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Stereoselective synthesis of L-[^{15}N] amino acids with glucose dehydrogenase and galactose mutarotase as NADH regenerating system

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We have developed an efficient stereospecific enzymatic synthesis of L-[^{15}N]-valine, L-[^{15}N]-leucine, L-[^{15}N]-norvaline, L-[^{15}N]-norleucine and L-[^{15}N]-isoleucine from the corresponding α-keto acids by coupling the reactions catalysed by leucine dehydrogenase and glucose dehydrogenase/galactose mutarotase. Giving high yields of L-amino acids, the procedure is economical and easy to perform and to monitor at a synthetically useful scale (1–10 g).

Keywords: amino acids; nitrogen 15; enzymatic synthesis; mass spectrometry

Introduction

Amino acids labelled with stable isotopes represent useful tools in medicine and agriculture to investigate the balance between protein synthesis and degradation.[1–3] On the other hand they have served for more than two decades in determining the three-dimensional structure of proteins by heteronuclear NMR spectroscopy.[4,5] Although several L-amino acids labelled with ^{15}N are commercially available, some of them such as L-[^{15}N]-methionine or L-[^{15}N]-serine are still prohibitively expensive.

Among the various methods proposed for the synthesis of naturally occurring amino acids, the enzymatic procedures, being stereospecific, are the most frequently used. Thus, by coupling the reaction catalysed by one of the well-characterized amino acid dehydrogenases (glutamate-, alanine-, leucine- or phenylalanine dehydrogenase) with a NADH generating reaction (glucose-6-phosphate/glucose-6-phosphate dehydrogenase; ethanol/alcohol dehydrogenase; lactate/lactate dehydrogenase; formic acid/formate dehydrogenase) ten proteinogenic L-amino acids labelled with ^{15}N can be produced from millimolar to molar scale.[6–10] The required specific enzymes could be obtained currently at low cost by recombinant DNA technology.[11]

In this paper we report an improved enzymatic procedure for preparation of L-[^{15}N]-valine, L-[^{15}N]-leucine, L-[^{15}N]-norvaline, L-[^{15}N]-norleucine and L-[^{15}N]-isoleucine from the corresponding α-keto acids, by using leucine dehydrogenase (LeuDH) as a catalyst, and glucose/glucose dehydrogenase (GlucDH) plus galactomutarotase (GalM) as NADH regeneration system, according to the following reactions:

(1) α keto acid + NADH + H^+ + $^{15}NH_3$ $\xrightarrow{LeuDH}$ L-[^{15}N] amino acid + NAD^+.

(2) β glucose + NAD^+ $\xrightarrow{GlucDH}$ gluconate + NADH + H^+.

(3) α glucose $\xrightleftharpoons{GalM}$ β glucose.

Overall:

α keto acid + $^{15}NH_3$ + α glucose $\xrightarrow[GlucDH,\ GalM]{NAD,\ LeuDH}$ L-[^{15}N]amino-acid + gluconate.

The substrate of GlucDH is the β-anomer of D-glucose and the 'spontaneous' anomerization rate of α-glucose to β-glucose is low (half-time of approximately 20 min at 30°C). Consequently, for a fast and quantitative conversion of the α-keto acid to the corresponding L-[^{15}N] amino acid using stoichiometric amounts of sugar, GalM is required as a complementary catalyst.

Experimental

Chemicals

$^{15}NH_4Cl$ 99 at.% ^{15}N was produced at the National Institute for Research and Development of Isotopic and Molecular Technologies, Cluj-Napoca, Romania. The various α-keto acids mentioned in this paper were purchased from Sigma. Nucleotides, restriction enzymes, T4 DNA ligase, Vent and *Tfu* DNA

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polymerases were purchased from Roche Applied Science, New England Biolabs, or Qbiogene Inc.

Recombinant DNA technology

General DNA manipulations were performed as described by Sambrook *et al.*[11] The gene encoding LeuDH from *Bacillus stearothermophillus* was amplified using the corresponding genomic DNA as template and the oligonucleotides listed in Table 1 as primers in a standard polymerase chain reaction (PCR) experiment. The amplified DNA was then digested with *Bam*HI and *Xho*I restriction enzymes, purified and inserted by ligation in the pET21b vector (Novagen) digested with the same restriction enzymes.

The genes encoding GlucDH from *B.subtilis* and GalM from *Escherichia coli* were amplified by PCR using chromosomal DNA from the corresponding bacteria as templates and the oligonucleotides indicated in Table 1 as primers. The amplified products were then digested with *Nde*I and *Bam*HI restriction enzymes, purified and inserted by ligation in the pET24a (Novagen) digested with the same restriction enzymes. After transformation of strain DH5α, the plasmid DNA was purified from several colonies and digested to check for the presence of an insert of the correct size. The plasmid with the good insert was used to transform strain BL21 (DE3). For overexpression of proteins, BL21 (DE3) containing either pET24a GlucDH, pET24a GalM or pET21b LeuDH was grown at 37°C in 2YT medium supplemented with kanamycin (30 μg/ml) and chloramphenicol (30 μg/ml) for the first two strains, and in Luria–Bertani medium supplemented with ampicillin (100 μg/ml) for the third one. When OD_{600} value 1.3 was reached isopropyl β-D-thiogalacto-pyranoside (IPTG) was added at a final concentration of 1 mM, and the cultures were further incubated at 37°C for 3 h, before being harvested by centrifugation.

Preparation of bacterial extracts and determination of enzyme activities

The bacterial pellet was stored at −80°C until used for amino acid synthesis. Bacteria were defrosted in 50 mM Tris-HCl (pH 7.4), then sonicated at 4°C and centrifuged at 10 000*g* for 30 min. The supernatant was tested for enzyme activity at 30°C and 340 nm by following either the formation or the consumption of NADH. One unit (1 U) of enzyme activity corresponds to 1 μmole of NADH formed or consumed in 1 min. LeuDH activity was determined in a medium containing 50 mM Tris-HCl (pH 8.0), 10 mM α-keto acid, 0.15 mM NADH and 1 M NH_4Cl. Under these conditions the specific activity of a purified enzyme preparation (U/mg protein) corresponds to the following numbers : 168 (α-ketovalerate), 46.5 (α-keto-β-methylvalerate), 36.8 (α-ketoisocaproate), 17.4 (α-ketocaproate), 7.8 (α-keto-γ-methylthiobutyrate) and 4.7 (α-ketobutyrate). In the crude bacterial extract, the specific activity of LeuDH with these α-keto acids is approximately four times lower. Since the enzyme activity is dependent on the chemical nature of the α-keto acid, the number of units used in each synthesis is calculated according to the corresponding α-keto acid. GlucDH activity was assayed in a medium containing 50 mM Tris HCl (pH 8.0), 10 mM glucose and 2 mM NAD. The specific activity of a crude bacterial extract in which GlucDH is overproduced is of about 200 U/mg of protein. GalM activity was determined in 50 mM Tris-HCl (pH 8.0), 2 mM NAD, 1 U of GlucDH and appropriately diluted bacterial extract containing overproduced GalM. Then freshly dissolved glucose in Tris-HCl buffer was added to the medium and the initial rate of NADH formation recorded at 340 nm. The rate of NADH formation in the absence of GalM was subtracted to determine the enzyme-catalysed anomerization. LeuDH, GlucDH and GalM can be stored at −20 or −80°C for months without significant loss of activity.

Synthesis and purification of L [^{15}N]-labelled amino acids

A typical synthesis of L[^{15}N]-labelled amino acid was performed as follows: 8 mmol glucose, 8 mmol α-keto acid (Na-salt) and 8 mmol [^{15}N] ammonium chloride (~99 at.% ^{15}N) were dissolved in 65 ml of bidistilled water maintained at 30°C and the pH adjusted to 8.0 with 1 N NaOH. Then 0.2 mmol NAD^+, followed by LeuDH (200 U), GlucDH (100 U) and GalM (100 U) were added and the volume adjusted to 70 ml. Under these conditions the formation of L[^{15}N]-labelled amino acid is accompanied by the release of gluconic acid and the pH decreases accordingly. By adding small volumes of 1 N NaOH the pH is restored to 8.0–8.2. The consumed NaOH indicates the amount of amino acid formed. Routinely after 2–3 h the reaction was complete and no further change in pH was observed. The disappearance of ammonia was tested colorimetrically; then the reaction mixtures were heated to 85°C for 15 min. After chilling, the denaturated proteins were removed by filtration or centrifugation. The solution was passed onto a 120 ml column (2.5 × 25 cm) of Dowex 50WX-8 in H^+ form. After washing the column with water, the adsorbed amino acid was eluded with 1 M ammonium hydroxide.

The fractions of the eluate containing the labelled amino acid were concentrated in a rotary vacuum evaporator at 50°C to approximately 5 ml. Ninety millilitre of 50/50 (v/v) and anhydrous ethanol/ether were added and the mixture was kept overnight in the refrigerator, while the amino acid crystallized. The precipitate was filtered off and dried. Table 2 shows the yield for various amino acids synthesized.

Table 1.

Enzyme	Primer	Template
LeuDH	5′-GGTGGATCCGATGGAATTGTTCAAATATATGG-3′ 5′-TATTCTCGAGTATTGCCGAAGCACCTGC-3′	*B. stearothermophilus*
GlucDH	5′-GGAATTCCATATGTATCCGGATTTAAAAGGAAAAG-3′ 5′-CGCGGATCCTCAACCGCGGCCTGCCTGG-3′	*B. subtilis*
GalM	5′-GGAATTCCATATGCTGAACGAAACTCCCGCAC-3′ 5′-CGCGGATCCTCACTCAGCAATAAACTGATATTCCG-3′	*E. coli*

Characterization of L[^{15}N]-labelled amino acids by mass spectrometry

A currently applied method for determining the percentage of labelled atoms is the analysis of amino acids by GC/MS. The isotope analysis requires derivatization of amino acids to more volatile compounds. The amino acids were converted to their N,O-tert-butyldimethylsilyl (TBDMS) derivatives using methyl, tert-butylsilyl trifluoracetamide (MTBSTFA) as TBDMS donor reagent and acetonitrile as solvent. Approximately 0.5–1.0 mg of labelled amino acid in 150 µl acetonitrile and 50 µl MTBSTFA with 1% DMTBCIS were reacted at 120°C for 20 min. Chromatographic separation of the TBDMS derivatives was performed on a DB5 capillary column, 30 m programmed from 55 to 250°C. The electron ionization mass spectra were recorded on a HP 5985 GC/MS system. The cromatogram of the five amino acid mixtures is shown in Figure 1. Elution is as follows:

Retention times: (1) Val = 25.54 min; (2) norVal = 26.16 min; (3) Leu = 27.12 min; (4) Ile = 28.15 min and (5) norLeu = 28.90 min.

The isoleucine peak is split into two components representing isomers due to its asymmetric carbon atom. The mass spectra corresponding to these isomers are identical. The mass spectra do not show molecular ions but may be recognized due to the characteristic fragmentation pattern. The spectra contain the following characteristic fragment ions:

$M_{val.deriv.}$ = 346.2 ($C_{17}H_{39}$ $^{15}NO_2Si_2$); The fragment ions characteristic for ^{15}N-valine: *m/z*: 187 (M-159), 261 (M-85), 289 (M-57), 303 (M-43).

$M_{norval\ deriv.}$ = 346.2 ($C_{17}H_{39}$ $^{15}NO_2Si_2$); The fragment ions characteristic for ^{15}N-norvaline: *m/z*: 187, 261, 289, 303.

$M_{leu.deriv.}$ = 360.3 ($C_{18}H_{41}$ $^{15}NO_2Si_2$); The fragment ions characteristic for ^{15}N-leucine: *m/z*: 201 (M-159), 275 (M-85), 303 (M-57).

$M_{Ile\ deriv.}$ = 360.3 ($C_{18}H_{41}$ $^{15}NO_2Si_2$); The fragment ions characteristic for ^{15}N-isoleucine: *m/z*: 201, 275, 303.

Table 2.

Substrate	Product	Yield (%)
α-Ketoisovalerate	l-Valine	90
α-Ketoisocaproate	l-Leucine	92.5
α-Ketovalerate	l-Norvaline	92
α-Ketocaproate	l-Norleucine	80
α-Keto, β-methyl valerate	l-Isoleucine	95

$M_{Norleu\ deriv.}$ = 360.3 ($C_{18}H_{41}$ $^{15}NO_2Si_2$); The fragment ions characteristic for ^{15}N-norleucine: *m/z*: 201, 275, 303.

The ^{15}N isotope content determined from the isotopic peaks of the main fragment ions remained the same as that of ammonium chloride, without isotopic dilution during synthesis, at least 99 at.% ^{15}N.

Discussion and concluding remarks

The use of bacterial aromatic or aliphatic amino acid dehydrogenases obtained from mesophylic or thermophylic organisms for preparative purposes is now well documented.[8–14] However, when amino acids labelled with stable isotopes are targeted, the choice of an appropriate enzyme and an efficient and cheap NADH regenerating system is still a critical issue to achieve maximal yields and undetectable isotopic dilution. GlucDH, like formate dehydrogenase-catalyzed reaction is irreversible and the overall yield approaches 100% when it is coupled to the reductive amination of various α-keto acids. Moreover, the catalytic efficiency of GlucDH expressed as the ratio of k_{cat}/K_m, is two orders of magnitude higher than the catalytic efficiency of formate dehydrogenase. The addition of GalM, an enzyme also having a high catalytic efficiency allows use of stoichiometric amounts of reactants, i.e. α-keto acids, labelled ammonia and glucose. On the other hand, the GlucDH/GalM couple can enlarge the possibility of synthesis of other L[^{15}N]-labelled amino acids by using the aliphatic and aromatic amino acid transaminases (AAT). In the latter case either L[^{15}N] aspartic acid or L[^{15}N] glutamic acid, easy to prepare from fumarate and, respectively, α-ketoglutarate,[6,15] might serve as starting material according to the following reactions:

(4)L [^{15}N] aspartate + α-keto acid $\xrightleftharpoons{AAT}$ oxaloacetate + L[^{15}N] amino acid.

(5)oxaloacetate + NADH + H^+ $\xrightleftharpoons{MDH}$ malate + NAD^+.

(6)glucose + NAD^+ $\xrightarrow{GlucDH,\ GalM}$ gluconate + NADH + H^+.

Overall:

L[^{15}N] aspartate + α-keto acid + glucose $\xrightarrow[GlucDH,\ GalM]{NAD,\ AAT,\ MDH}$ L[^{15}N] amino acid + malate + gluconate.

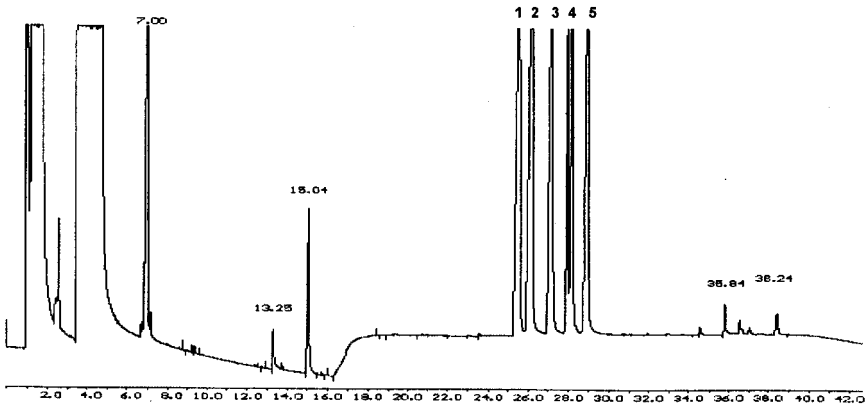

Figure 1. Amino acid separation. Retention times: (1) Val = 25.54 min; (2) norVal = 26.16 min; (3) Leu = 27.12 min; (4) Ile = 28.15 min and (5) norLeu = 28.90 min.

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REFERENCES

[1] D. E. Matthews, M. Bier, *Ann. Rev. Nutr.* **1983**, *3*, 309–339.
[2] M. Beylot, *Curr. Opin. Nutr. Metab. Care* **2006**, *9*, 734–739.
[3] N. Knobe, J. Vogl, W. Pritzkow, U. Panne, H. Fry, H. M. Lochotzke, A. Preiss-Weigel, *Anal. Bioanal. Chem.* **2006**, *386*, 104–108.
[4] K. Ozawa, P. S. Wu, N. E. Dixon, G. Otting, *FEBS J.* **2006**, *273*, 4154–4159.
[5] M. P. Foster, C. A. McElroy, C. D. Amero, *Biochemistry* **2007**, *46*, 331–340.
[6] O. Bojan, M. Bologa, G. Niac, N. Palibroda, E. Vargha, O. Barzu, *Anal. Biochem.* **1980**, *101*, 23–25.
[7] E. Presecan, A. Ivanof, A. Mocanu, N. Palibroda, M. Bologa, V. Gorun, M. Oarga, O. Barzu, *Enzyme Microb. Technol.* **1987**, *9*, 663–667.
[8] F. Hasumi, K. Fukuoka, S. Adachi, Y. Miyamoto, I. Okura, *Appl. Biochem. Biotechnol.* **1996**, *56*, 341–344.
[9] N. M. Kelly, B. C. O'Neill, J. Probert, G. Reid, R. Stephen, T. Wang, C. L. Willis, P. Winton, *Tetrahedron Lett.* **1994**, *35*, 6533–6536.
[10] J. A. Chantry, P. H. Schafer, M. S. Kim, D. M. LeMaster, *Anal. Biochem.* **1993**, *213*, 147–151.
[11] J. Sambrook, E. F. Fritsch, T. Maniatis, *Molecular Cloning*: *A Laboratory Manual*, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, **1989**.
[12] A. Galkin, L. Kulakova, T. Yoshimura, K. Soda, N. Esaki, *Appl. Environ. Microbiol.* **1997**, *63*, 4651–4656.
[13] T. Ohshima, K. Soda, *Trends Biotechnol.* **1989**, *7*, 210–214.
[14] W. Hummel, M. R. Kula, *Eur. J. Biochem.* **1989**, *184*, 1–13.
[15] A. Ivanof, L. Muresan, L. Quai, M. Bologa, N. Palibroda, A. Mocanu, E. Vargha, O. Barzu, *Anal. Biochem.* **1981**, *110*, 267–269.