Enzymatic Production of Urocanic Acid by *Achromobacter liquidum*

TAKEJI SHIBATANI, NORIYUKI NISHIMURA, KOICHI NABE, TOSHIO KAKIMOTO, AND ICHIRO CHIBATA

Research Laboratory of Applied Biochemistry, Tanabe Seiyaku Company, Limited, 962, Kashima-cho, Higashiyodogawa-ku, Osaka, Japan

Received for publication 26 December 1973

To develop an efficient method for the production of urocanic acid, optimal conditions for the production of microbial L-histidine ammonia lyase and for the conversion of L-histidine to urocanic acid by this enzyme were studied. A number of microorganisms were screened to test their ability to form and accumulate urocanic acid from L-histidine. *Achromobacter liquidum* was selected as the best organism. With this organism, enzyme activity as high as 2.0 units/ml could be produced by a shaking culture at 30 C in a medium containing glucose, urea, potassium phosphate, L-histidine, yeast extract, peptone, and inorganic salts. Appropriate addition of a surface-active agent to the reaction mixture shortened the time required for the conversion. A large amount of L-histidine was converted stoichiometrically to urocanic acid in 48 h at 40 C. Accumulated urocanic acid was readily isolated in pure form by ordinary procedures with isoelectric precipitation. Yields of isolated urocanic acid of over 92% from L-histidine were easily attainable. When the culture of *Achromobacter liquidum* was added to DL-histidine, D-histidine and urocanic acid were simultaneously obtained in high yields.

L-Histidine ammonia lyase, which converts L-histidine to urocanic acid, is widely distributed in bacteria such as *Pseudomonas fluorescens* (4, 8, 20, 21), *P. aeruginosa* (11), *P. testosteroni* (A. K. Soutar and H. Hassall, Biochem J. 114:79P–80P, 1969), *P. putida* (6, 7), *Aerobacter aerogenes* (9, 13), *Bacillus subtilis* (2, 5), *B. cereus* (19), *Mycobacterium avium* (22), *Salmonella typhimurium* (12), *Serratia marcescens* (13), and *Vibrio cholerae* (10). The enzyme occurs also in the liver (1), blood serum (14), and stratum corneum (24) of higher animals and in plants such as spinach and sunflowers (18).

There is only one report on the application of this enzyme in *P. fluorescens* ATCC 11299b (16) for the biochemical preparation of urocanic acid.

In this paper we present an efficient industrial method of production of urocanic acid, a chemical used as a sun-screening agent in the pharmaceutical and cosmetics fields.

**MATERIALS AND METHODS**

Organisms. Screening tests were performed on 106 strains of bacteria, all from the collection in this laboratory. *Achromobacter liquidum* IAM 1667 was selected for the fermentation experiments.

Screening experiments. Slant cultures of bacteria were grown on a medium containing 1% glucose, 0.25% peptone, 0.25% meat extract (Difco), 0.25% yeast extract, and 0.5% sodium chloride. All organisms were grown for 24 h at their respective optimal temperatures for growth. The screening medium contained 0.2% L-histidine hydrochloride monohydrate, 0.1% yeast extract, 0.15% K$_2$HPO$_4$, 0.06% KH$_2$PO$_4$, and 0.02% MgSO$_4$, 7H$_2$O. The medium was adjusted to pH 7.0 with NaOH, distributed in 3-ml amounts to test tubes, and sterilized. After inoculation with test organisms from the slant cultures, shaking incubation was carried out for 24 h at 30 C. To 1 ml of the culture broth obtained was added 1 ml of 6% L-histidine hydrochloride monohydrate adjusted to pH 9.0 with 5 N NaOH. The mixture was incubated for 24 h at 37 C.

Routine identification and rough quantitative estimation of the urocanic acids and amino acids formed were made by paper chromatography. The ascending method was used with Toyo filter paper no. 50 and n-butanol-acetic acid-water (4:1:1). After development, the chromatograms were sprayed with Pauly reagent or 0.2% ninhydrin in 80% ethanol.

Fermentation experiments. Unless otherwise noted, fermentation experiments for enzyme formation were carried out as follows. The respective media were distributed in 100-ml amounts to shaking flasks (500 ml), sterilized, and inoculated with one loopful of the selected organism. The cultures were incubated at 30 C for 24 h with reciprocal shaking (140 rpm, 8-cm stroke).
Methods of analysis. The assay of L-histidine ammonia lyase was carried out by spectrophotometric measurements of urocanic acid formed from L-histidine. The cells harvested by centrifugation from 2 ml of the broth cultured for 24 h were washed with saline and suspended in reaction mixture to a final volume of 4 ml. The reaction mixture contained 0.15 M L-histidine hydrochloride monohydrate adjusted to pH 9.0 with 5 N NaOH, and was incubated for 1 h at 37 C. After appropriate dilution with 0.01 M potassium phosphate (pH 7.4), the amount of urocanic acid formed was assayed by measuring the optical density at 277 nm, taking advantage of the extinction coefficient of urocanic acid, 1.88 x 10^4 mol^-1 cm^-1 (15). One unit of L-histidine ammonia lyase activity was defined as that activity which converted 1 μmol of L-histidine to urocanic acid per min under the conditions of the assay. Total enzyme activity, i.e., amount of formed enzyme, was expressed in terms of units per millilitre of unfiltered broth, and specific activity was expressed in terms of units per milligram of dried cells.

No activity of L-histidine ammonia lyase was found in the filtered broth of *A. liquidum*.

The assay of histidine was carried out by a spectrophotofluorometric method using o-phthalaldehyde (17).

Ammonia was assayed by the nesslerization method.

For the estimation of growth, the fermentation broth was diluted 1:20 with saline, and optical density was measured at 660 nm with a Hitachi photoelectric photometer model 101. Dried-cell weight was estimated from a standard curve which correlates optical density to weight of lyophilized cells.

RESULTS AND DISCUSSION

Screening experiments. Urocanic acid formation from L-histidine was observed in a considerable number of bacteria when the reaction mixtures were incubated for 24 h at 37 C (Table 1). Marked accumulation was found among strains of the genera *Achromobacter, Agrobacterium, Bacillus, Bacterium, Flavobacterium, Micrococcus, Pseudomonas, Sarcina,* and *Xanthomonas.* L-Histidine ammonia lyase and urocanase activities were compared in the strains which produced high levels of urocanic acid (Table 2). Of the tested organisms, *Achromobacter liquidum* possessed the highest L-histidine ammonia lyase activity and was used for the subsequent experiments.

Strains of genus *Micrococcus* had no urocanase activity (Table 2); therefore, urocanic acid would not be degraded further by these bacteria. Thus, conversion to urocanic acid of 100% of the L-histidine supplied to *Micrococcus* sp. is theoretically possible. In the genus *Achromobacter,* weak urocanase activities led to degradation of formed urocanic acid, and glutamate, which is a final product of the degradation pathway, was detected in the reaction mixture. The *Achromobacter* urocanase activity, however, could be destroyed by heat treatment without inactivation of L-histidine ammonia lyase, so strains of *Achromobacter* were used instead of *Micrococcus* for the production of urocanic acid.

A heat-treated and washed cell suspension of *A. liquidum* converted L-histidine to equimolar amounts of urocanic acid and ammonia.

Cultural conditions for formation of L-histidine ammonia lyase. To establish the most advantageous cultural conditions for the formation of L-histidine ammonia lyase, various conditions were investigated with *A. liquidum* IAM 1667.

Among the carbon sources tested at a concent-

| Genus              | No. of tested strains | No. of strains producing >1 mg of urocanic acid/ml |
|--------------------|-----------------------|-----------------------------------------------|
| *Achromobacter*     | 14                    | 8                                             |
| *Aerobacter*        | 1                     | 1                                             |
| *Agrobacterium*     | 2                     | 2                                             |
| *Alcaligenes*       | 2                     | 2                                             |
| *Arthurbacter*      | 1                     | 0                                             |
| *Bacillus*          | 14                    | 9                                             |
| *Bacterium*         | 2                     | 2                                             |
| *Brevibacterium*    | 4                     | 3                                             |
| *Corynebacterium*   | 4                     | 0                                             |
| *Erwinia*           | 1                     | 0                                             |
| *Escherichia*       | 2                     | 0                                             |
| *Flavobacterium*    | 4                     | 3                                             |
| *Kurthia*           | 2                     | 2                                             |
| *Microbacterium*    | 2                     | 0                                             |
| *Micrococcus*       | 10                    | 6                                             |
| *Proteus*           | 3                     | 0                                             |
| *Pseudomonas*       | 20                    | 20                                            |
| *Sarcina*           | 14                    | 10                                            |
| *Serratia*          | 2                     | 2                                             |
| *Staphylococcus*    | 1                     | 1                                             |
| *Xanthomonas*       | 2                     | 1                                             |

| Bacteria                         | L-Histidine ammonia lyasea | Urocanasea |
|----------------------------------|---------------------------|-----------|
| *Achromobacter liquidum*         | 84                        | 2.5       |
| *A. superficialis*               | 62                        | 1.5       |
| *Micrococcus luteus*             | 14                        | 0.0       |
| *M. ureae*                       | 45                        | 0.0       |
| *Pseudomonas fluorescens*        | 58                        | 0.8       |

a x 10^3 units/ml of broth.
tration of 1%, glucose was the most effective in the formation of the enzyme. Thus, glucose was used as a main carbon source for subsequent experiments. The highest total activity was obtained at 2% glucose (Fig. 1).

L-Histidine ammonia lyase was induced most effectively at an L-histidine concentration of 1% (Fig. 1). When L-histidine was omitted from the medium, enzyme activity of 0.01 unit per ml of broth was obtained, indicating that low levels of the enzyme were produced constitutively. L-Histidine at a concentration of less than 1% was enough to obtain high levels of the enzyme (Fig. 3). The addition of 0.2% L-histidine at the final period of log phase induced the immediate increase of the enzyme, and at 16 h of induction time the same amount of the enzyme as the control culture was obtained.

In preliminary experiments, shaking cultures gave much higher enzyme activity than did stationary cultures. The effect of aeration was studied by varying the amount of media in the flasks. Maximal growth and enzyme formation were attained with less than 100 ml of medium in a 500-ml flask (Fig. 4).

The effect of temperature was also studied at 25, 30, and 37 C. The optimal temperature for enzyme formation was 30 C. At 37 or 25 C, both growth and enzyme formation were inferior to those observed at 30 C.

**Typical changes during fermentation.** The data for a typical fermentation under optimal conditions are given in Fig. 5. A 500-ml flask containing 100 ml of medium composed of 2% glucose, 0.2% yeast extract, 0.2% peptone, 0.2% L-histidine hydrochloride monohydrate, 0.2% urea, 0.2% K₂HPO₄, 0.05% KH₂PO₄, 0.02% NaCl, 0.02% MgSO₄.7H₂O, 0.01% MnSO₄.4H₂O, and 0.001% FeSO₄.7H₂O (pH 7.0) was inoculated with one loopful of the organism, and shaking incubation was carried out at 30 C. Enzyme formation started at the final period of

---

**Fig. 1.** Effect of glucose on L-histidine ammonia lyase formation by Achromobacter liquidum. Symbols: O, L-histidine ammonia lyase activity; Δ, growth; △, terminal pH.

**Fig. 2.** Effect of L-histidine on L-histidine ammonia lyase formation by Achromobacter liquidum. Symbols are as in Fig. 1.

**Fig. 3.** Induction of L-histidine ammonia lyase by L-histidine. In case of "+L-His medium," 0.2% L-histidine was added at the time of inoculation. Furthermore, 0.2% L-histidine was added to both cultures at 24 h, as indicated by the arrows.

**Fig. 4.** Effect of aeration on L-histidine ammonia lyase formation by Achromobacter liquidum. Symbols are as in Fig. 1.
log phase, and additions of 0.2% L-histidine at 24, 32, and 48 h greatly accelerated the formation of L-histidine ammonia lyase. At 72 h, the enzyme level reached its maximal point.

Throughout the culture period, the enzyme activity was found in the cells and not in the filtered broth. Maximal activity, 0.62 units per ml of medium and 0.059 units per mg of dried cells, was observed in this culture of A. liquidum.

**Conditions for the enzymatic formation of urocanic acid.** The most advantageous conditions for the enzymatic conversion of L-histidine to urocanic acid by L-histidine ammonia lyase were investigated.

L-Histidine ammonia lyase of A. liquidum is heat stable, and urocanase of this bacteria is heat labile. Heat treatment of the broth at 70 °C for 30 min completely inactivated urocanase and had no effect on L-histidine ammonia lyase (Fig. 6). The slight increase of L-histidine ammonia lyase activity at the first 13 min of heating was ascribed to an increase in permeability of the cell membrane.

To obtain a greater increase in permeability of the cell membrane, the effects of various surfactants were examined. Of the tested surfactants, nonionic types showed no effect, but anionic, cationic, and amphotheric types were very effective. Among them, triethanolamine lauryl sulfate (TEALS) was most effective. None of the tested surfactants inhibited enzyme activity at a concentration of 0.02%. After the addition of TEALS to the reaction mixture at concentrations of 0.02 to 0.2%, the enzyme activity increased by about threefold (Fig. 7).

The effects of pH and temperature on the enzymatic conversion of L-histidine to urocanic acid were investigated. Maximal activity was obtained at pH 9.0 and 60 °C (Fig. 8). At 60 °C, however, the enzyme was gradually inactivated, and the conversion of 0.5 M L-histidine to urocanic acid was inferior at temperatures higher than 50 °C (Fig. 9), whereas at temperatures lower than 40 °C, the enzyme was stable for at least 10 days in the reaction mixtures.

---

**Fig. 5.** Changes during fermentation for L-histidine ammonia lyase formation by Achromobacter liquidum. Symbols are as in Fig. 1. Additions of 0.2% L-histidine were made at 0, 24, 32, and 48 h, as indicated by the arrows.

**Fig. 6.** Different heat stabilities at 70 °C of the L-histidine ammonia lyase and urocanase of Achromobacter liquidum. Symbols: O, L-histidine ammonia lyase activity; ●, urocanase activity.

**Fig. 7.** Effect of surfactant on L-histidine ammonia lyase activity of Achromobacter liquidum.

**Fig. 8.** Effect of pH and temperature on L-histidine ammonia lyase activity of Achromobacter liquidum. The pH of reaction mixtures containing 0.15 M L-histidine was adjusted with NaOH or HCl. Effect of temperature was studied at pH 9.0.
Therefore, it is most advantageous to carry out the reaction at pH 9.0 and 40 C. Thus, the maximal enzyme activity, 1.92 units per ml of broth, observed in this reaction system is much higher than that previously reported in Pseudomonas fluorescens ATCC 11299b (16), which showed an activity of 0.88 units per ml of medium.

When the reaction mixture was agitated, the velocity of the enzymatic reaction appeared to be almost the same as that attained without agitation.

The initial velocity decreased at L-histidine concentrations higher than 0.5 M (Fig. 10), and the percentage of conversion did not reach 100% when the level of L-histidine exceeded 0.8 M (Fig. 11). Therefore, it is necessary to feed less than 0.8 M L-histidine.

Urocanic acid formation under optimal conditions. A typical bioconversion of L-histidine to urocanic acid is illustrated in Fig. 12.

The incubation mixture contained 100 ml of the heat-treated broth cultured under optimal conditions as enzyme source, 33.6 g of L-histidine hydrochloride monohydrate, and 0.2 ml of 50% aqueous solution of TEALS to a final volume of 200 ml. The incubation mixture was adjusted to pH 9.0 with 5 N NaOH and incubated at 40 C. Urocanic acid increased linearly with the consumption of L-histidine. During the reaction, the pH was maintained at around 9, which is optimal for the enzymatic reaction.

In this enzymatic procedure, neither formation of amino acids and organic compounds other than urocanic acid nor decomposition of accumulated urocanic acid occurred, even with prolonged incubation. The urocanic acid formed was easily isolated by an ordinary procedure as follows. (i) The reaction mixture was heated, adjusted to pH 4.8 with concentrated HCl, and chilled to 0 C. (ii) The precipitated urocanic acid was collected with filtration and recrystallized from hot water to yield 25.6 g of pine leaf-like crystals of urocanic acid. Some characteristics of the crystals were: melting point, 225 C; analysis: C_{13}H_{16}N_{4}O_{2}·2H_{2}O; calculated: C, 41.37; H, 5.78; N, 16.08; found: C, 41.13; H, 5.91; N, 15.88.
Yields of urocanic acid of over 90% from L-histidine were obtained. Amino acids and Pauly-positive compounds other than urocanic acid were not detected in the product by paper chromatography.

Simultaneous production of D-histidine and urocanic acid from DL-histidine. Since the L-histidine ammonia lyase of A. liquidum is specific for L-histidine, D-histidine and urocanic acid can be easily obtained from DL-histidine. Fig. 13 illustrates the typical reaction for the formation of urocanic acid and D-histidine from DL-histidine. The reaction conditions were the same as in the previous experiment, except that the concentration of DL-histidine was 0.24 M. The urocanic acid and D-histidine were isolated in high yields from the reaction mixture (Fig. 14).

ACKNOWLEDGMENTS

We are grateful to T. Takayanagi, managing director of Tanabe Seiyaku Co., Ltd., for his helpful advice and encouragement in this study, and to T. Kawahara for her technical assistance.

LITERATURE CITED

1. Auerbach, V. H., and H. A. Waisman. 1959. Tryptophan peroxidase-oxidase, histidase, and transaminase activity in the liver of the developing rat. J. Biol. Chem. 234:304–306.
2. Chasin, L., and B. Magasanik. 1968. Induction and repression of the histidine-degrading enzymes of Bacillus subtilis. J. Biol. Chem. 243:5165–5178.
3. Cruz-Camarillo, R. 1961. Effect of some uncouplers of oxidative phosphorylation on the induced biosynthesis of histidase and on the respiration of Serratia marcescens. Rev. Latinoam. Microbiol. Parasitol. 4:167–182.
4. Frankfater, A., and I. Fridovich. 1970. The purification and properties of oxidized derivatives of L-histidine ammonia-lyase. Biochem. Biophys. Acta 206:457–472.
5. Hartwell, L. H., and B. Magasanik. 1963. The molecular basis of histidase induction in Bacillus subtilis. J. Mol. Biol. 7:401–420.
6. Hassall, H., P. J. Lunn, and J. Ryall-Wilson. 1970. Detection of histidase and urocanase after disc electrophoresis on polyacrylamide gels. Anal. Biochem. 35:326–334.
7. Hug, D. H., and D. Roth. 1968. Inhibition of histidine deaminase by L-tyrosine and D-hydroxyphenylpyruvate. Biochem. Biophys. Res. Commun. 30:249–254.
8. Jacoby, G. A. 1964. The induction and repression of amino acid oxidation in Pseudomonas fluorescens. Biochem. J. 92:1–8.
9. Jensen, D. E., and F. C. Neidhardt. 1969. Effect of growth rate on histidine catabolism and histidase synthesis in Aerobacter aerogenes. J. Bacteriol. 96:131–142.
10. Kidwai, J. R., and C. R. K. Murti. 1964. Enzyme induction in Vibrio cholerae. Indian J. Biochem. 1:66–70.
11. Lessie, T. G., and F. C. Neidhardt. 1967. Formation and operation of the histidine-degrading pathway in Pseudomonas aeruginosa. J. Bacteriol. 93:1800–1810.
12. Magasanik, B. 1963. The genetic and molecular basis of catabolite repression. In H. J. Vogel, V. Bryson, and J. O. Lampen (ed.), Informational macromolecules. Academic Press Inc., New York.
13. Magasanik, B., P. Lund, F. C. Neidhardt, and D. T. Schwarz. 1965. Induction and repression of the histidine-degrading enzymes in Aerobacter aerogenes. J. Biol. Chem. 240:4320–4324.
14. Mansurova, I. D., and L. G. Kaletkina. 1972. Histidine-ammonia-lyase and urocanase activity of blood serum, liver, and its subcellular structures in rats. Biokhimka 37:1302–1305.
15. Mehler, A. H., and H. Tabor. 1953. Deamination of histidine to form urocanic acid in liver. J. Biol. Chem. 201:775–784.
16. Mehler, A. H., H. Tabor, and O. Hayashi. 1955. Urocanic acid (4(or 5)-imidazoacrylic acid). Biochem. Prep. 4:50–53.
17. Pisano, J. J., J. D. Wilson, L. Cohen, D. Abraham, and S. Udenfriend. 1961. Isolation of γ-aminobutylihistidine (homocarnosine) from brain. J. Biol. Chem. 236:499–502.
18. Ruis, H., and H. Kindl. 1971. Formation of α,β-unsaturated carboxylic acids from amino acids in plant peroxisomes. Phytochemistry 10:2627–2631.
19. Steinberg, W., and H. O. Halvorson. 1968. Timing of enzyme synthesis during outgrowth of spores of Bacillus cereus. I. Ordered enzyme synthesis. J. Bacteriol. 95:469–478.
20. Tabor, H., and A. H. Mehler. 1955. Histidase and urocanase, p. 228-231. In S. P. Colowick and N. O. Kaplan (ed.), Methods in Enzymology, vol. 2. Academic Press Inc., New York.

21. Tabor, H., A. H. Mehler, O. Hayaishi, and J. White. 1952. Urocanic acid as an intermediate in the enzymatic conversion of histidine to glutamic and formic acids. J. Biol. Chem. 196:121-128.

22. Wachi, T. 1958. Intermediate metabolism of amino acids by Mycobacterium avium. I. Histidine deaminase. Med. J. Osaka Univ. 10:741-746.

23. Zannoni, V. G., and B. N. Ladu. 1963. Determination of histidine α-deaminase in human stratum corneum and its absence in histidinaemia. Biochem. J. 88:160-162.