) subgroup [18]. Due to this new re-assignment of the enzyme, from now on it will be referred to as *N*-succinylamino acid racemase (NSAAR).

To obtain a new NSAAR with superior industrial properties to those previously described, our laboratory started to screen thermophilic microorganisms. Thermostable enzymes isolated mainly from thermophilic microorganisms have received attention due to their potential commercial applications because of their overall inherent stability and high reaction rates at high temperatures [19]. These elevated temperatures also increase mass transfer effects, improve solubility of substrates in water and reduce risk of cross contamination [20]. Many thermophiles and hyperthermophiles have been isolated from hot springs and other thermal environments. The complete genome sequences of 20 thermophilic or hyperthermophilic prokaryotic species have been determined [21]. One of these is *G. kaustophilus*, a thermophilic *Bacillus*-related species, whose complete nucleotide genome sequence was recently determined [21]. The genome is composed of a 3.54 Mb single circular chromosome and a 47.9 kb plasmid. These authors assigned a biological role to 1914 of the 3498 predicted protein-coding genes and one of them corresponded to a putative NSAAR.

Here we describe the cloning, purification and biochemical characterization of a NSAAR enzyme encoded by a gene located on the circular chromosome of *G. kaustophilus*. The biochemical characteristics of this enzyme have been compared with those of NSAARs present in actinomycetes and *D. radiodurans*.

2. Materials and methods

2.1. General protocols and reagents

Standard methods were used for cloning and expression [22,23]. Restriction enzymes, T4 DNA ligase and thermostable *Pwo* polymerase for PCR were purchased from Roche Diagnostic S.L. (Barcelona, Spain). Optically pure *N*-acetylamino acids used in this work were purchased from Sigma–Aldrich (Madrid, Spain). D- and L-carbamoyl-methionine were synthesized as previously described [24]. *N*-Succinylamino acids were synthesized as previously described with minor changes [18], by the addition of succinic anhydride to the corresponding enantiopure amino acid (alanine and phenylalanine) in a solvent of acetic acid. The reaction mixture was heated under reflux for 12 h at 55 °C. Substrates were obtained as a white powder after recrystallization.

2.2. Microbes and culture conditions

G. kaustophilus CECT4264, acquired from the Spanish Type Culture Collection (CECT), was used as possible donor of the *N*-succinylamino acid racemase gene (*nsaar*). This strain was cultivated at 55 °C for 20 h in Luria–Bertani medium (LB) (1% tryptone, 0.5% yeast extract, 0.5% NaCl, pH 7.2). *E. coli* BL21 (DE3) [25] was used to clone and express the *N*-succinylamino acid racemase gene.

2.3. Cloning and sequence analysis of GkNSAAR

A single-colony isolate of *G. kaustophilus* CECT4264 was chosen for DNA isolation using a similar method to that described for *Sinorhizobium meliloti* CECT4114 [26]. Using a sterile inoculating loop, the bacterial colony was transferred from the LB plate to 50 μl double-distilled water. The cells were lysed by boiling at 100 °C for 10 min followed by immediate chilling on ice. After cooling for 5 min, cell debris was removed by centrifugation. A sample of 5 μl of the supernatant containing genomic DNA was used to amplify the gene encoding the GkNSAAR by PCR.

The primers used were designed based on GenBank sequence accession no. AP006508 [21]. These were Gknsaar5 (5′-GAGCTCGTGACAGGAA-AGAGGAATGGC-GATCAACA-3′) and Gknsaar3 (5′-CTCGAGGGATCCACGCGG-AACCAGTGCCGTCGCCG-TACGATGAA-3′). The latter included the thrombin recognition sequence (Leu-Val-Pro-Arg-Gly-Ser) before the stop codon. The *Sac*II and *Xho*I digested 1128 bp fragment was purified from agarose gel using QIAquick (Qiagen) and ligated into pET21b plasmid (Novagen, Barcelona, Spain), to create plasmid pJPD25.

Once the fragment had been cloned, it was sequenced using the dye dideoxy nucleotide sequencing method in an ABI 310 DNA Sequencer (Applied Biosystems). Sequencing was carried out at least twice using standard T3 and T7 primers, edited with Microsoft Word 2003 and assembled using CLUSTALW [27]. The assembled sequences were aligned and compared with all of the amino acid sequence databases available from Internet using Basic Local Alignment Search Tool (BLAST) [28]. Manual comparisons of the sequences were performed with CLUSTALW and drawn with ESPript [29]. The percentage of homology between fragments was calculated with BLAST and SEAVIEW [30] programmes.

2.4. Expression of the GkNSAAR

The transformant in BL21 (DE3) strain was grown in LB medium supplemented with 100 μg ml^{-1} of ampicillin. A single-colony was transferred into 10 ml of LB medium with ampicillin at the above-mentioned concentration in a 100 ml flask. This culture was incubated overnight at 37 °C with shaking. In a 2 l flask 500 ml of LB with the appropriate concentration of ampicillin was inoculated with 5 ml of the overnight culture. After 2 h of incubation at 37 °C with vigorous shaking, the OD_{600} of the resulting culture was 0.3–0.5. For expression induction of the *nsaar* gene, isopropyl-β-D-thiogalactoside (IPTG) was added to a final concentration of 0.2 mM and the culture was incubated at 34 °C for an additional 5 h. The cells were collected by centrifugation (Beckman JA2-21, 7000 × *g*, 4 °C, 10 min), washed twice and resuspended in 50 ml wash buffer (300 mM NaCl, 20 mM imidazole, 50 mM sodium phosphate; pH 7.0). The cell walls were disrupted in ice by sonication using a UP 200 S Ultrasonic Processor (Dr. Hielscher GmbH, Germany) for six periods of 60 s, pulse mode 0.5 and sonic power 60%. The pellet was precipitated by centrifugation (Beckman JA2-21, 10,000 × *g*, 4 °C, 20 min) and discarded. The supernatant was applied to a column with TALON™ metal affinity resin (CLONTECH Laboratories, Inc., Nucliber, Madrid, Spain) and then washed three or four times with wash buffer. After washing, GkNSAAR was eluted with elution buffer (100 mM NaCl, 150 mM imidazole, 2 mM Tris, pH 8.0). The purified enzyme was dialysed against 0.1 M borate/HCl pH 8.0 and stored at 4 °C until use.

2.5. Enzyme assay

Standard enzymatic reaction was carried out with the purified GkNSAAR (at a final concentration of 0.09 μM) together with *N*-acetyl-D-methionine as substrate (20 mM) dissolved in 100 mM borate/HCl (pH 8.0) in 500 μl reaction volume. The reaction mixture was incubated at 55 °C for 20 min and stopped by boiling at 100 °C for 5 min. After centrifugation, the resulting supernatants were analyzed by high performance liquid chromatography (HPLC). The HPLC system (Breeze HPLC System, Waters Cromatografia S.A., Barcelona, Spain) equipped with a Chirobiotic T column (4.6 mm × 250 mm, ASTEC Inc., USA) was used to separate the D- and L-forms of the *N*-acetylamino acid. The mobile phase was 70% methanol, 30% ammonium acetate (0.01 M), and 0.5 ml acetic acid per litre [11], pumped at a flow rate of 0.75 ml min^{-1} and measured at 200 nm. The specific activity of the enzyme was defined as the amount of enzyme that catalyzed the formation of 1 μmol of D- or L-acetylamino acid at 55 °C min^{-1} mg^{-1} of protein.

2.6. Molecular mass analysis

Size exclusion chromatography–HPLC (SEC–HPLC) analysis was performed to estimate the molecular mass of the native enzyme using a non-denatured protein molecular weight marker kit (Biorad, Madrid, Spain). The enzyme was eluted with 0.1 M buffers from pH 6.0 to 9.0 at a flow rate of 0.5 ml min^{-1} and measured at 280 nm in an HPLC System with a Biosep-SEC-S2000 column (Phenomenex, Madrid, Spain). Molecular mass of the monomeric form was estimated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) by the Laemmli method [31] using a low molecular weight marker kit (Amersham Biosciences, Barcelona, Spain).

2.7. Protein characterization

The thermal stability of the GkNSAAR enzyme was measured after 30 and 60 min of preincubation at temperatures from 4 to 95 °C in 100 mM borate/HCl buffer (pH 8.0). Enzyme assay was then carried out at 55 °C for 20 min with the *N*-acetyl-D-methionine substrate together with the purified GkNSAAR enzyme. To analyze the effect on the enzyme of 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), β-mercaptoethanol, dithiothreitol (DTT), 8-hydroxy-5-quinolinesulfonic acid (HQSA), $HgCl_2$,

$NiCl_2$, $MgCl_2$, $MnCl_2$, $CoCl_2$, $CuCl_2$, $ZnCl_2$, $CaCl_2$, $PbCl_2$, $FeCl_2$, $FeCl_3$, LiCl, NaCl, KCl, RbCl and CsCl, 0.5 mM of each metal and DTNB, and 10 mM of β-mercaptoethanol, DTT and HQSA were added to the reaction without previous incubation. The specific activity for the effect of metals was determined by standard enzyme assay.

2.8. Nucleotide sequence accession number

The nucleotide sequence of GkNSAAR has been deposited in the GenBank database under accession no. EU427322.

3. Results and discussion

3.1. Sequence comparison of N-succinylamino acid racemase

G. kaustophilus CECT4264 presents a specific fragment for the nsaar gene (Gknsaar). The deduced amino acid sequence of the *G. kaustophilus* enzyme was compared to NSAAR sequences of proven activity from different sources (Fig. 1). The highest amino acid sequence identity (48.39%) was found with the NSAAR from *D. radiodurans* (DrNSAAR) [13]. When compared with the three *Amycolatopsis* strains, the identity percentage was similar but slightly lower: 44.74%, 43.78% and 43.67% for *Amycolatopsis* sp. TS-1-60 (AsNSAAR) [11], *A. orientalis* subsp. *lurida* (AoNSAAR) [7], and *Amycolatopsis azurea* (AaNSAAR) [12], respectively.

3.2. Functional expression and purification of GkNSAAR

The *N*-succinylamino acid racemase gene was expressed in *E. coli* BL21 (DE3). *N*-Acylamino acid racemase activity was determined in crude extracts by HPLC using *N*-acetyl-D-methionine as substrate (see Section 2). A one-step purification procedure of the recombinant GkNSAAR fused to His6 tag was employed using immobilized cobalt affinity chromatography followed by

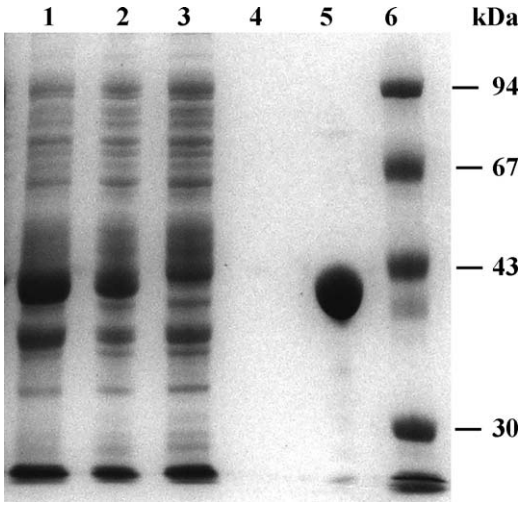

Fig. 2. SDS-PAGE analysis of each purification step of GkNSAAR from *E. coli* BL21 (DE3) harbouring the pJPD25 plasmid. Lanes 1 and 2: pellet and supernatant of the resuspended crude extract after cell sonication; lane 3: eluate after adding the sonicated supernatant to the metal affinity column; lane 4: flow-through after washing the metal affinity column with buffer; lane 5: purified enzyme; lane 6: low molecular weight marker.

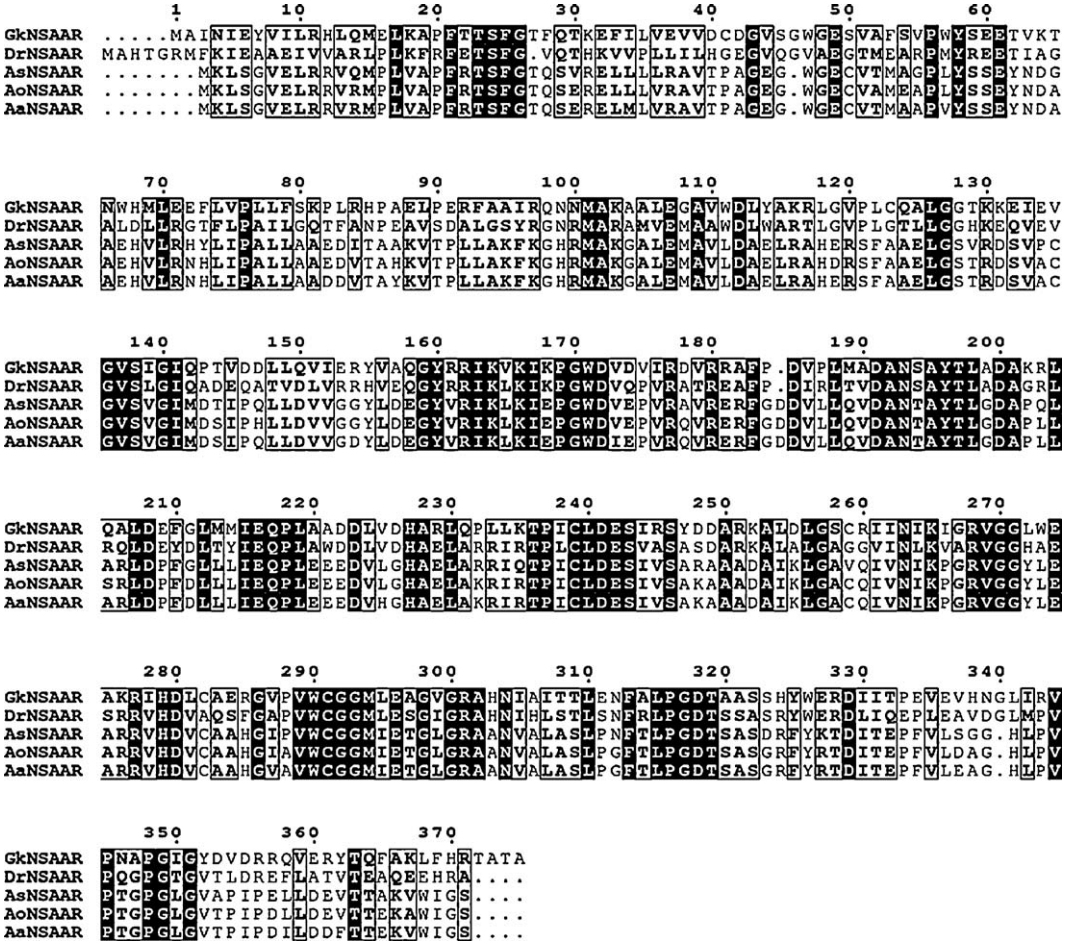

Fig. 1. Multiple alignments of the amino acid sequences of NSAARs. NSAAR from *Geobacillus kaustophilus* (GkNSAAR), GenBank accession no. EU427322; NSAAR from *Deinococcus radiodurans* (DrNSAAR), GenBank accession no. Q9RYA6; NSAAR from *Amycolatopsis* sp. TS-1-60 (AsNSAAR), GenBank accession no. D30738; NSAAR from *Amycolatopsis orientalis* subsp. *lurida* (AoNSAAR), GenBank accession no. AJ292519; NSAAR from *Amycolatopsis azurea* (AaNSAAR), GenBank accession no. AY271627.

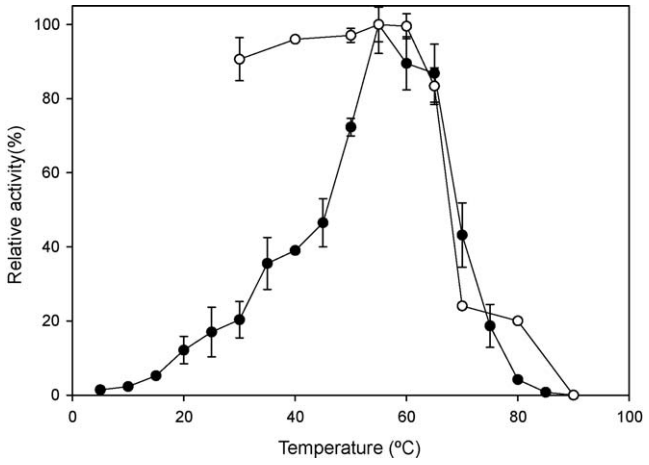

Fig. 3. Effect of temperature on *N*-acylamino acid racemase activity of GkNSAAR. Activity measured at different temperatures using standard enzyme assay (black circles). Activity after incubation at the indicated temperatures for 60 min (white circles). The remaining activity was measured at 55 °C for 20 min using *N*-acetyl-D-methionine substrate.

proteolytic digestion with thrombin (see Section 2). SDS-PAGE analysis indicated that the purified enzyme was over 95% pure after elution of the affinity column (Fig. 2). Specific activity was calculated for the purified enzyme including His6 tag, and also after removing it by thrombin dependent cleavage, showing no differences in enzymatic activity or biochemical characteristics (results not shown). The purified enzyme could be stored in 100 mM borate/HCl (pH 8.0) at 4 °C for over 1 month without noticeable loss of activity.

3.3. Molecular mass and subunit structure of GkNSAAR

Apparent molecular mass of the GkNSAAR subunit calculated by SDS-PAGE analysis was about 40–43 kDa (Fig. 2), similar to that deduced from the amino acid sequence (42,294 Da). This molecular mass of the subunit is highly conserved in all the recombinant and native NSAARs purified to date, with values of 40–41 kDa [7,9–13]. Likewise, the molecular mass deduced from the amino acid sequence was nearly the same for all the recombinant NSAARs (data not shown). However, the similar subunit molecular mass among the different NSAARs was not found after comparing the relative molecular mass of the native enzymes. Thus, for GkNSAAR the molecular mass estimated by SEC–HPLC on a Biosep-SEC-S2000 column was about 152,000 Da. The molecular mass is considerably

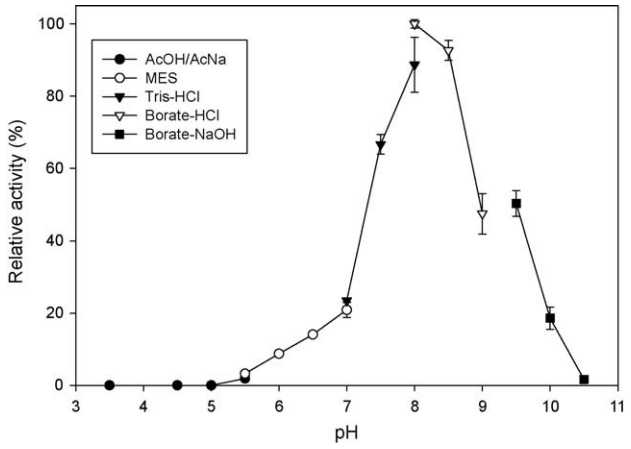

Fig. 4. Effect of pH on *N*-acylamino acid racemase activity of GkNSAAR calculated at different pHs using enzyme assays at 55 °C for 20 min with *N*-acetyl-D-methionine substrate.

Table 1
Effect of metal ions and chemical agents on the *N*-acylamino acid racemase activity of GkNSAAR.

Compound	Specific activity (U mg^{-1})	Relative activity (%)
None	0.05 ± 0.00	0
Pb^{2+}	0.06 ± 0.00	0
Ca^{2+}	0.15 ± 0.02	1
Fe^{3+}	0.24 ± 0.04	2
Fe^{2+}	0.22 ± 0.01	2
Hg^{2+}	0.04 ± 0.01	0
Mn^{2+}	6.16 ± 0.12	48
Zn^{2+}	0.51 ± 0.03	4
Co^{2+}	12.83 ± 0.10	100
Cu^{2+}	0.03 ± 0.00	0
Mg^{2+}	1.27 ± 0.08	10
Ni^{2+}	4.63 ± 0.26	36
Na^{+}	0.07± 0.01	1
K^{+}	0.07 ± 0.00	1
Cs^{+}	0.07 ± 0.00	1
Li^{+}	0.07 ± 0.00	1
Rb^{+}	0.08 ± 0.00	1
Co^{2+} + DTT	0.01 ± 0.00	0
Co^{2+} + β-mercaptoethanol	0.03 ± 0.00	0
Co^{2+} + HQSA	0.03 ± 0.00	0
Co^{2+} + HQSA + Co^{2+}	12.78 ± 0.53	100
Co^{2+} + HQSA + Mn^{2+}	5.22 ± 0.04	41
Co^{2+} + HQSA + Ni^{2+}	3.98 ± 0.37	31
Co^{2+} + HQSA + Mg^{2+}	1.04 ± 0.05	8
Co^{2+} + HQSA + Zn^{2+}	0.52 ± 0.03	4

The chloride salt of the metal ions, reducing, sulfydryl or chelating reagents was added directly to the reaction. The restoration of *N*-acylamino acid racemase activity of GkNSAAR by metal ions after treatment with HQSA chelating agent was also studied. The enzyme was incubated overnight with 10 mM of HQSA at 4 °C. The chelating agent was removed by dialysis in four stages at 12-h intervals, all at 4 °C. Divalent metal salt solutions were added directly to the reaction. Activity assays were carried out in triplicate as described in Section 2 with *N*-acetyl-D-methionine as substrate.

lower than those of NSAARs previously described from *S. atratus* Y-53 (SaNSAAR) with 244,000 Da [9], DrNSAAR, AsNSAAR and AoNSAAR with 300,000 Da [7,10,13], or AaNSAAR with 320,000 Da [12]. This phenomenon shows the heterogenic structural distribution of the subunits that make up the whole protein; four NSAARs are homooctamers, one is a homohexamer and the NSAAR described in this study is a homotetramer.

3.4. Influence of temperature and pH on GkNSAAR activity

The temperature activity profile of purified GkNSAAR was determined at values between 5 and 90 °C using the physiological

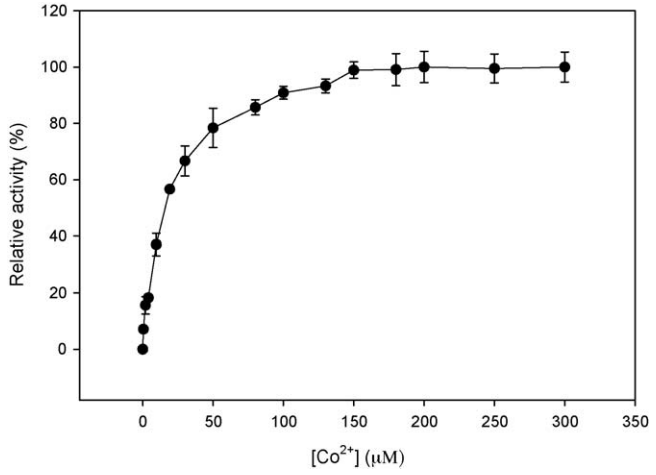

Fig. 5. Effect of Co^{2+} on *N*-acylamino acid racemase activity of GkNSAAR calculated at different Co^{2+} concentrations using enzyme assays at 55 °C for 20 min with *N*-acetyl-D-methionine substrate and protein concentration of 0.09 μM.

pH of 7.5. Maximum enzymatic activity occurred at 55 °C (Fig. 3). At this temperature, the optimum pH was determined for racemization of *N*-acetyl-D-methionine as substrate in sodium acetate–acetic acid, MES, Tris–HCl, borate/HCl and borate/NaOH buffers at a concentration of 100 mM and pH values of 3.0–5.5, 5.5–7.0, 7.0–8.0, 8.0–9.0 and 9.5–10.5, respectively. Optimal pH to obtain the maximum enzymatic activity at 55 °C was pH 8.0 (Fig. 4). Thermal stability studies showed a gradual loss of activity on preincubation for 1 h at temperatures over 60 °C (Fig. 3). These parameters are the same as those obtained for *D. radiodurans* and *Amycolaptosis* sp. NSAARs for thermal stability and optimum reaction temperature [10,13], suggesting that they are thermostable proteins. Thermal stability of AoNSAAR was practically the same (50 °C), but surprisingly the optimum reaction temperature fell to 30 °C [7]. AaNSAAR and SaNSAAR have proven to be the most thermosensitive proteins (40–45 °C) and they have a low optimum reaction temperature (40 °C) [9,12]. Optimal pH values were very similar for all the NSAARs, ranging from pH 7.4 for *A. azurea* to 8.0 for the one studied in this work, *D. radiodurans* and *A. orientalis*.

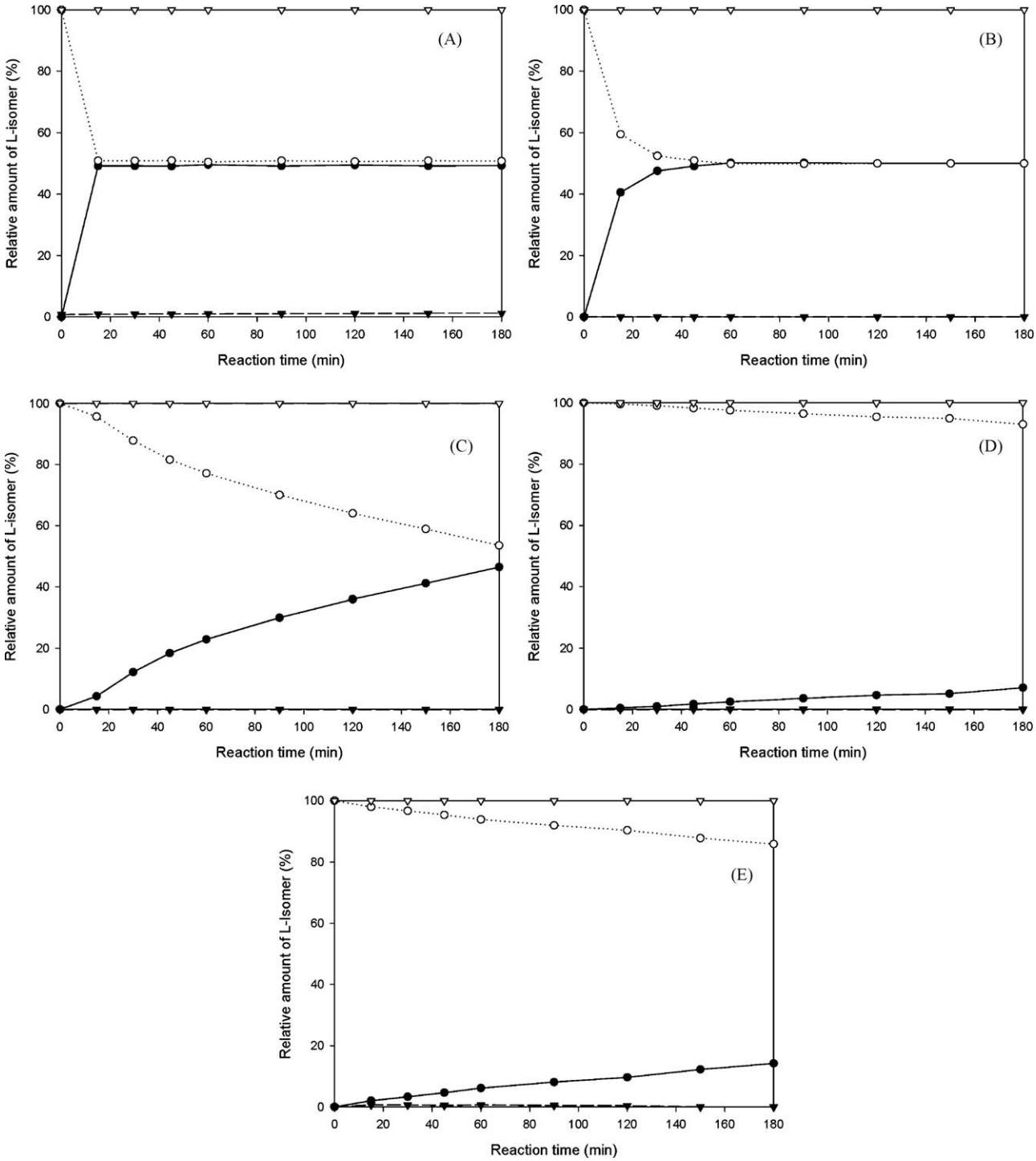

Fig. 6. Enzymatic racemization of the D-isomer (●) and L-isomer (○) of different *N*-acetylamino acids by recombinant GkNSAAR. The reaction was followed by chiral-HPLC at the points shown in the graphs. Chemical racemization of the D-isomer (▼) and L-isomer (▽) of each substrate was also measured at the same intervals. (A) *N*-Acetyl-methionine; (B) *N*-acetyl-phenylalanine; (C) *N*-acetyl-alanine; (D) *N*-acetyl-asparagine; (E) *N*-acetyl-tryptophan.

3.5. Effects of metal ions and chemical agents

N-Acylamino acid racemase activity of the purified GkNSAAR enzyme was assayed in the presence of 2 mM of different metal ions using *N*-acetyl-D-methionine as substrate (Table 1). Pre-incubation of the enzyme with metal ions for several hours before the reaction produced the same results as directly adding the metal ions to the reaction (data not shown). The metal ions were therefore included as a component of the reaction. The protein was nearly inactive after elution from the metal affinity column. Several metal ions such as Co^{2+}, Mn^{2+}, Ni^{2+} and Mg^{2+} greatly enhanced the activity, Co^{2+} proving the most effective. However, enzyme activity was not affected by the other metal ions. Inhibition effect was studied in the active protein incubated with Co^{2+} (2 mM, at 4 °C for 12 h). The reducing compounds dithiothreitol (DTT) and β-mercaptoethanol, and the chelating agent 8-hydroxy-5-quinolinesulfonic acid caused total inhibition of the enzyme. However, activity restoration was possible after removing the chelating agent by dialysis and adding 2 mM of Co^{2+}, Mn^{2+}, Ni^{2+} and Mg^{2+}. Fig. 5 shows the Co^{2+}-dependent activity profile of GkNSAAR, with maximum enzymatic activity at 200 μM of Co^{2+} in the reaction, for a protein concentration of 0.09 μM (Co^{2+}:protein ratio of about 2000:1), with no noticeable inhibition at higher metal ion concentrations (5 mM). Our results agree with previous works that suggested NSAAR as a metalloenzyme and Co^{2+} as the best cofactor [7,9–13]. The present work has also demonstrated that *N*-acylamino acid racemase activity can be restored in the enzyme previously treated with chelating agents. Although previous studies on AoNSAAR had reported that higher concentrations of metal ions enhanced the enzyme activity [7], this is the first work that reports the metal ion requirements for maximum enzymatic activity.

3.6. Substrate enantioselectivity and kinetic characterization of GkNSAAR

The ability of the purified GkNSAAR enzyme to racemize different *N*-acetylamino acids was examined. The D- and L-isomers of *N*-acetylamino acids were completely racemized (Fig. 6). A high rate of *N*-acylamino acid racemase activity was detected for *N*-acetylamino acids of methionine and phenylalanine compared to those of alanine, asparagine and tryptophan, which racemized slowly. These results were corroborated by examining the kinetic parameters for different substrates. These parameters were obtained from hyperbolic saturation curves by least-square fit of the data to the Michaelis–Menten equation, using protomeric unit as protein concentration (Table 2). Reactions were carried out at different concentrations of optically pure substrates at 55 °C at pH 8.0 in the presence of 1 mM Co^{2+}. GkNSAAR showed activity towards both aliphatic and aromatic *N*-acetylamino acids, such as *N*-acetyl-methionine and *N*-acetyl-phenylalanine, respectively, with k_{cat}/K_m values from 0.4×10^3 to 3×10^3 s^{-1} M^{-1}. Apparent K_m values were similar for *N*-acetyl-tryptophan and *N*-acetyl-methionine (Table 2), but k_{cat} values for the former were considerably lower (two orders of magnitude). GkNSAAR was very active towards *N*-carbamoyl-methionine with k_{cat}/K_m values of over 10^3 s^{-1} M^{-1}. Kinetic parameters were better for *N*-acetyl-D-amino acids than for *N*-acetyl-L-specific ones. However, for *N*-carbamoyl-methionine, the k_{cat}/K_m value was higher for *N*-carbamoyl-D-specific amino acid. The highest kinetic parameters of the enzyme were presented using both isomers of *N*-succinylalanine and *N*-succinylphenylalanine as substrates, thus agreeing with the new nomenclature proposed by the Gerlt group [18].

Table 2
Kinetic parameters of GkNSAAR.

Substrate	K_m (mM)	k_{cat} (s^{-1})[a]	k_{cat}/K_m (s^{-1} M^{-1})
N-Acetyl-L-methionine	8 ± 1	22 ± 1	$(2.8 \pm 0.5) \times 10^3$
N-Acetyl-D-methionine	7 ± 0	20 ± 1	$(3.0 \pm 0.1) \times 10^3$
N-Acetyl-L-phenylalanine	43 ± 4	16 ± 1	370 ± 67
N-Acetyl-D-phenylalanine	23 ± 3	10 ± 0	467 ± 67
N-Acetyl-L-alanine	41 ± 3	2 ± 0.0	39 ± 4
N-Acetyl-D-alanine	17 ± 2	0.8 ± 0.0	47 ± 6
N-Acetyl-L-tryptophan	2 ± 0.3	0.15 ± 0.00	60 ± 9
N-Acetyl-D-tryptophan	2 ± 0.2	0.09 ± 0.00	61 ± 9
N-Acetyl-L-asparagine	27 ± 2	0.06 ± 0.00	2 ± 0.2
N-Acetyl-D-asparagine	18 ± 2	0.07 ± 0.00	4 ± 0.3
N-Carbamoyl-L-methionine	5 ± 0.9	2 ± 0.0	$(1.1 \pm 0.2) \times 10^3$
N-Carbamoyl-D-methionine	2 ± 0.1	2 ± 0.0	$(1.2 \pm 0.1) \times 10^3$
N-Succinyl-L-alanine	0.12 ± 0.01	43 ± 8	$(35.20 \pm 4.22) \times 10^4$
N-Succinyl-D-alanine	0.13 ± 0.01	15 ± 1	$(11.93 \pm 0.53) \times 10^4$
N-Succinyl-L-phenylalanine	0.13 ± 0.02	5 ± 0.0	$(3.51 \pm 0.05) \times 10^4$
N-Succinyl-D-phenylalanine	0.04 ± 0.00	2 ± 0.1	$(3.81 \pm 0.53) \times 10^4$

The kinetic parameters of GkNSAAR were determined at 55 °C for 20 min at pH 8.0 in the presence of 1 mM Co^{2+}. Reactions were performed in triplicate and the maximum substrate concentration was 100 mM for all substrates except for *N*-acetyl-tryptophan, for which it was 60 mM.

[a] Protein concentration was expressed as protomeric units.

4. Conclusion

We have cloned, overexpressed, purified and characterized a novel NSAAR from *G. kaustophilus* CECT4264 with *N*-acylamino acid racemase activity. The enzyme has shown a broad substrate range with activity towards aliphatic and aromatic *N*-acetylamino acids, as well as high thermostability with gradual loss of activity at preincubation temperatures over 60 °C. These results make this enzyme an excellent candidate for a multienzymatic system together with an *N*-aminoacylase for the production of optically pure amino acids from racemic mixtures of *N*-acetylamino acids.

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References

[1] Breuer M, Kitrich K, Habicher T, Hauer B, Keseler M, Stümer R, et al. Industrial methods for the production of optically active intermediates. Angew Chem Int Ed 2004;43:788–824.
[2] Bommarius AS, Schwarm M, Drauz K. Biocatalysis to amino acid-based chiral pharmaceuticals—examples and perspectives. J Mol Catal B Enzym 1998;5:1–11.
[3] Clemente-Jimenez JM, Martinez-Rodriguez S, Rodriguez-Vico F, Las Heras-Vazquez FJ. Optically pure α-amino acids production by the "Hydantoinase Process". Recent Pat Biotechnol 2008;2:35–46.
[4] Wolf LB, Sonke T, Tjen KCMF, Kaptein B, Broxterman QB, Schoemaker HE, et al. A biocatalytic route to enantiomerically pure unsaturated α-H-α-amino acids. Adv Synth Catal 2001;343:662–7.
[5] Asano Y. Enzymes acting on D-amino acid amides. In: Konno R, Brückner H, D'Aniello A, Fisher GH, Fujii N, Homma H, editors. D-amino acids: a new frontier in amino acid and protein research—practical methods and protocols. New York: Nova Biomedical Books; 2007. p. 579–89.
[6] May O, Verseck S, Bommarius A, Drauz K. Development of dynamic kinetic resolution processes for biocatalytic production of natural and nonnatural L-amino acids. Org Process Res Dev 2002;6:452–7.
[7] Verseck S, Bommarius A, Kula MR. Screening, overexpression and characterization of an *N*-acylamino acid racemase from *Amycolatopsis orientalis* subsp. *lurida*. Appl Microbiol Biotechnol 2001;55:354–61.

[8] Tokuyama S, Hatano K, Takahashi T. Discovery of a novel enzyme, *N*-acylamino acid racemase in an actinomycete: screening, isolation and identification. Biosci Biotechnol Biochem 1994;58:24–7.
[9] Tokuyama S, Miya H, Hatano K, Takahashi T. Purification and properties of a novel enzyme, *N*-acylamino acid racemase, from *Streptomyces atratus* Y-53. Appl Microbiol Biotechnol 1994;40:835–40.
[10] Tokuyama S, Hatano K. Purification and properties of thermostable *N*-acylamino acid racemase from *Amycolatopsis* sp. TS-1-60. Appl Microbiol Biotechnol 1995;42:853–9.
[11] Tokuyama S, Hatano K. Cloning, DNA sequencing and heterologous expression of the gene for thermostable N-acylamino acid racemase from *Amycolatopsis* sp. TS-1-60 in *Escherichia coli*. Appl Microbiol Biotechnol 1995;42:884–9.
[12] Su SC, Lee CY. Cloning of the *N*-acylamino acid racemase gene from *Amycolatopsis azurea* and biochemical characterization of the gene product. Enzyme Microb Technol 2002;30:647–55.
[13] Wang WC, Chiu WC, Hsu SK, Wu CL, Chen CY, Liu JS, et al. Structural basis for catalytic racemization and substrate specificity of an *N*-acylamino acid racemase homologue from *Deinococcus radiodurans*. J Mol Biol 2004;342:155–69.
[14] Tokuyama S. Discovery and application of a new enzyme *N*-acylamino racemase. J Mol Catal B Enzym 2001;12:3–14.
[15] Palmer DR, Garrett JB, Sharma V, Meganathan R, Babbitt PC, Gerlt JA. Unexpected divergence of enzyme function and sequence: "*N*-acylamino acid racemase" is o-succinylbenzoate synthase. Biochemistry 1999;38:4252–8.
[16] Tokuyama S, Hatano K. Overexpression of the gene for *N*-acylamino acid racemase from *Amycolaptosis* sp. T-1-60 in *Escherichia coli* and continuous production of optically active methionine by a bioreactor. Appl Microbiol Biotechnol 1996;44:774–7.
[17] Taylor Ringia EA, Garrett JB, Thoden JB, Holden HM, Rayment I, Gerlt JA. Evolution of enzymatic activity in the enolase superfamily: functional studies of the promiscuous o-succinylbenzoate synthase from *Amycolatopsis*. Biochemistry 2004;43:224–9.
[18] Sakai A, Xiang DF, Xu C, Song L, Yew WS, Raushel FM, et al. Evolution of enzymatic activities in the enolase superfamily: *N*-succinylamino acid racemase and a new pathway for the irreversible conversion of D- to L-amino acids. Biochemistry 2006;45:4455–62.
[19] Dermirjian DC, Moris-Vargas F, Cassidy CS. Enzymes from extremophiles. Curr Opin Chem Biol 2001;5:144–51.
[20] Becker P, Abu-Reesh I, Markossian S, Antranikian G, Maerkl H. Determination of the kinetic parameters during continuous cultivation of the lipase-producing thermophile *Bacillus* sp. IHI-91 on olive oil. Appl Microbiol Biotechnol 1997;48:184–90.
[21] Takami H, Takaki Y, Chee G-J, Nishi S, Shimamura S, Suzuki H, et al. Thermoadaptation trait revealed by the genome sequence of thermophilic *Geobacillus kaustophilus*. Nucleic Acids Res 2004;32:6292–303.
[22] Ausubel F, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, et al. Short protocols in molecular biology. Hoboken: Wiley; 1992.
[23] Sambrook J, Fritsch EF, Maniatis T. Molecular cloning: a laboratory manual. New York: Cold Spring Harbour Laboratory Press; 1989.
[24] Martinez-Rodriguez S, Clemente-Jimenez JM, Rodriguez-Vico F, Las Heras-Vazquez FJ. Molecular cloning and biochemical characterization of L-*N*-carbamoylase from *Sinorhizobium meliloti* CECT4114. J Mol Microbiol Biotechnol 2005;9:16–25.
[25] Studier FW, Moffatt BA. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. J Mol Biol 1986;189:113–30.
[26] Martinez-Rodriguez S, Gonzalez-Ramirez LA, Clemente-Jimenez JM, Rodriguez-Vico F, Las Heras-Vazquez FJ, Gavira JA, et al. Crystallization and preliminary crystallographic studies of the recombinant dihydropyrimidinase from *Sinorhizobium meliloti* CECT4114. Acta Crystallogr Sect F Struct Biol Cryst Commun 2006;62:1223–6.
[27] Jeanmougin F, Thompson JD, Gouy M, Higgins DG, Gibson TJ. Multiple sequence alignment with Clustal X. Trend Biochem Sci 1998;10:403–5.
[28] Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol 1990;5:403–10.
[29] Gouet P, Courcelle E, Stuart DI, Metoz F. ESPript: multiple sequence alignments in Postscript. Bioinformatics 1999;15:305–8.
[30] Galtier N, Gouy M, Gautier C. Seaview and PHYLO_WIN: two graphic tools for sequence alignment and molecular phylogeny. Comput Appl Biosci 1996;12:543–8.
[31] Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T. Nature 1970;227:680–5.