Enzymes Relevant to Kojic Acid Biosynthesis in *Aspergillus flavus*

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Hexokinase, glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, glucose dehydrogenase, glucose oxidase and gluconate dehydrogenase have been demonstrated in mycelial homogenates from a strain of *Aspergillus flavus* that produces considerable amounts of kojic acid. The enzymes were assayed after different periods of growth under different experimental conditions and their activities were compared with the rate of kojic acid production. Glucose oxidase activity was too low to account for the formation of kojic acid but the other enzymes had sufficient activities. Correlation of the pattern of enzyme activities under different experimental conditions with kojic acid concentrations provided evidence for the involvement of glucose dehydrogenase and gluconate dehydrogenase in kojic acid biosynthesis. Based on these data a possible pathway for the biosynthesis of kojic acid is presented. Gluconic acid- δ -lactone and at least one of the three compounds, 3-ketogluconic acid lactone, 3-ketoglucose and oxykojic acid, are believed to be intermediates in this pathway.

INTRODUCTION

Kojic acid is produced aerobically from carbohydrate sources by a variety of micro-organisms. Hypotheses have been put forward from time to time to explain the biosynthesis of kojic acid (Beelik, 1956), but the exact pathway of conversion of glucose to kojic acid and the enzymes involved are not known. In the present investigation an attempt has been made to elucidate the mechanism of kojic acid biosynthesis. Certain enzymes which may be involved in kojic acid biosynthesis have been assayed at different times under different experimental conditions and an attempt has been made to correlate the data obtained with the extent of kojic acid production under different conditions.

METHODS

Organism. *Aspergillus flavus* strain B, isolated in this laboratory, was used (Bajpai, 1978). It was maintained on potato dextrose agar slants by subculturing at monthly intervals. The methods pertaining to the cultivation of the organism, cultural conditions, resuspension experiments and kojic acid estimation have been reported previously (Bajpai *et al.*, 1977).

Enzyme assays. Different enzymes were assayed in mycelium after different periods of growth or resuspension. The mycelium plus medium was chilled in the refrigerator for 30 min, and then the medium was decanted and the mycelium was washed three times with 50 ml portions of the appropriate ice-cold buffer. A suitable quantity of the mycelium was ground in the cold with a chilled mortar and pestle with five times its weight of acid-washed sand and 5 ml of ice-cold buffer, and made up to a known volume to give a 10% (w/v) homogenate on a wet weight basis. The homogenate was centrifuged at 15000 *g* for 15 min. The clear supernatant was stored in the cold at 4 °C till further use for determining the protein content and enzyme activity which were assayed within 24 h. The buffers

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used for the preparation of homogenate for each enzyme assay, and the assay methods, were as follows: hexokinase (EC 2.7.1.1) was assayed in 0.1 M-glycylglycine buffer, pH 9.0, by the method of Darrow & Colowick (1962); 6-phosphogluconate dehydrogenase (EC 1.1.1.44) in 0.1 M-triethanolamine/HCl buffer, pH 7.6, by the method of Pontremoli & Grazi (1966); glucose-6-phosphate dehydrogenase (EC 1.1.1.49) in 0.1 M-glycylglycine buffer, pH 8.0, by the method of Kuby & Noltman (1966); glucose oxidase (EC 1.1.3.4) in 0.1 M-phosphate buffer, pH 7.0, by the method of Hugget & Nixon (1957); glucose dehydrogenase (EC 1.1.1.47) in 0.1 M-phosphate buffer, pH 6.0, by the method of Hauge (1966); gluconate dehydrogenase (EC 1.1.99.3) in 0.5 M-sodium acetate buffer, pH 5.5, by the method of Wood *et al.* (1962). One unit of enzyme activity was defined as the amount of enzyme which catalysed the conversion of 1 μ mol substrate into product min^{-1} . Specific activity was expressed in terms of units $(\text{mg protein})^{-1}$. Protein was estimated by the Lowry method.

Paper chromatography. Samples (0.5 ml) of the mycelial homogenate in 0.1 M-phosphate buffer, pH 6.0, or in 0.1 M-glycylglycine buffer, pH 8.0, were incubated, respectively, with 0.2 ml 0.6 M-glucose for 15 min or with 0.4 ml 0.2 M-glucose plus 0.05 ml 0.1 M-ATP for 15 or 30 min at 25 °C. The reaction mixtures were concentrated in vacuo and then subjected to descending chromatography on Whatman no. 1 filter paper in the following three solvent systems: butan-1-ol/acetic acid/water (50:25:25, by vol.), upper phase from a mixture of water/butan-2-ol/2-methylpropan-2-ol (48.4:34:17.6, by vol.) and propan-1-ol/conc. HCl/water (65:18.4:16.6, by vol.). The chromatograms were air dried and sprayed with hydroxylamine reagent or Hanes & Isherwood reagent as required (Abdel Akher & Smith, 1951; Hanes & Isherwood, 1949). Authentic samples of gluconic acid- δ -lactone, glucose 6-phosphate and 6-phosphogluconic acid were chromatographed for comparison.

RESULTS AND DISCUSSION

In the absence of any information on the enzymes involved in the pathway of kojic acid biosynthesis, the enzymes to be studied were selected on the basis of several indirect lines of evidence. The studies of Arnstein & Bentley (1953) established that the glucose molecule is converted directly into kojic acid without a cleavage of the carbon chain into smaller fragments. Theoretically, starting from the pyranose form of glucose, such a conversion should involve a minimum of three reactions, namely, oxidation of a secondary hydroxyl group and the introduction of two double bonds by removing the elements of water:

Enzymes of glucose metabolism mentioned below have been reported in micro-organisms and especially in fungi (Blakely & Ciferri, 1961; Gibak, 1967; Horecker *et al.*, 1955; Bentley, 1955; Darrow & Colowick, 1962). Therefore their possible participation in kojic acid biosynthesis had to be considered. Further, earlier studies on the effect of different inhibitors on kojic acid production under different experimental conditions also provided indirect evidence for their possible participation in the synthesis of kojic acid (Bajpai, 1978). The relevant enzymes are glucose oxidase, glucose dehydrogenase, gluconate dehydrogenase, hexokinase, glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase. Also, preliminary experiments indicated the presence of the above enzyme activities in crude homogenates of *A. flavus* mycelium. Paper chromatography studies provided evidence for the formation of gluconic acid- δ -lactone, glucose 6-phosphate and 6-phosphogluconic acid from glucose in such homogenates.

During growth on YES (yeast extract/sucrose) medium, the specific activities of hexokinase, glucose dehydrogenase and gluconate dehydrogenase reached a maximum after 10 d growth (Table 1), at about the same time as the kojic acid concentration in the medium reached a maximum (Table 2; Bajpai *et al.*, 1977). On the other hand, the specific activities of glucose oxidase and hexosemonophosphate dehydrogenases decreased with time (Table 1). These data suggest that glucose oxidase and hexose monophosphate dehydrogenases are the least relevant to kojic acid production and hexokinase, glucose dehydrogenase and gluconate dehydrogenase are the most relevant. In experiments in which 10 d old mycelium, 3 week old mycelium and 10 d old preincubated mycelium were resuspended in phosphate buffer supplemented with glucose, kojic acid concentrations reached a maximum after 8, 12 or 10 d,

Table 1. *Enzyme activities in A. flavus mycelium grown on YES medium, and in 10 d old mycelium resuspended in phosphate buffer plus glucose*

Activities are expressed as units (mg protein)⁻¹; one unit represents the conversion of 1 μ mol substrate into product min⁻¹.

Enzyme	Mycelium grown on YES medium					10 d old mycelium resuspended in 0.2 M-phosphate buffer, pH 6.5, supplemented with 20% (w/v) glucose				
	Period of incubation (d):					Period after resuspension (d):				
	3	7	10	15		0	1	3	7	9
Glucose oxidase	0.05	0.04	0.03	0.02		0.08	0.09	0.08	0.01	0.004
Glucose dehydrogenase	0.05	0.285	0.61	0.27		0.19	0.30	0.56	0.78	0.44
Gluconate dehydrogenase	0.09	0.37	0.99	0.44		0.20	0.60	0.82	1.10	0.54
Hexokinase	9.89	18.04	19.83	9.90		9.90	15.64	12.30	5.80	3.40
Glucose-6-phosphate dehydrogenase	0.25	0.24	0.16	0.09		0.52	0.70	0.47	0.23	0.12
6-Phosphogluconate dehydrogenase	0.24	0.20	0.13	0.086		0.26	0.37	0.20	0.12	0.04

Table 2. *Kojic acid synthesis in A. flavus mycelium grown on YES medium, and in different types of mycelium resuspended in phosphate buffer plus glucose*

Type of mycelium	Kojic acid (mg ml ⁻¹)					
	Period of incubation (d):					
	4	6	8	10	12	16
Mycelium grown on YES medium	10.2	37.0	66.3	81.5	55.2	17.2
10 d old mycelium resuspended in 0.2 M-phosphate buffer supplemented with 20% (w/v) glucose	20.2	55.2	78.4	62.4	50.2	14.2
3 week old mycelium resuspended in 0.2 M-phosphate buffer supplemented with 20% (w/v) glucose	15.5	30.2	50.2	60.1	76.4	42.2
10 d old but preincubated mycelium resuspended in 0.2 M-phosphate buffer supplemented with 20% (w/v) glucose	17.8	32.4	60.4	77.2	57.2	25.2

respectively, (Table 2; Bajpai *et al.*, 1977). The activities of glucose oxidase, hexokinase and hexosemonophosphate dehydrogenases remained high for 1 to 3 d and then decreased to low values by 9 d, whereas those of glucose dehydrogenase and gluconate dehydrogenase generally increased with the period of incubation and were comparatively high at around 9 d. The data for the enzyme activities in 10 d old mycelium resuspended in phosphate buffer supplemented with glucose are presented in Table 1. These again implicate glucose dehydrogenase and gluconate dehydrogenase in kojic acid biosynthesis.

The activities exhibited *in vitro* by the different enzymes were compared with the rate of kojic acid production to ascertain if there was any correlation between these two parameters. The maximum rates of kojic acid production were calculated to be 0.047, 0.053, 0.023 and 0.030 $\mu\text{mol min}^{-1}$ (100 mg wet wt mycelium)⁻¹ during growth on YES medium, or after resuspension of 10 d old mycelium, 3 week old mycelium or 10 d old preincubated mycelium, respectively. These rates were compared with the activities of the relevant enzymes at the time at which kojic acid concentration reached a maximum. Of the six enzymes studied, hexokinase had the highest activity and may be involved in kojic acid biosynthesis. Glucose oxidase had insufficient activity to account for the observed rate of kojic acid production. The activities of hexosemonophosphate dehydrogenases in most of the resuspension systems were just sufficient to account for the rates of kojic acid synthesis. However, the activities of glucose dehydrogenase and gluconate dehydrogenase were higher than those required to account for observed rates of kojic acid synthesis. It is very likely that glucose dehydrogenase and gluconate dehydrogenase are components of the biosynthetic pathway. Also, the pattern of changes in enzyme activities during resuspension strongly supported the involvement of the above two enzymes in kojic acid biosynthesis. The pathway based on theoretical grounds presented earlier in this paper must therefore be modified to include gluconic acid as an intermediate. The product formed by gluconate dehydrogenase has not been identified in this system. Possible products are 2-ketogluconate, 5-ketogluconate, 2,5-diketogluconate and 3-ketogluconate. The first three are known to be produced in different enzyme systems such as gluconate dehydrogenase, 5-ketogluconate reductase and ketogluconate dehydrogenase. Some evidence also exists for 3-keto compounds (Preiss & Ashwell, 1963; Horecker & Smyrniotis, 1950; Moses & Hansen, 1959). A ketogluconic acid which is not the usual 2- or 5-keto acid and is probably 3-ketogluconic acid has been isolated from *Acetobacter melanogenum* (Tumova & Bernhauer, 1950). This seems especially significant since the acetic acid bacteria are the only group of bacteria known to produce kojic acid. Therefore 3-ketogluconic acid has been included in the proposed pathway. The pathway beyond this is purely hypothetical but the reactions suggested have similarities to other well known enzymic

Fig. 1. Proposed biosynthetic pathways for kojic acid biosynthesis in *A. flavus*.

reactions. The proposed biosynthetic pathway is given in Fig. 1: three alternative routes have been suggested.

Though the evidence for the roles of glucose dehydrogenase and gluconate dehydrogenase is quite strong both the data on the enzyme activities in resuspended mycelium and on the effect of inhibitors do not rule out the involvement of phosphorylated intermediates in kojic acid biosynthesis. If such intermediates are involved, the above postulated pathways are still tenable provided gluconic acid- δ -lactone and later intermediates are replaced by 6-phosphogluconic acid and other phosphorylated derivatives.

Further detailed investigations are needed to check the validity of the proposed pathways.

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REFERENCES

- ABDEL AKHER, M. & SMITH, F. (1951). Detection of carbohydrate esters and lactones after separation by paper chromatography. *Journal of the American Chemical Society* **73**, 5859–5860.
- ARNSTEIN, H. R. V. & BENTLEY, R. (1953). The biosynthesis of kojic acid 1. Production from 1 ^{14}C and 3:4 $^{14}\text{C}_2$ glucose and 2 ^{14}C 1:3 dihydroxy acetone. *Biochemical Journal* **54**, 493–508.
- BAJPAL, P. (1978). *Studies on kojic acid biosynthesis*. Ph.D. thesis, Kanpur University, India.
- BAJPAL, P., AGRAWALA, P. K. & VISHWANATHAN, L. (1977). Kojic acid synthesis by resuspended mycelia of *A. flavus*. *Proceedings of the Sixth Joint Convention of Sugar Technologists' Association of India, The Deccan Sugar Technologists' Association of India and the South India Sugar and Sugarcane Technologists' Association*, G17–23.
- BELLIK, A. (1956). Kojic acid. *Advances in Carbohydrate Chemistry* **11**, 145–164.
- BENTLEY, R. (1955). Glucose aerodehydrogenase. *Methods in Enzymology* **1**, 340–345.
- BLAKELY, E. R. & CIFERRI, O. (1961). Oxidation of gluconate, 2-deoxy-D-glucose and lactic acid by an enzyme preparation from *Aerobacter aerogenes*. *Canadian Journal of Microbiology* **5**, 61–73.
- DARROW, R. A. & COLOWICK, S. P. (1962). Hexokinase from baker's yeast. *Methods in Enzymology* **5**, 226–235.
- GIBAK, T. (1967). Studies on glucose dehydrogenase of *Aspergillus oryzae* III. General enzymatic properties. *Biochimica et biophysica acta* **146**, 317–328.
- HANES, C. S. & ISHERWOOD, F. A. (1949). Separation of phosphoric esters on the filter paper chromatogram. *Nature, London* **164**, 1107–1112.
- HAUGE, J. G. (1966). Glucose dehydrogenase, soluble II. *Bacterium anitratum*. *Methods in Enzymology* **9**, 107–111.
- HORECKER, B. L. & SMYRNIOTIS, P. Z. (1950). The enzymic production of ribose-5-phosphate from 6-phosphogluconate. *Archives of Biochemistry* **29**, 232–233.
- HORECKER, B. L., KORNBERG, A. & SMYRNIOTIS, P. Z. (1955). (a) Glucose-6-phosphate dehydrogenase and (b) 6-phosphogluconate dehydrogenase. *Methods in Enzymology* **1**, 323–327.
- HUGGET, S. G. A. & NIXON, D. A. (1957). Enzymic determination of blood glucose. *Biochemical Journal* **66**, 12P.
- KUBY, S. A. & NOLTMAN, E. A. (1966). Glucose-6-phosphate dehydrogenase from brewer's yeast. *Methods in Enzymology* **9**, 116–125.
- MOSES, V. & HANSEN, O. (1959). Non photosynthetic fixation of carbon dioxide by three microorganisms. *Journal of Bacteriology* **77**, 70–78.
- PONTREMOLI, S. & GRAZI, E. (1966). 6-Phosphogluconate dehydrogenase, crystalline. *Methods in Enzymology* **9**, 137–141.
- PREISS, J. & ASHWELL, G. (1963). Polygalacturonic

- acid metabolism in bacteria 2. Formation and metabolism of 3-deoxy-D-glycero-2,5-hexodiulsonic acid. *Journal of Biological Chemistry* **238**, 1571–1576.
- TUMOVA, R. E. & BERNHAUER, K. (1950). Oxidation by acetic acid bacteria. VIII. Method of bacterial oxidations in submerged cultures. *Biochemische Zeitschrift* **320**, 466–471.
- WOOD, W. A., FETTING, R. A. & HERTLEIN, B. C. (1962). Gluconic acid dehydrogenase from *Pseudomonas fluorescens*. *Methods in Enzymology* **5**, 287–291.