Enzymatically catalysed decarboxylation of β-carboxyaspartic acid (Asa)

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A better synthetic route to β-carboxyaspartic acid (Asa) was achieved through condensation of sodium dibenzyl malonate and benzyl 2-bromoacetate as an alternative to two steps in the method of Koch *et al.* Catalytic hydrogenation at 5 atm pressure in a shaken stainless steel vessel removed protecting carbobenzyloxy groups. Asa was purified on a cation exchange column. An extract of *E. coli* was found to catalyse the decarboxylation of Asa in a doubly coupled assay designed to be specific for produced aspartic acid. Evidence for enzymatic decarboxylation includes complete loss of activity on boiling and progressive loss of activity on repeated freeze–thawing of the extract.

Key words: decarboxylation, β-carboxyaspartic acid, Asa decarboxylase.

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On a développé une meilleure méthode de synthèse de l'acide β-carboxyaspartique (Asa) qui fait appel à une condensation du sel de sodium du malonate de dibenzyle avec le 2-bromoacétate de benzyle, à la place de deux étapes de la méthode de Koch et ses collaborateurs. Pour éliminer les groupements protecteurs carbobenzyloxy, on a effectué une hydrogénation catalytique, à une pression de 5 atmosphères, dans des contenants en acier inoxydable qui étaient agités. On a purifié l'Asa par un échange cationique sur colonne. Lors d'essais doublement couplés effectués dans le but spécifique d'évaluer l'acide aspartique produit, on a trouvé qu'un extrait d'*E. coli* catalyse la décarboxylation de l'Asa. Parmi les données suggérant l'existence du décarboxylation enzymatique, on doit mentionner la perte totale d'activité qui est observée lorsqu'on fait bouillir l'extrait ainsi que la perte progressive de son activité lorsqu'on le soumet d'une façon répétée à des congélations suivies de périodes de réchauffement.

Mots clés : décarboxylation, l'acide β-carboxyaspartique, Asa-décarboxylase.

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Introduction

Discovery of the carboxylated amino acids, aminomalonic acid (Ama),[2] γ-carboxyglutamic acid (Gla), and Asa in various tissues including atherosclerotic plaque (1), osteocalcin (2), and *E. coli* ribosomal proteins (3) has generated considerable interest in their synthesis and properties. Several methods for racemic Asa (3–5) and one for D-β-Asa (6) have been reported. All prescribe millimolar quantities, with suggestions for scaling up offered in one procedure (4).

Carboxylations of the amino acids are thought to take place post-translationally and, in the case of Gla, a Vitamin K-dependent carboxylase has been characterized (7). The kinetics of thermal decarboxylation of the three synthetically prepared carboxy amino acids have been compared (8). Enzymes catalysing the decarboxylation of Ama have been described (9, 10). We wish to report the demonstration of Asa decarboxylase activity in an extract of *E. coli*.

Experimental

Asa was prepared essentially by the scaled-up method of Koch *et al.* (4) with the following modifications. The first two steps involving the nucleophilic substitution of sodium diethylmalonate with methyl chloroacetate to produce 1,1,2-tris(carbomethoxy)ethane followed by transesterification to 1,1,2-tris(carbobenzyloxy)ethane were replaced by a single reaction. The consideration of equimolar amounts of sodium dibenzyl malonate and benzyl 2-bromoacetate result in a larger yield (84% overall) of product.

$$BnO_2CCH_2Br + NaCH(CO_2Bn)_2 \rightarrow BnO_2CCH_2CH(CO_2Bn)_2 + NaBr$$

In a typical experiment 1.65 g of Na was added in small pieces to 20.36 g of dibenzyl malonate in 163 mL of dry methanol at 0°C and stirred for 1 h. After addition of 16.41 g of benzyl 2-bromoacetate, stirring was continued at room temperature overnight. Following rotary evaporation of the methanol an orange oil was obtained; this oil was placed in a separatory funnel with 100 mL of ether, 200 mL of deionized water, and 7.5 mL of 6 M HCl. After extraction and separation of the ether layer the water layer was back-extracted twice with 100-mL portions of ether. The ether layers were combined, dried over anhydrous $MgSO_4$, and rotary evaporated. The residue was a light yellow oil weighing 25.98 g.

The catalytic hydrogenation of the azide, tribenzyl 1-azidoethane-1,2,2-tricarboxylate, to reduce the protective carbobenzyloxy groups to toluene and yield Asa was carried out by placing 0.70 g of azide, 0.35 g of $PdCl_2$, and 150 mL of dry methanol in a stainless steel vessel (inner diameter 54 mm, height 78 mm), purging, then pressurizing to 5 atm with H_2 (1 atm = 101.3 kPa). After shaking mechanically for 48 h the reaction mixture was filtered and rotary evaporated. Three additions of benzene were followed by rotary evaporation to remove traces of toluene. The yellow solid remaining was washed five times with ether, then dissolved in 4 mL of deionized water, pH 7.0. A small amount of insoluble material was removed by centrifugation at 10 000 g for 15 min. The supernatant was passed through a 5-cm column of Amberlite IRC-50 cation exchange resin. After freeze-drying of the clear eluate, there remained 0.112 g of a white crystalline powder (yield 43%), which was shown to be chromatographically pure and was identified as Asa monohydrate by NMR, IR, and MS.

For the enzyme assay an *E. coli* extract was prepared by suspending late log phase cells harvested from 30 mL of nutrient broth in 5 mL of H_2O. After ultrasonic disruption at 5°C, cellular debris was removed centrifugally. Commercial preparations of GOT (1667 units/mL) and MDH (10 000 units/mL) were dialysed against deionized water and diluted to working solutions with a 0.1 M potassium phosphate

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[2]The abbreviations used are Asa, β-carboxyaspartic acid; Ama, aminomalonic acid; Gla, γ-carboxyglutamic acid; L-Asp, L-aspartic acid; GOT, glutamate–oxalacetate transaminase; OAA, oxalacetic acid; MDH, malate dehydrogenase; NADH, nicotinamide adenine dinucleotide.

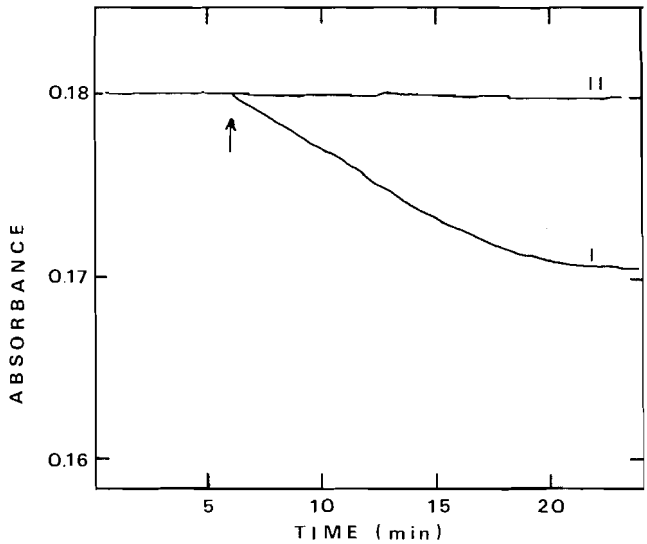


FIG. 1. Assay of Asa decarboxylase activity in an extract of *E. coli*. (I) Complete assay mixture. (II) Boiled extract. (↑) Point of addition of extract.

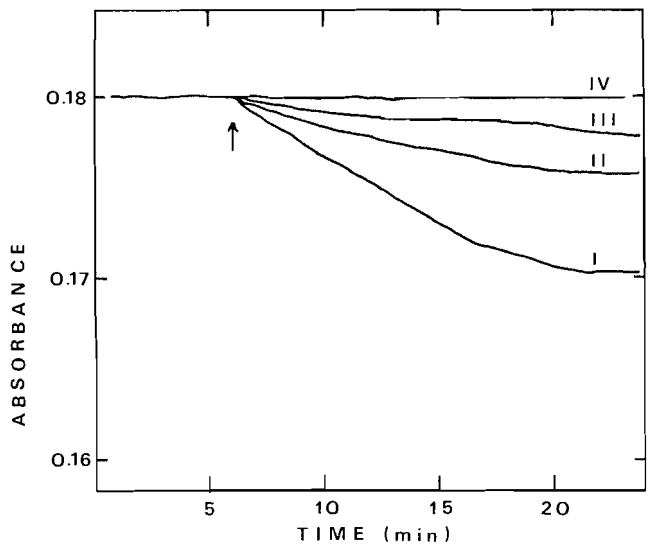


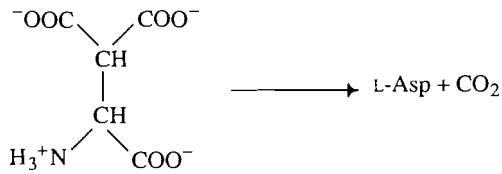
FIG. 2. Effect of freeze-thawing on Asa decarboxylase activity. (I) Complete assay mixture. (II), (III), (IV) Subsequent freezings and thawings of extract. (↑) Point of addition of extract.

buffer solution, pH 7.6, containing 1 mg bovine serum albumin per mL.

The assay mixture was as follows: 0.1 mL 10^{-2} M α-ketoglutarate, 0.1 mL 1/10 diluted GOT, 0.1 mL 5/1000 diluted MDH, 0.1 mL 10^{-3} M NADH, 0.3 mL 0.1 M potassium phosphate buffer pH 7.6, 1.6 mL H_2O, 0.1 mL 10^{-2} M Asa, and 0.1 mL *E. coli* extract.

Results

The assay procedure is a doubly coupled one, with the first appearance of L-aspartic acid (L-Asp) being scavenged by the glutamate–oxalacetate transaminase (GOT) reaction, using a suitable excess of α-ketoglutarate and GOT. The oxalacetate (OAA) produced is coupled to the malate dehydrogenase (MDH) reaction. The disappearance of coenzyme NADH is followed spectrophotometrically at 340 nm, and is a direct measure of the rate-limiting decarboxylase step:



$$\alpha\text{-ketoglutarate} + \text{L-Asp} \xrightarrow{\text{GOT}} \text{L-glutamate} + \text{OAA}$$

$$\text{OAA} + \text{NADH} + \text{H}^+ \xrightarrow{\text{MDH}} \text{L-malate} + \text{NAD}^+$$

Evidence for enzymatic decarboxylation of Asa is shown in Fig. 1. When GOT is left out of the assay mixture no absorbance decrease is observed, suggesting the effective absence of any interfering levels of dehydrogenases. MDH is known to be highly specific for OAA, making the doubly coupled assay a true indicator of Asa decarboxylation. Boiling the *E. coli* extract for 5 min results in a total loss of enzyme activity. Further evidence of enzyme character (Fig. 2) is the progressive loss of activity due to repeated freezing and thawing of the extract.

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