Biotechnology Letters Vol 13 No 6 405-410 (1991)
Received 21st May

Enzymic Synthesis of Argininosuccinate Catalyzed by δ-Crystallin

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Summary: A fast and practical procedure has been developed for the synthesis of argininosuccinate using immobilized duck lens δ-crystallin as catalyst.

Introduction

Argininosuccinate (ArS) is used clinically in measuring enzymic activity of argininosuccinate lyase (ASase EC 4.3.2.1.) and argininosuccinate synthetase (EC 6.3.4.5.) for the diagnosis of the diseases of argininosuccinic aciduric and argininaemia, respectively [Bastone, 1990; Donn, 1985; Qureshi, 1984.]. ArS was first isolated from the condensation of citrulline and aspartate in a reaction catalyzed by argininosuccinate synthetase and shown to be identical with the condensation product of arginine (Arg) and fumarate (Fum) by the ASase-catalyzed reaction [Ratner, 1951; Ratmer. 1953]. In principle, ArS can be synthesized enzymatically from either reaction. However, neither enzymatic method is suitable for a large-scale preparation of this intermediate due to the fact that the former reaction needs an efficient phosphate-regenerating system and both enzymes are present in various tissue only in small amounts [Ratner, 1972]. The recent report on the synthesis of N^{α}-benzyloxycarbonyl-arginosuccinic acid trimethyl ester from N^{α}-benzyloxycarbonyl-L-citrulline with dimethyl aspartate required three steps with a total yield of only 33% [Ranganthan, 1990]. A further deprotecting step is needed to give ArS. Here, we have found an efficient enzymatic method using δ-crystallin isolated from duck lens.

δ-Crystallin, the most abundant crystallin in lenses of some avian species, was shown to possess an endogenenous ASase activity with high stability suitable for the application to enzyme technology [Chiou, 1991]. This crystallin is especially rich in the lenses of birds belonging to the family Anatidae which includes ducks, swans and

geese [Chiou, 1989]. It comprised about 50% of total protein in the duck lens and could be isolated easily by single-step purification [Chiou, 1990]. In this study the endogenous ASase of duck lens is found to be very stable ($t_{1/2}$ at pH 7.5 and r.t. is about 20 days) and can also maintain its activity in organic cosolvent.

Experimental.

Material and reagents: The duck (also call Kaiya duck, a cross-bred hybrid between Cairina moschata and Anas platyrhynchos var domestica) lenses were obtained from the Taiwan Livestock Research Institute, Yinan, Taiwan. Arginine and fumarate are obtained from Sigma Chemical Company (USA). The amino acid analyses were performed on a Beckman 6300 amino acid analyser. The optical rotation was measured on a Universal Polarimeter (Schmidt & Haensch). The HPLC system consisted of two Waters Model 6000 pumps, a Waters Model 450 UV detector, and an M-660 solvent programmer. High purity solvent were obtained from ALPS Chemical Company, Taiwan.

Enzyme preparation. The batch preparations of crystallins were routinely carried out from the pooled lenses of about 50 duck eyeballs. They were freshly collected from the above Institute without freezing, were decapsulated, and homogenized in 20-30 mL of 0.05 M-ammonium bicarbonate buffer, pH 7.7, containing 5 mM EDTA. The clear solution of the enzyme in buffer was lyophilized to give 250 mg of the crude enzyme powder which was used directly without further purification.

Enzyme assay. Twenty-two mg of lyophilized enzyme powder in 20 mL of phosphate buffer (pH7.5) was stirred at room temperature to maintain homogeneity. A 100 µL portion of the enzyme solution was added to a cuvette containing 0.25 mM of ArS in same buffer. The initial reaction rates were determined from time dependent plots of the increasing absorbance at 240 nm. (ε=0.81 x 10^3)

Synthesis of ArS. Arg (5.24 g, 30 mmol), and Fum (6.96 g, 60 mmol) in a mixture of water/ethanol (3:1, v/v 600 mL) was added to a dialysis bag containing ASase (330 mg in 25 mL of reaction solution) and reacted for 12 h. The enzyme in the bag was removed, $BaCl_2$ (6.24g, 30 mmol) was added, and pH adjusted to 9.5 (1N KOH). The resulting mixture was lyophilized and then ethanol (80%, 100 mL) was added. The suspension was filtered and washed with ethanol (80% , 2x 50 mL) to yield crude Ba-salt of ArS (14.48 g,). For further purification, the crude salt (0.42 g, 1.0 mmol) in water (2 mL) was eluted with a gel filtration column (packed with TSK-HW40S, 2.5x 470 mm) and the desired fractions pooled, and 0.5 N H_2SO_4 (1.26 mL) added . The supernatant lyophilized to yield ArS (0.182g, 71.1% yield from crude salt), $[\alpha]_D$:+ 16.56, c=2 H_2O; ^{1}H-NMR(300 MHz, D_2O),δ: 4.21 (m, 1H), 3.54 (m 1H), 3.23 (t, 2H), 2.66, (t, 2H), 1.70 (m, 4H); Lit [Ratner,1965]: $[\alpha]_D$:+16.4, in 2.9% H_2O; ^{1}H-NMR(D_2O) δ: 4.21 (m, 1H), 3.54 (m 1H), 3.23 (t, 2H), 2.66, (t, 2H), 1.70 (m, 4H).

Results and discussion.

ASase was prepared from the pooled lenses of eyeballs, by being decapsulated, homogenized, and lypholized to give the crude enzyme which has a specific activity of 59.8×10^{-2} U/mg as assayed spectrophotometrically according to the procedure of Havir et al.[Havier, 1965]. This activity is 9.4 times the activity of an enzyme that was isolated from Steer liver (15.3 U/mg of a 240x purified enzyme). Figure 1 shows the stability of ASase in buffer, pure water, and 30% ethanol (v/v), respectively. The rate of inactivation of the enzyme was measured on the basis of the remaining activity (using a 30 second-incubated ASase as the reference of 100% activity). The enzyme can maintain its activity in both phosphate buffer and in pure water. The latter condition is very

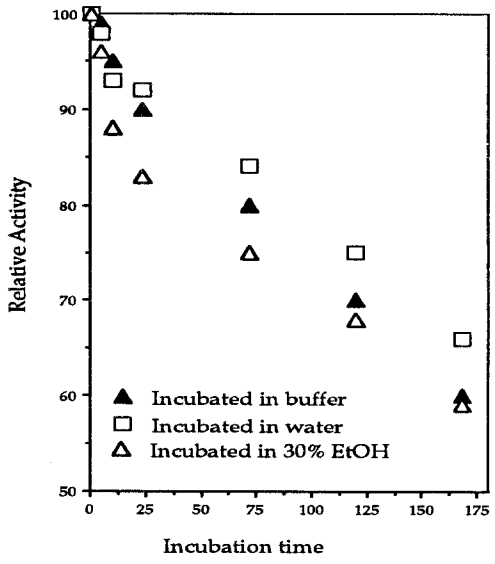
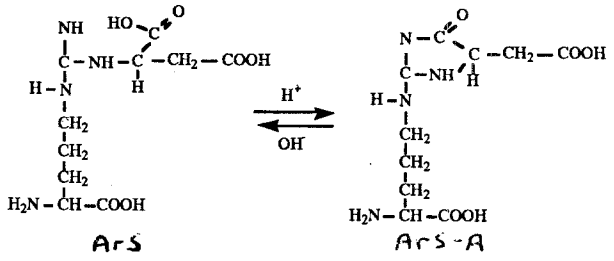


Figure 1: Stability of argininosuccinate lyase in phosphate buffer (pH 8.5), water, and 30% ethanol (v/v), respectively. The 100% activity was defined as the lyase activity of an aliquot of the mixture towards the argininosuccinate formation as measured in the decrease of uv absorbance at 240 nm, 30 s after addition of the incubated enzyme solution to the reaction mixture.

Scheme I



helpful for large scale synthesis because the buffer in the reaction solution will contaminate the product in the isolation while the water is evaporated. Further investigation has found that the enzyme can also maintain activity in a mixture of water:ethanol (7:3, v/v) for more than 7 days.

The ASase catalysed reaction is favorable for ArS formation. In a preliminary test, Arg (1.0 mmol), Fum (1.0 mmol), and crude powder of ASase (22 mg) in water (20 mL, pH 7.5 adjusted with NaOH) was stirred at room temperature for 24 h. It

found to contain ArS (61.82%), Arg (28.85%), and argininosuccinate anhydride (ArS-A) (9.33%) by amino acid analysis (Keq = 1.35×10^{-4} for [Arg].[Fum]/[ArS]). A similar result was also obtained in using water:ethanol (3:1, v/v) as the solvent. We found both substrates could serve as a buffer in solution. Once the pH of the reaction solution was adjusted to 7.5, it will not change during the reaction. An early work has shown that ArS readily undergoes anhydride formation in acid solution (see scheme I). The structure of ArS-A is confirmed as a five membered-ring [Ratner,1965]. We found that the ArS-A also forms at pH 7.5. Further study was done by reacting two and four equivalents of Fum in water:ethanol (3:1, v/v). The time course of the product formation and the reactant disappearance was followed by amino acid analysis in each time interval. The results are shown in figure 2.

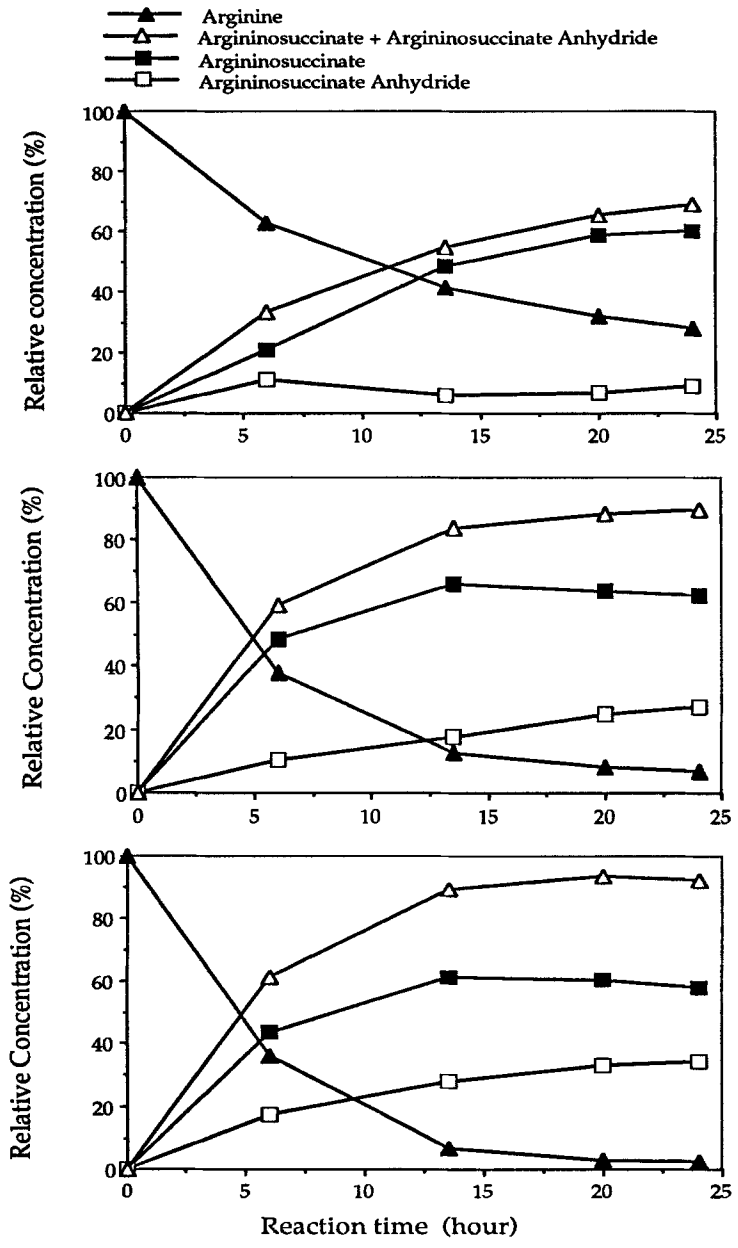


Figure 2: The time course of argininosuccinate, argininosuccinate anhydride formation and of arginine disappearence in various ratios of arginine and fumarate. Fig. 2-a, arginine:fumarate=1:1, fig. 2-b, arginine:fumarate=1:2, fig. 2c, arginine:fumarate=1:4. The concentrations of compounds in each time interval were calculated using a Beckman amino acid analyzer with 6300 Na-column. The retention time of each compound is argininosuccinate: 17.52 min, argininosuccinate anhydride:24.87 min, and arginine: 37.60 min.

Using four equivalents of Fum in reaction, only 2.84% of Arg was left in solution. The formation of ArS-A increased when a high concentration of Fum present in the reaction solution. As shown in figure 2, the ratio of ArS/ArS-A was 62/9, 62/27, 58/34 in the reactions which contained 1, 2. and 4 equivalents of Fum, respectively. The enzyme in solution, which will contaminate product in isolation, can be separated by the membrane enclosed enzyme immobilization (MEEI) method [Bendnarski, 1987]. In a practical preparative scale reaction, Arg (30 mmol), and Fum (60 mmol) in a mixture of water/ethanol (3:1, v/v 600 mL) were added to a dialysis bag containing ASase (330 mg in 25 mL of reaction solution) and reacted for 12 h. The enzyme in the bag was removed, $BaCl_2$ (30 mmol) was added, and pH adjusted to 9.5 (1N KOH). The resulting mixture was lyophilized and then ethanol (80%, 100 mL) was added. The suspension was filtered and washed with ethanol (80% , 2x 50 mL) to yield crude Ba-salt of ArS. The crude salt was further purified by gel filtration column with TSK-HW40S, the desired fractions pooled, and lyophilized to yield ArS (63% of total yield).

That the duck lens crystilline does not contain protease activity may be one reason for the extra-ordinary stability of δ-crystallin. The δ-crystallin catalysed the reaction in a Keq 12 times more favorable for the ArS formation than the enzyme from Liver. For further study the catalytic properties and specifity of ASase, malate and D-Arg are used as substrates respectively; neither is a substrate for ASase. In 80% ethanol, the Arg is more soluble than Fum, and ArS is insoluble. All three substrates are hygroscopic but the barium salt of ArS is not. The crude product was found to contain 39% of Fum (δ-5.67 ppm by nmr) but no Arg, and the product purified by gel filtration was >98% pure (based on nmr) [Kowasky, 1969]. The formation of ArS-A in the reaction solution is interesting. It shifts the reaction in the direction of ArS formation, but does not affect the K_{eq}. The present of ArS-A in solution does not interfere with product isolation, because the ArS and ArS-A readily undergo interconversion at room temperature (scheme 1) and the ArS predominates at high pH. In summary, the procedure illustrated here describes the practical application of ASase in ArS synthesis. It involved use of an abundant natural crystallin to achieve the C-N bond formation and C=C bond addition between Arg and Fum. The immobilization of enzyme makes it possible for the ASase to be repeatedly used and makes the isolation of product very simple. The compound prepared in this study is very pure and useful for clinical diagnosis.

Acknowledgement

Support for this Research provided by the National Science Council of the ROC is gratefully acknowledged. Thanks are due for the kind provision of duck lens by the Taiwan Livestock Research Institute.

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