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Hydrolysis of the Nitrile group in α-Aminophenylacetonitrile by Nitrilase; Development of a New Biotechnology for Stereospecific Production of *S*-α-Phenylglycine

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Abstract□Phenylglycine was obtained as the sole metabolite when α-aminophenylacetonitrile was fed to the culture broth of *Aspergillus furmigatus*. The isolated phenylglycine showed L-configuration with 80% optical purity. Examination of the hydrolysis of the substrate to phenylglycine with cell free extracts, and the supernatant fraction and the particulate fraction both of which were obtained after ultracentrifugation of the cell free extract at 100,000g, indicated that the nitrile group hydrolyzing enzymes, nitrilase existed not only in cytoplasm, but in microsome fractions.

Keywords□α-Aminophenylacetonitrile, Phenylglycine, Nitrile Group Hydrolyzing Enzymes, Nitrilase.

Many amino acids such as L-glutamic acid (Kanjaki *et al.*, 1972), L-valine (Kisumi *et al.*, 1973), phenylalanie (Sugimoto et al., 1973; Hagino and Nakayama, 1974), tryptophane (Terui, 1972; Miozzari, *et al.*, 1978) *etc.* are produced by fermentation with various microorganisms and some are obtained by microbial transformations of precusors (Fukumura, 1976; Esaki *et al.*, 1980; Yamada *et al.*, 1975) which are synthetically easily accessible (Hirose and Okada, 1979). The latter method is usually characterized by stereospecific conversion of certain functional groups to produce optically active compounds and it is usually regarded as one of the best methods for production of certain biologically important enantiomers (Kitahara *et al.*, 1959; Nakazawa *et al.*, 1972). We are interested in production of amino acids by conversion of certain precusors to the desired products by employing microorganisms or enzymes (Goo, 1986). Since D-phenylglycine is the raw material for oral cephalosporins and penicilins (Goo, 1983), it is consumed in a large quantity every year. Furthermore, since its precusor, α-aminophenylacetonitrile can be easily obtained from benzaldehyde by cyanoamination reaction (Steiger, 1955), it is interesting to examine the conversion of α-aminophenylacetionitrile to phenylglycine by the biochemical hydrolysis of its nitrile group (Goo, 1986).

α-Aminophenylacetonitrile was fed to many bacteria, fungi, yeast and Streptomycetes and their growth and their accumulation of certain metabolites in the cultured brothes were studied (Choi, 1985; Goo, 1986). It was found that most of the microorganisms had accumulated a metabolite in their growth media. To confirm the structure of the metabolite, we fed α-aminophenylacetonitrile in a large quantity to *Asperigillus furmigatus* and isolated the accumulated product. *A. furmigatus* was inoculated to 25 ml of Theriault's medium, which contained dextrose (5%), soy bean flour (5%), yeast extract (5%), NaCl (5%), K_2HPO_4 (1%) and KH_2PO_4 (1%) and grown for 48 hrs at 27 °C on a rotary shaker (170 rpm). Then, it was reinoculated to 500 ml of the same medium and further incubated for 48 hrs. After the pH of the medium was then adjusted to 6.5 by adding diluted HCl solution, α-aminophenylacetonitrile (0.3 g) was added and the cultured broth was shaken further for 12 hrs at the same temperature. The broth was filtered through a Buchner funnel to remove cells. The filtrate was rotary evaporated and dried under vacuum to give a thick oily residue (4.6 g). The residue was dissolved in water and chromatographed through a column packed with Sephadex G-25. The fraction containing the metabolite was collected and evaporated to give a yellowish powder. Further chromatography of this fraction on an anion exchange column (Amberlite IR-410) by elution with ammonia water (5%) gave a fraction containing the metabolite, evaporation of which gave a white powder (60 mg). Crystallization of this powder in hot ethanol gave a crystal (45 mg) whcih showed λ_{max} at 261 nm

(ε = 236 M^{-1} cm^{-1} in EtOH at 20°C) in the UV spectrum and bands at 3200, 3000, 1610, 1510, 1400 cm^{-1} in its IR spectrum (KBr pellet) and peaks at 4.90 (s , 1-H) and 7.4 ppm (b, Ph) in its ^{1}H NMR spectrum (DMSO-d_6). The isolated product was identified to be phenylglycine when compared with an authentic sample. The isolated phenylglycine showed an optical rotation value, $[\alpha]_D^{20}$ + 140° (c=0.1, 2 N HCl) which confirmed that the isolated product had *S*-configuration and the isolated product showed about 80% of optical purity when compared with the reported value for *S*-(+)-α-phenylglycine ($[\alpha]_D^{20}$ + 156.4°, C = 1, 1 N HCl) (Ingersoll and Adams, 1922.). Currently, it is not obvious whether one of the enantiomer of the racemic mixture of the substrate, or both enantiomers are hydrolyzed *S*-α-phenylglycine. In the latter, during hydrolysis of the substrate, a configurational change should be accompanied and we would investigate further for this possibility.

When hydrolysis of the nitrile group in α-aminophenylacetonitrile was further examined by a cell contact method, it was found that the substrate is completely converted to phenylglycine. For this study *A. furmigatus* was cultured in 50 ml of Theriault's medium and reinoculated to 500 ml of the same medium and incubated for 48 hrs at 28°C on a rotary shaker (170 rpm). Then, α-aminophenylacetonitrile (300 mg) was added to the culture and it was incubated further for 24 hrs. Then, cells (4.9 g) was harvested by centrifugation at 3,000 g for 10min. at 4°C, washed twice with potassium phosphate buffer (100 mM, pH = 7.0), and resuspended in the same buffer (10 ml). Part of this cell suspension (0.5 ml) was mixed with the substrate solution (131 mg in 10 ml of the same phosphate buffer) and incubated on a reciprocal shaker at 37°C. When the consumption of the substrate and the appearance of phenylglycine were examined by TLC (Silica gel, 0.25 mm, glass plates, purchased from E. Merck, developed with *n*-BuOH-AcOH-water = 4:1:1) after 4, 8 and 12 hours' incubation, the substrate was found to be converted to phenylglycine completely in 12 hours. In this study, it was noticed that the substrate was hydrolyzed only to phenylglycine completely without production of any other side products.

When the harvest cells (0.9 g) suspended in potassium phosphate buffer (10 ml) was added with mercaptoethanol (7.0 μl), sonicated (100 amplitude, 30 sec × 2), and centrifuged at 6,000g for 20 min at 0 – 4°C to obtain a cell free extract. The cell free extract was further centrifuged at 100,000 g for 60 min at 0°C to give a supernatant and a particulate fraction. When α-aminophenylacetonitrile (0.5 ml: 30 mM solution) dissolved in potassium phosphate buffer (100 mM, pH = 6.5) was incubated with these enzyme preparations (0.5 ml), it was hydrolyzed to phenylglycine in all three preparations, as shown in Figure 1, This study suggested that the

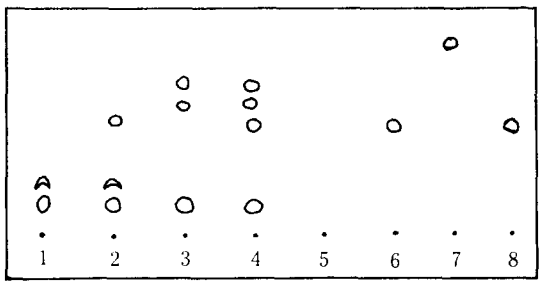

Fig. 1. Thin layer chromatographic (TLC, silica gel) analyses of the metabolites produced form α-aminophenylacetonitrile by cell fractions of *A.furmigatus*.

The bottom numbers represent cell free extract (1), cell free extract incubated with α-aminophenylacetonitrile (2), the particulate fraction (3), the particulate fraction incubated with α-aminophenylacetonitrile (4), the supernatant fraction (5), the supernatant fraction incubated with α-aminophenylacetonitrile (6), α-aminophenylacetonitrile (7) and phenylglycine (8). The TLC plate was developed with *n-butanol*-acetic acid-water (4:1:1) and the spots were detected by spray ninhydrin solution (1.5% in acetone).

nitrilase responsible for the hydrolysis of the substrate to phenylglycine should exist in the cytoplasm, as well as in microsomal fractions.

Organic chemically it is known that a nitrile group is hydrolyzed to an acid through an amide intermediate, but since there exist two kinds of enzymes for nitrile group hydrolysis in biological systems, it seems that the nitrile group in α-aminophenylacetonitrile is hydrolyzed directly to an acid by nitrilase. Although there are several reports on nitrile group hydrolyzing enzymes' activity, but none have ever studied on the stereospecificity of these enzymes. From this study we have confirmed the conversion of α-aminophenylacetonitrile to S-α-phenylglycine with very high efficiency. Since R-α-phenylglycine is currently produced organic chemically by resolution of the racemic mixture produced by acid hydrolysis of α-aminophenylacetonitrile, stereospecific production of *R*-α-phenylglycine, further characterization of nitrilase and ultilization of microbial cells or enzymes for production of *R*-α-phenylglycine is currently under investigation.

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