Preparation of ^{15}N-labeled L-alanine by coupling the alanine dehydrogenase and alcohol dehydrogenase reactions

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1. INTRODUCTION

Amino acids labeled with ^{15}N are useful in investigating protein balance in human medicine because the patients are not exposed to the hazards associated with the administration of radioactive tracers [1,2]. The technical precision and sensitivity achieved by mass spectrometers coupled to gas chromatographs makes possible the study of ^{15}N distribution from different amino acids in newly synthesized proteins and nucleic acids.

Here we report a simple enzymatic procedure for the preparation of L-[^{15}N]alanine, one of the metabolically most active amino acids in all types of cells [3,4]. The procedure is based on the coupling of two reactions, one catalyzed by bacterial alanine dehydrogenase, the second catalyzed by yeast alcohol dehydrogenase:

$$\text{Pyruvic acid} + \text{NADH} + {}^{15}NH_4^+ \overset{\text{AlaDH}}{\rightleftharpoons} \text{L-[}^{15}\text{N]alanine} + \text{NAD}^+ + H_2O \quad (1)$$

$$\text{Ethanol} + \text{NAD}^+ \overset{\text{ADH}}{\rightleftharpoons} \text{acetaldehyde} + \text{NADH} + H^+ \quad (2)$$

$$\text{Pyruvic acid} + \text{ethanol} + {}^{15}NH_3 \overset{\text{AlaDH, ADH, NAD}^+}{\rightleftharpoons} \text{L-[}^{15}\text{N]alanine} + \text{acetaldehyde} + H_2O \quad (\text{sum})$$

An impediment in the use of this procedure could be the high cost of commercial AlaDH. However, the enzyme is widespread in the *Bacillus* species [5] and partially purified samples, adequate preparative purposes, could be obtained relatively easily by chromatography on blue–Sepharose [6].

Abbreviations: AlaDH, L-alanine dehydrogenase (EC 1.4.1.1); ADH, alcohol dehydrogenase (EC 1.1.1.1); blue–Sepharose, Cibacron blue 3G-A–Sepharose 4B-CL.

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2. MATERIALS AND METHODS

2.1. *Chemicals*

All commercial nucleotides, substrates and enzymes were products of Boehringer Mannheim (a generous gift of Professor H.F. Schmidt). Sepharose 4B obtained from Pharmacia (Uppsala) was crosslinked according to [7], omitting $NaBH_4$ from the reaction medium. Cibacron blue 3G-A, a product of Ciba-Geigy (Basel) was coupled to the crosslinked Sepharose as in [8]. $^{15}NH_4Cl$ (98.2% isotopic enrichment) was prepared from $H^{15}NO_3$ at the Institute of Isotopic and Molecular Technology (Cluj-Napoca).

2.2. *Partial purification of AlaDH from* Bacillus cereus

The bacteria (strain 11 548 from the Institute 'Dr. Cantacuzino', Bucharest), were grown at 37°C on broth with bactopeptone Difco and meat extract (pH 7.2) up to the late logarithmic phase. The cellular mass (5 g wet wt from 2 l nutritive medium) was harvested by centrifugation then suspended in 50 ml 50 mM phosphate buffer (pH 7.2) and disrupted by sonication. The unbroken cells (5%), membrane fragments and ribosomes were removed by centrifugation at 100 000 × *g* for 30 min. The clear yellow extract containing 10–12 mg protein/ml and 1 unit AlaDH/mg protein (with L-alanine as substrate at pH 10.5 and 25°C) was adjusted to pH 6.0 and loaded on a blue–Sepharose column (1.2 × 25 cm), at 2 ml/min and +4°C. The column

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was washed with 50 ml 50 mM phosphate buffer (pH 6.0), then AlaDH was specifically eluted with 40 ml 1 mM NADH in the same buffer. The enzyme contained in ~20 ml had spec. act. 30–50 units/mg protein with 70–80% yield. The partial purification of AlaDH lasted for 2 h and the procedure was performed just before the enzymatic synthesis of alanine.

2.3. *Analytical procedures*

The activity of AlaDH was determined in both senses as in [9]. The isotope content of L-[^{15}N]alanine was measured with an Atlas M86 mass spectrometer with molecular inlet system for gases. The sample was converted to nitrogen gas by microcombustion with CuO in the presence of Cu and CaO, after achieving a vacuum of 10^{-4} Torr [10]. The molecular mass spectrum of L-[^{15}N]alanine was obtained after derivatization of the amino acid as *N*(*O*)-trifluoroacetyl *n*-butyl ester [11]. The mass spectrum was recorded on a Varian MAT 311 double-focussing mass spectrometer under electron impact ionization at 70 eV.

3. RESULTS

3.1. *Theoretical premises*

Since the formation of L-[^{15}N]alanine involves two reactions it would be interesting to know the equilibrium concentration of all intermediates. From the equilibrium concentrations it should be possible to estimate the excess reagent necessary for the most favourable use of $^{15}NH^+$, representing the most expensive ingredient. Furthermore, the knowledge of the kinetic parameters of the enzymes should enable us to choice the optimal AlaDH/ADH ratio as well as the optimum pH. From the equilibrium constants of the reactions (1) and (2) taken in the sense of NADH formation (3.1×10^{-14} and 1.15×10^{-11}, respectively) [12,13] and the initial concentration of pyruvic acid (120 mM), ethanol (300 mM), $^{15}NH_4Cl$ (100 mM), and NAD^+ (0.25 mM), a 95% conversion of the ammonium ion to L-alanine is calculated at all pH-values (fig.1). However, the concentrations of NADH and NAD^+ are strongly pH dependent: at pH 9 which is close to the optimal pH of both AlaDH and ADH, the ratio NADH/NAD^+ at equilibrium is ~ 1/40; pH > 9, although favouring higher equilibrium and stationary concentrations of NADH (which would increase the rate of L-alanine formation) would nevertheless result in a faster denaturation of the enzymes involved in the synthesis.

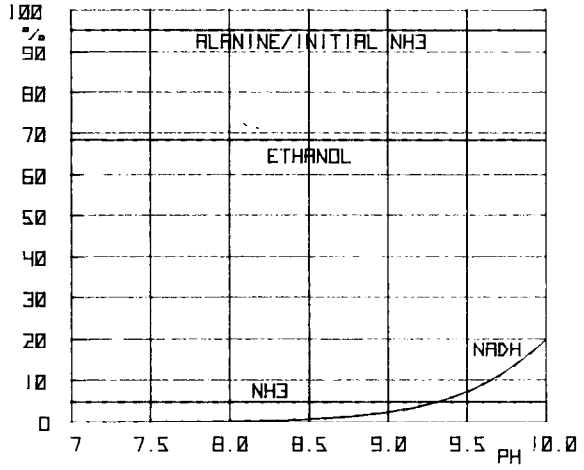

Fig.1. Equilibrium concentrations of L-alanine, ethanol, NH_3 and NADH resulted from the coupled alanine dehydrogenase and alcohol dehydrogenase reactions.

3.2. *Synthesis of L-[^{15}N]alanine*

In a typical experiment the following reagents were mixed in 450 ml bidistilled water: 2.73 g (50 mmol) $^{15}NH_4Cl$, 6.6 g (60 mmol) sodium pyruvate, 10 ml (~ 150 mmol) 95% ethanol and 100 mg (~ 0.15 mmol) NAD^+. Having adjusted the pH to 9 with 1 N KOH, the freshly obtained AlaDH preparation containing 300–350 units was added. The reaction was started with 1000 units ADH and was allowed to procede for several hours at 30°C while sampling the pH, and the rates of disappearance of $^{15}NH_4Cl$ and pyruvate, respectively. When [NH_4^+] in the reaction medium dropped by > 85% (5 h), the pH was brought to 6.0 with 2 N HCl, the mixture was boiled for 5 min and the precipitated proteins removed by filtration.

3.3. *Purification and properties of L-[^{15}N]alanine*

The deproteinized solution was passed through a column (2.5 × 30 cm) of Amberlite IR-120 ion-exchange resin (H^+ form) at a rate of 25–30 ml/h. The absorbed L-[^{15}N]alanine was then eluted with 400 ml 2 N NH_4OH. The solution was cleared by 5 min contact with active charcoal powder followed by filtration. It was then concentrated to dryness at low pressure (18 Torr) and 50°C. The solid residue

was dissolved in 15 ml hot bidistilled water (80°C) after which 40 ml cold methanol were added. Crystalline L-[^{15}N]alanine was obtained after 24 h at +4°C, which was recovered by filtration, then washed 3 times with 10 ml methanol. The final yield was 3.1 g pure product (34.4 mmol), that is 69% as compared to the starting $^{15}NH_4Cl$. The compound has a melting temperature of 298–300°C (with decomposition) and showed a single band by thin-layer chromatography, which corresponded to the pure commercial L-alanine (Merck, Darmstadt). No inorganic ions were traced in the preparation. The rotation of polarized light for a 1% amino acid solution in 6 N HCl (α^{20}_{546} = + 17.0) corresponded to a purity >99%. The isotope content of L-[^{15}N]alanine was found to be 97.76 ± 0.05 atom % ^{15}N. The mass spectrum also showed a high degree of ^{15}N labeling, as deduced from the intensities of the fragment ions 140 and 141 (fig.2). There was a good agreement among the relative intensities when compared to the mass spectrum of the unlabeled compound. All heavy fragment ions showed a mass shift of 1 mass unit corresponding to the substitution $^{14}N \rightarrow {}^{15}N$.

4. DISCUSSION

The enzymatic synthesis of amino acids labeled with ^{15}N starting from ammonium salts has several advantages over the chemical methods of synthesis, mainly a higher yield in the use of $^{15}NH_4^+$ accompanied by the formation of naturally occuring isomers [14–18]. However, these advantages could be seriously limited by the high cost of some enzymes required for large scale preparative purposes, as well as by the formation of secondary products. In [16] L-[^{15}N]alanine was made by a linked-enzyme system involving glutamate dehydrogenase, glutamate–pyruvate transaminase and glucose 6-phosphate dehydrogenase. In addition, glutamate decarboxylase was required to remove the glutamic acid, a by-product. The procedure described here offers the following advantages over other enzymatic or fermentative methods [16,19]:

(i) The enzymes required in the synthesis are easily obtained either commercially (ADH) or by partial purification from bacteria of the *Bacillus* species (AlaDH). Even a spec. act. of 10 U/mg protein (which corresponds to

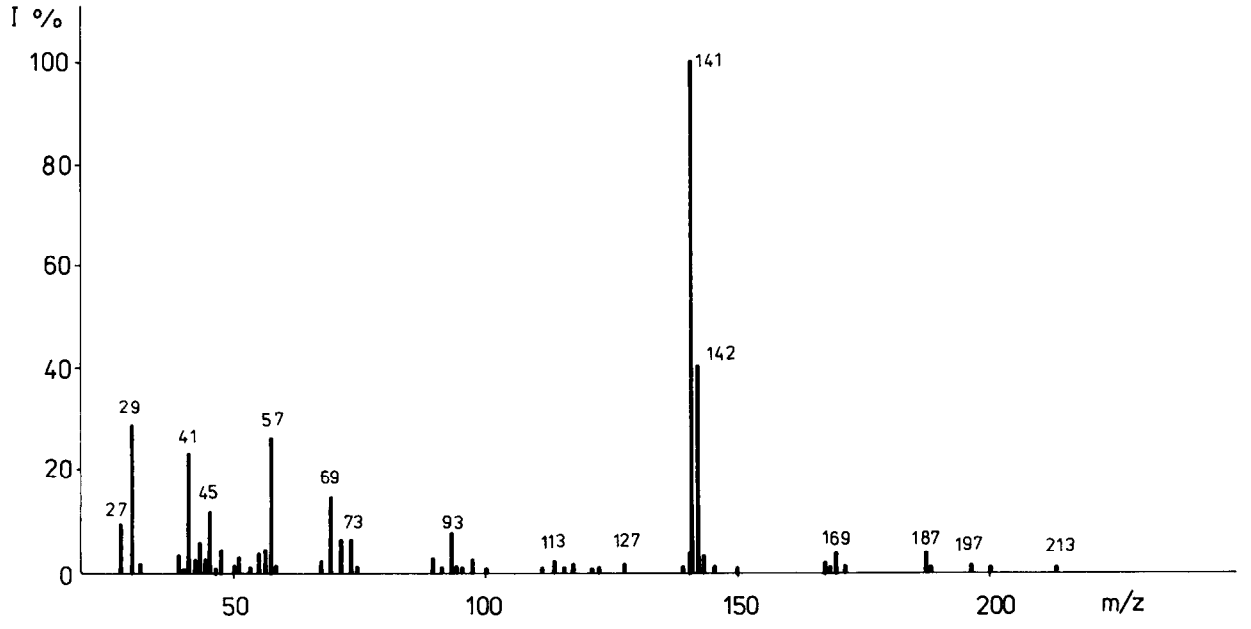

Fig.2. Mass spectrum of the *N*(*O*)-trifluoracetyl *n*-butyl ester of L-[^{15}N]alanine.

30 U/mg protein in the amination reaction) is sufficient for preparative purposes;

(ii) The regeneration of NADH by the conversion of ethanol to acetaldehyde (a highly volatile compound, b.p. 21°C) can displace the equilibrium (1) + (2) quasi-completely to the right by removing the acetaldehyde;

(iii) The purification and crystallization of L-[^{15}N]alanine, as the single amino group containing compound, are straightforward. Unlike with the synthesis of L-[^{15}N]glutamic acid [17], it is essential that the unreacted ketoacid be eliminated from the reaction medium by ion-exchange chromatography;

(iv) The experimental yield of conversion of NH_4^+ to L-alanine is high;

(v) The procedure can be easily adapted for large-scale preparation purposes by immobilization of AlaDH on a solid matrix (in preparation).

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REFERENCES

[1] Golden, M.H.N. and Waterlow, J.C. (1977) Clin. Sci. Mol. Med. 53, 277–288.

[2] Nissim, Y., Yudkoff, M., Yang, W., Terwilliger, T. and Segal, S. (1981) Clin. Chim. Acta 109, 295–304.

[3] Sallach, H.J. and Fahien, L.A. (1969) in: Metabolic Pathways (Greenberg, D.M. ed) 3rd edn, vol.3, pp. 1–94, Academic Press, New York.

[4] Umbarger, H.E. (1978) Annu. Rev. Biochem. 47, 533–606.

[5] Ohshima, T. and Soda, K. (1979) Eur. J. Biochem. 100, 29–39.

[6] Váli, Z., Kilár, F., Lakatos, S., Venyaminov, S.A. and Zádvodsky, P. (1980) Biochim. Biophys. Acta 615, 34–47.

[7] Kristiansen, T. (1974) Methods Enzymol. 34, 331–341.

[8] Böhme, H.J., Kopperschlänger, G., Schulz, J. and Hoffman, E. (1972) J.Chromatogr. 69, 209–214.

[9] Yoshida, A. and Freese, E. (1964) Biochim. Biophys. Acta 92, 33–43.

[10] Faust, H. (1967) Isotopenpraxis 3, 100–103.

[11] Metz, J., Ebert, W. and Weicker, H. (1971) Chromatographia 4, 259–265.

[12] Yoshida, A. and Freese, E. (1965) Biochim. Biophys. Acta 96, 248–262.

[13] Racker, E. (1950) J.Biol.Chem. 184, 313–319.

[14] Greenway, W. and Whatley, F.R. (1975) J. Labelled Compd. 11, 395–400.

[15] Greenway, W. and Whatley, F.R. (1977) FEBS Lett. 75, 41–43.

[16] Greenway, W., Whatley, F.R. and Ward, S. (1977) FEBS Lett. 81, 286–288.

[17] Bojan, O., Bologa, M., Niac, G., Palibroda, N., Vargha, E. and Bârzu, O. (1980) Anal. Biochem. 101, 23–25.

[18] Ivanof, A., Muresan, L., Quai, l., Bologa, M., Palibroda, N., Mocanu, A., Vargha, E. and Bârzu, O. (1981) Anal. Biochem. 110, 267–269.

[19] Kitai, A., Kitamura, T. and Miyachi, N. (1962) in: Amino acids (Hakko To Taisha, ed.) vol. 5, pp. 61–65 (Japanese, English Summ.) Assoc. Amino acid fermentation, Tokyo.