Phytochemistry, 1967, Vol. 6, pp. 625 to 632. Pergamon Press Ltd. Printed in England

PURIFICATION AND PROPERTIES OF CHALCONE-FLAVANONE ISOMERASE FROM SOYA BEAN SEED

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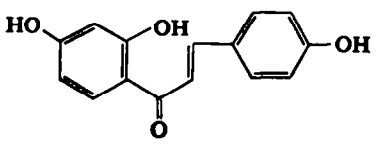
(*Received* 20 *September* 1966)

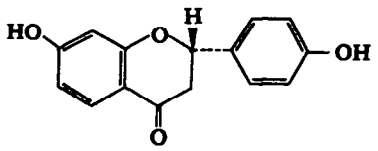
Abstract—An enzyme catalysing the conversion of chalcones to the corresponding flavanones has been purified about 150-fold from soaked soya bean seed (*Soja hispida*). This enzyme is stable when stored as a freeze-dried preparation at 2–4°. It is not inhibited by azide, cyanide, diethyldithiocarbamate or EDTA but is strongly inhibited by low concentrations of *p*-hydroxymercuribenzoate. The pH optimum is about 7·5. The enzymic reaction is not dependent on coenzymes or activators. Substrate specificity of the enzyme was studied using seven chalcones.

INTRODUCTION

RECENT studies have shown that flavanones occupy a central position in flavonoid biosynthesis.[1–3] It is very probable that they represent the primary heterocyclic intermediate from which the biosynthetic pathways to the different classes of flavonoid compounds diverge. Although isomerization of most chalcones to the corresponding flavanones occurs spontaneously in solution, there must be an enzyme catalysing this reaction, since natural flavanones occur in optically active forms. Preliminary evidence for the presence of such an enzyme in lemon peel has been presented by Shimokoriyama.[4] We have recently reported the existence in soya bean seedlings (*Soja hispida*) of an enzyme which catalyses the conversion of 2′,4,4′-trihydroxychalcone (isoliquiritigenin) (I) to (−) 4′,7-dihydroxyflavanone (liquiritigenin) (II).[5] In this paper we report a procedure for purifying the enzyme from soaked soya bean together with some of its properties.



(I)



(II)

RESULTS

Enzyme Purification

After acid precipitation, ammonium sulphate fractionation and phosphate gel treatment (see Experimental section) the enzyme was passed through a Sephadex G 200 column (35 ×

[1] L. PATSCHKE, W. BARZ and H. GRISEBACH, *Z. Naturforsch.* **19b**, 1110 (1964).
[2] H. GRISEBACH and S. KELLNER, *Z. Naturforsch.* **20b**, 446 (1965).
[3] L. PATSCHKE, W. BARZ and H. GRISEBACH, *Z. Naturforsch.* (In press.) Personal communication.
[4] M. SHIMOKORIYAMA, *J. Am. Chem. Soc.* **79**, 4199 (1957).
[5] E. WONG and E. MOUSTAFA, *Tetrahedron Letters* 3021 (1966).

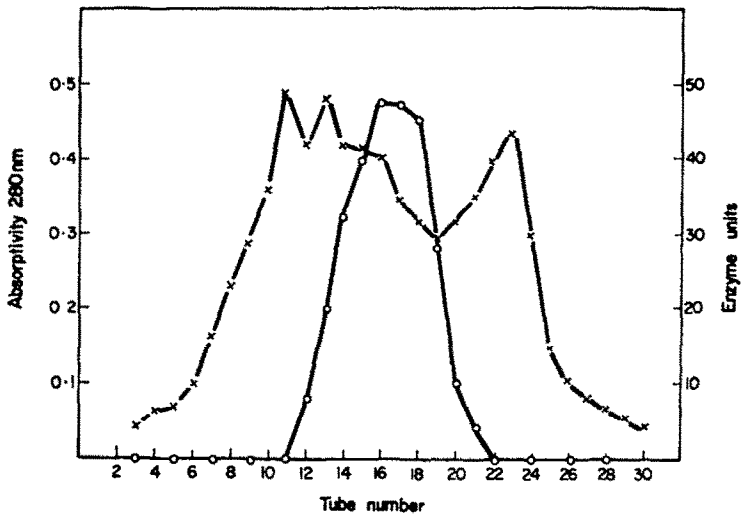


FIG. 1. FIRST SEPHADEX COLUMN FRACTIONATION OF CHALCONE-FLAVANONE ISOMERASE.

× Absorptivity at 280 nm.
○ Enzyme units per 15 ml fraction.

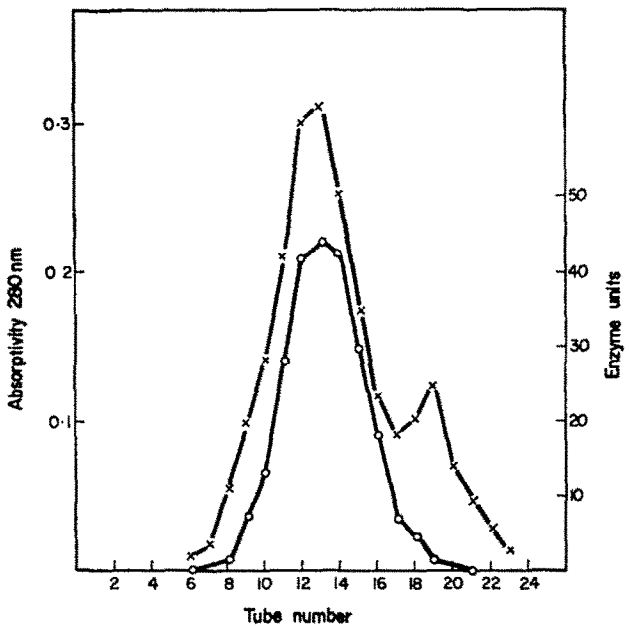


FIG. 2. SECOND SEPHADEX COLUMN FRACTIONATION OF CHALCONE-FLAVANONE ISOMERASE.

× Absorptivity at 280 nm.
○ Enzyme units per 15 ml fraction.

3 cm). Absorptivity at 280 nm and enzyme activity were determined in each of the 15 ml fractions (Fig. 1). Fractions 13–19 were dialysed against two changes of 4 l. of distilled water at 2–4° and freeze-dried. The freeze-dried protein (about 30 mg) was dissolved in 4 ml of 0·02 M tris HCl buffer at pH 7·5 and fractionated through a second Sephadex G 200 column. The results of the second column fractionation showed the presence of two protein components one of which contained the enzyme (Fig. 2). Fractions 11–15 were collected, dialysed against water and freeze-dried. The colourless, amorphous powder was kept over $CaCl_2$ at 2–4° for several weeks without losing significant activity.

The progressive purification and yield of the enzyme are shown in Table 1. It will be seen that the final enzyme preparation was purified approximately 150-fold.

TABLE 1. PURIFICATION OF CHALCONE-FLAVANONE ISOMERASE FROM SOAKED SOYA BEAN SEED

Fractions	Protein (mg)	Specific activity*	Purity	Units†	Yield %
Crude dialysed extract	5760	0·08	1	460·8	100
Acid-treated fraction	1764	0·21	2·6	370·4	80·3
$(NH_4)_2SO_4$ fraction	272	1·20	15·0	326·4	70·8
Phosphate gel fraction	70·3	4·20	52·5	295·2	64·0
Second Sephadex column	15·0	12·52	156·5	187·8	40·7

* μmoles chalcone converted per mg protein per hour.
† One enzyme unit = amount of enzyme that catalyses the conversion of 1 μmole of chalcone to flavanone per hour.

When the enzyme was subjected to centrifugation in an artificial boundary cell in the model E Spinco Ultracentrifuge at 59,780 rpm a single symmetrical component was observed. The observed sedimentation constant was 1·6–2 S.

Reactions Catalysed by the Enzyme

When the purified enzyme was incubated with isoliquiritigenin (I), the latter was converted to liquiritigenin (II) which earlier was shown to be the laevorotatory enantiomer.[5] The disappearance of the chalcone (as measured by loss of absorption at 375 nm) and the formation of flavanone (measured by colorimetry after $NaBH_4$ reduction, see Experimental) are shown in Fig. 3. It will be seen that the rate of formation of flavanone in the presence of heat-inactivated enzyme was only about 5 per cent of the rate in the presence of the enzyme.

The crude extract used in this work contains an enzyme system which catalyses the conversion of isoliquiritigenin to the corresponding aurone.[6, 7] The final enzyme preparation used here was found to be free of this enzyme system as demonstrated by two-dimensional paper chromatographic examination[6] of the products of the enzymic reaction.

Effect of pH

The pH optimum of the enzymic reaction is 7·3–7·8.

[6] E. WONG, *Phytochem.* 5, 463 (1966).
[7] E. WONG, *Chem. Ind.* 589 (1966).

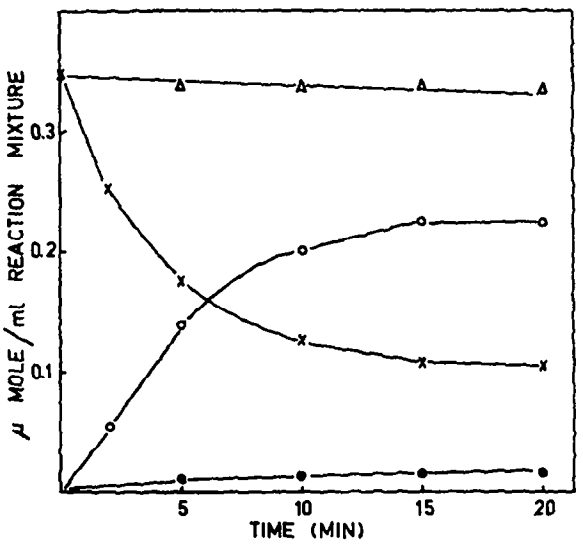


FIG. 3. REACTIONS CATALYSED BY CHALCONE-FLAVANONE ISOMERASE.

× Disappearance of chalcone (measured by the decrease in optical density at 375 nm) in the presence of enzyme.
△ Disappearance of chalcone in the presence of heat-inactivated enzyme.
○ Formation of flavanone in the presence of enzyme.
● Formation of flavanone in the presence of heat-inactivated enzyme.

Effect of Inhibitors and Activators

The effect of some enzyme inhibitors on the rate of reaction was studied and the results are shown in Table 2. It will be seen that the reaction is not, or very slightly, inhibited by cyanide, azide, diethyldithiocarbamate or EDTA, suggesting that the enzyme is not dependent on any of the metals chelated by these inhibitors. The reaction is strongly inhibited by low concentrations of *p*-hydroxymercuribenzoate (Table 2) which indicates that the enzyme is dependent on a sulphydryl group. As sulphydryl compounds such as cysteine, glutathione

TABLE 2. EFFECT OF SOME ENZYME ACTIVATORS AND INHIBITORS ON THE ENZYMIC CONVERSION OF CHALCONE TO FLAVANONE

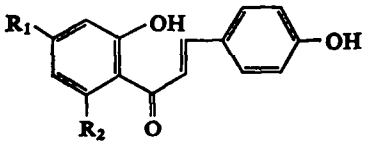
Additions	Conversion of chalcone (% in 5 min)
None	34·4
0·005 M diethyldithiocarbamate	33·3
0·01 M diethyldithiocarbamate	33·8
0·001 M EDTA	36·0
0·005 M EDTA	34·8
0·001 M Sodium azide	34·5
0·005 M sodium azide	35·7
0·001 M KCN	33·9
0·005 M KCN	34·9
0·1 mM *p*-hydroxymercuribenzoate	4·4
0·5 mM *p*-hydroxymercuribenzoate	0
0·016 M $MgCl_2$	36·0
0·016 M $MnCl_2$	35·0

and mercaptoethanol were found to react with chalcone it was not possible to use any of these compounds to restore the enzymic activity after incubation with *p*-hydroxymercuribenzoate. When chalcone was incubated with cysteine, glutathione or mercaptoethanol loss of absorption at 375 nm occurred but without the formation of flavanone.

Extensive dialysis did not cause a decrease in the rate of enzymic conversion of chalcone to flavanone. Furthermore, the rate of reaction was not increased by the addition of $MgCl_2$ or $MnCl_2$ (Table 2).

Substrate Specificity

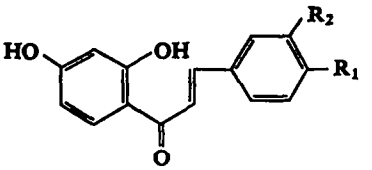
The enzyme seems to have a low specificity. Six other chalcones (IIIa, b and IVa–c) with different substitution patterns in ring A or ring B were tested with the enzyme. The rates of conversion of these chalcones to flavanones in the presence and absence of the enzyme are given in Table 3. It will be seen that the chalcone with no hydroxyl group in the position 4′ (III b) fails to act as substrate for the enzyme. It will also be seen that increased hydroxyl-



(III)

(a) $R_1=R_2=OH$

(b) $R_1=R_2=H$



(IV)

(a) $R_1=OCH_3$ $R_2=H$

(b) $R_1=OCH_3$ $R_2=OH$

(c) $R_1=R_2=OCH_3$

(d) $R_1=R_2=H$

ation in ring A results in a higher rate of enzymic reaction. The fact that 2′,4′-dihydroxychalcone (IV d) is enzymically converted to flavanone (Table 3) indicates that hydroxylation in the ring B is not one of the specificity requirements of the enzyme. Compounds with methoxyl groups attached to the ring B are not enzymically isomerized or undergo enzymic isomerization at a very low rate.

TABLE 3. SPECIFICITY OF CHALCONE-FLAVANONE ISOMERASE

Substitution pattern	% conversion of chalcone to flavanone*		
	Total	Non-enzymic	Enzymic
2′,4,4′,6′-Tetrahydroxy (III a)	99	47	52
2′,4,4′-Trihydroxy (I)	38	0	38
2′,4-Dihydroxy (III b)	20	20	0
2′,4′-Dihydroxy-4-methoxy (IV a)	7	2	5
2′,3,4′-Trihydroxy-4-methoxy (IV b)	25	18	7
2′,4′-Dihydroxy,3,4-dimethoxy (IV c)	0	0	0
2′,4′-Dihydroxy (IV d)	28	0	28

* % conversion was measured after 5 min incubation.

Similar relative rates of reactions were obtained when the seven chalcones were tested using enzyme preparations from different stages of purification (Table 1). This suggests that there is, in the crude extract, one enzyme with low substrate specificity rather than several enzymes catalysing the conversion of chalcones to flavanones.

DISCUSSION

The isolation of the chalcone-flavanone isomerase described in the present work provides the first direct evidence, at the enzymic level, of a discrete step in the conversion of chalcones to other classes of flavonoid compounds. The central position of chalcones as the primary C_{15} intermediate in flavonoid biosynthesis is indicated by evidence from isotope-labelling experiments.[2, 6, 8–10]

Recent results from tracer studies indicate that a flavanone may be the primary heterocyclic compound from which other classes of flavonoids are formed.[1–3] Grisebach and his co-workers[3] have shown that the naturally occurring (−) form of 5,7,4′-trihydroxyflavanone (III a) is stereospecifically incorporated into the isoflavone, anthocyanin and flavanol. The formation of flavanone from chalcone would thus represent one of the key steps common to the biosynthetic pathways to different classes of flavanoid compounds.

The substrate specificity exhibited by the enzyme can be discussed in terms of the probable sequence of biosynthetic steps leading to the flavonoid compounds. In 5-deoxy flavonoid compounds, the absence of the 5-OH group reflects removal of an oxygen function from the normal phloroglucinol type ring A resulting from acetate condensation. It is generally considered that this step takes place at an early stage of the elaboration of the C_{15} skeleton, probably prior to aromatization of ring A.[11, 12] That the reduction takes place prior to the chalcone stage is supported by results from tracer studies.[13] In view of these considerations, chalcones of the types exemplified by (I) and (III a) are expected to be precursors of the 5-deoxy and normal series of flavonoid respectively. Results from the present study (Table 3) indicate that the same enzyme is involved in the isomerization step to the flavanone in both series.

The fact that chalcone (III b) is not isomerized by the enzyme is noteworthy since with few exceptions, naturally occurring flavonoid compounds possess oxygenation at C_7 in ring A.[14]

Comparison of the activities of the chalcones (IV a)–(IV d) toward the isomerizing enzyme shows that hydroxylation in the ring B is not essential for activity, but methylation of an hydroxyl group greatly decreases activity (Table 3). Genetical studies indicate that methylation is a very late step in flavonoid biosynthesis.[15] Methoxylated chalcones therefore

[8] H. GRISEBACH, In *Chemistry and Biochemistry of Plant Pigments* (Edited by T. W. GOODWIN), p. 279. Academic Press, New York (1965).
[9] L. PATSCHKE and H. GRISEBACH, *Z. Naturforsch.* **20b**, 137 (1965).
[10] E. WONG, *Chem. Ind.* 1895 (1964).
[11] R. W. RICKARDS, In *Recent Developments in the Chemistry of Natural Phenolic Compounds* (Edited by W. D. OLLIS), p. 4. Pergamon Press, Oxford (1961).
[12] T. SWAIN and E. C. BATE-SMITH, In *Comparative Biochemistry*, Vol. 3A (Edited by M. FLORKIN and H. S. MASON), p. 755. Academic Press, New York (1962).
[13] H. GRISEBACH and G. BRANDNER, *Z. Naturforsch.* **16b**. 2 (1961).
[14] J. H. RICHARDS and J. B. HENDRICKSON, *The Biosynthesis of Steroids, Terpenes and Acetogenins*, p. 52. Benjamin Press, New York (1964).
[15] J. B. HARBORNE, In *The Chemistry of Flavonoid Compounds* (Edited by T. A. GEISSMAN), p. 593. Pergamon Press, Oxford (1962).

are probably not natural intermediates for further transformation. The low activities found for chalcones (IV a)–(IV c) support this view.

EXPERIMENTAL

Soya Beans

Seed were obtained from the local market, soaked in tap water for 16 hr and spread on damp filter paper for 8 hr before extraction.

Enzyme Preparation

Crude extract. Soaked soya beans (360 g) were ground with 240 ml of 0·05 M tris-HCl buffer (pH 7·5, 37°) in a Waring blender for three periods of 30 sec each. The paste was passed through several layers of cheese-cloth and centrifuged at 27,000 *g* for 30 min. The supernatant (about 160 ml) was dialysed against 3 l. of the same buffer at 2–4° for 24 hr. All subsequent steps were carried out at 2–4°.

Acid fractionation. The crude dialysed extract was made 0·05 M with mercaptoethanol. 50 ml aliquots of the extract were brought to pH 5 with 0·5 N HCl and the solution was centrifuged at 30,000 *g* for 2 min. The supernatant was brought quickly to pH 7·5 using 0·5 N KOH.

Ammonium sulphate fractionation. The combined supernatants from the acid treatment (about 120 ml) were fractionated with solid $(NH_4)_2SO_4$ and the fraction obtained between 45 and 60% saturation (pH 7·5) was collected and dissolved in 12 ml of 0·05 M tris-HCl buffer (pH 7·5).

Phosphate gel treatment. To the above fraction, 3 ml of calcium phosphate gel were added dropwise with stirring. The mixture was centrifuged at 20,000 *g* and the precipitate discarded.

First Sephadex column fractionation. A column (35 × 3 cm) was made of Sephadex G 200 in 0·02 M tris-HCl buffer (pH 7·5). The phosphate gel treated enzyme was poured on the column which was developed with the same buffer collecting 15 ml fractions every 20 min. The fractions containing the enzyme (Fig. 1) were combined, dialysed against two changes of 4 l. of distilled water and freeze-dried.

Second Sephadex column fractionation. The freeze-dried enzyme was dissolved in 4 ml of 0·02 M tris HCl buffer (pH 7·5) and subjected to Sephadex G 200 fractionation as shown above. Two protein components were obtained (Fig. 2). The fractions containing the enzyme were dialysed and freeze-dried as before.

Preparation of Chalcones

2′,4,4′,6′-Tetrahydroxychalcone (III a) was prepared from naringenin by heating at 100° for 2 min with 50% KOH. Acidification and recrystallization from aqueous alcohol gave the yellow chalcone.

All the other chalcones were prepared by alkaline condensation of the appropriate hydroxyacetophenone with the appropriate aldehyde.[16] The products were purified by polyamide column chromatography using ethanol as solvent, and recrystallized from aqueous alcohol. Melting points of the products correspond with those reported in the

[16] T. A. GEISSMAN and R. O. CLINTON, *J. Am. Chem. Soc.* **68**, 697 (1946).

literature: 2'-4-dihydroxychalcone[16] (III b) m.p. 158–160°; 2',4'-dihydroxy-4-methoxychalcone[17] (IV a) m.p. 186–190°; 2',3,4'-trihydroxy-4-methoxychalcone[18] (IV b) m.p. 196–199°; 2',4'-dihydroxy-3,4-dimethoxychalcone[17] (IV c) m.p. 127–128°; 2',4'-dihydroxychalcone[19] (IV d) m.p. 150–152°.

Preparation of Phosphate Gel

The method described by Kunitz[20] was used.

Enzyme Assay

The reaction mixture (10 ml) contained 3 ml of 0·05 M tris-HCl buffer (pH 7·5) and 4μ-moles of chalcone (Na salt). Incubation was at 37° and 1 ml samples were withdrawn and pipetted in 2 ml of ethanol. Chalcone in the supernatant obtained after centrifugation was determined by measuring absorptivity at 375 nm, or flavanone by the borohydride reduction to a flavylium salt. One enzyme unit was calculated as amount of enzyme which catalysed the conversion of 1μmole of chalcone to flavanone per hour.

Determination of Flavanone

This assay method was developed from the qualitative test for flavanone using $NaBH_4$.[21] To 2 ml of solution was added 0·2 ml of 1% $NaBH_4$ in isopropanol; the mixture was left 10 min and 0·2 ml of 2 N HCl were then added. The mixture was left for a further 10 min and made up to 5 ml with conc. HCl. After 10 min the absorptivity at 560 nm was measured. A standard curve was made with a sample of (−) 4',7,-dihydroxyflavanone prepared enzymically.[5]

Determination of Protein

The method of Lowry *et al.*[22] was used.

[17] A. BELLINI and P. VENTURELLA, *Ann. Chim.* (*Rome*) **48**, 111 (1958).
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[22] O. H. LOWRY, N. J. ROSENBROUGH, A. L. FARR and R. J. RANDALL, *J. Biol. Chem.* **193**, 265 (1951).