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COMPARISON OF CHALCONE–FLAVANONE ISOMERASE HETEROENZYMES AND ISOENZYMES

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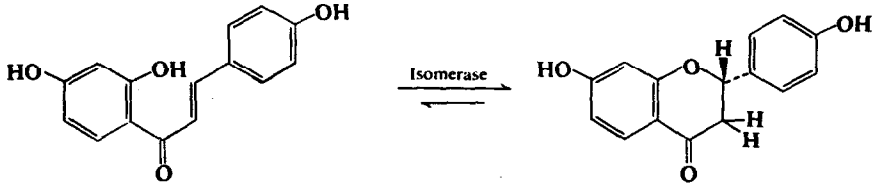
Abstract—Chalcone–flavanone isomerases from mung bean seedlings (*Phaseolus aureus* Roxb.), garbanzo bean seedlings (*Cicer arietinum* L.) and parsley leaves (*Petroselinum hortense* Hoffm.) have been separated into a number of isoenzymes by means of polyacrylamide gel electrophoresis. Some of these isoenzymes have been purified and their pH optima and Michaelis constants for a number of differently substituted chalcones determined. Whereas the purified isomerases from mung bean and garbanzo bean have a relatively low substrate specificity, the parsley enzyme appears to be inactive with chalcones lacking a 6′-hydroxyl group. A possible correlation between isomerase specificity and substitution patterns of the naturally occurring flavonoid compounds in the plant is discussed. Chalcone glucosides were not isomerized by any of the enzymes studied.

INTRODUCTION

A CHALCONE–flavanone isomerase was first isolated and purified from soya bean seed (*Soja hispida*).[1] This enzyme catalyses the cyclization of 2′,4,4′-trihydroxychalcone (I) to (−)-4′,7-dihydroxyflavanone (II) and of a number of other chalcones to the corresponding flavanones. Chalcone–flavanone isomerase activity has since been found in a number of



2′,4,4′-Trihydroxychalcone (I) (−)-(2S)-4′,7-Dihydroxyflavanone (II)

other plants. Purification of these enzymes using polyacrylamide gel electrophoresis has shown that the isomerases exist in a varying number of isoenzyme modifications in these plants. In the present paper we report the isolation of four of these isomerase enzymes from mung bean (*Phaseolus aureus*), garbanzo bean (*Cicer arietinum*) and parsley (*Petroselinum hortense*). The properties of these heteroenzymes and isoenzymes have been studied and compared.

RESULTS

Enzyme Purification

The purification procedure for two mung bean isoenzymes is summarized in Table 1. Up through the Sephadex G-200 chromatography step there was no separation of isoenzymes observed, as judged from the single symmetrical peaks of enzyme activity in the fractions

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[1] E. MOUSTAFA and E. WONG, *Phytochem.* 6, 625 (1967).

TABLE 1. PURIFICATION OF ISOMERASES FROM MUNG BEAN

Purification step	Protein (mg)	Specific activity*	Purity
Crude extract	25,000	9	1
Acid precipitation	3,700	21	2
Ammonium sulfate fractionation	1,700	30	3
DEAE cellulose column	340	110	12
1st Sephadex G-200 column	110	230	26
2nd Sephadex G-200 column	66	290	32
Preparative gel electrophoresis			
Peak I	10	640	71
Peak II	16	450	50

* mμmoles flavanone produced per mg protein per min.

obtained after DEAE cellulose (Fig. 1) and Sephadex G-200 chromatography (Fig. 2). However, when the enzyme preparation from the second Sephadex G-200 step was subjected to preparative gel electrophoresis on polyacrylamide, it was separated into two enzymatically active fractions, which in the following will be referred to as MB I and MB II (Fig. 3). The efficiency of this method of separating the two isoenzymes was demonstrated by electrophoresis on polyacrylamide gel plates. This analytical method proved that neither of these enzymes was contaminated by the other (Fig. 4*a*).

The garbanzo bean isomerase was purified by a similar series of techniques as was used for the mung bean enzyme. Gel electrophoresis revealed the presence of at least three isoenzymes in the crude isomerase preparation. These could not be separated on Sephadex G-200, but subsequent chromatography on DEAE cellulose gave two enzymatically active peaks (Fig. 5). Two enzyme peaks were also obtained on preparative gel electrophoresis. When freshly prepared by either of the two procedures these enzyme fractions each gave rise to a single spot on gel electrophoresis (GB I or GB II, Fig. 4*b*), but reverted back to a mixture of GB I and GB II on standing. These interconversions are presumably due to conformational changes.[2] GB II represented the major isoenzyme component of garbanzo isomerase. A freshly prepared sample of GB II was used for the determination of pH maximum and K_m values. GB I was not isolated in sufficient quantity in fresh condition for further study in this work.

Except for minor differences (see Experimental section) parsley isomerase "P" was purified by the procedure described for purification of the two mung bean isomerases, MB I and MB II. Electrophoretic studies on polyacrylamide gel plates of the Sephadex G-200 fraction showed that there were at least five isoenzymes partly overlapping one another (Fig. 4*c*). The bulk of this fraction was concentrated and separated by preparative gel electrophoresis into several enzymatically active peaks. Figure 6 shows that only one of these (P) appeared satisfactorily symmetrical to be used as a parsley isoenzyme. There was not enough material to check the purity of this enzyme on a gel plate.

Effect of pH

Mung bean isoenzymes I and II differ markedly in regard to their pH optima (Fig. 7), whereas both the garbanzo bean and parsley enzymes have a pH optimum in the the same range found for MB II. The following pH optima were observed: MB I, pH 7·5; MB II, pH 8·4; GB II, pH 8·5; P, pH 8·4.

[2] C. L. MARKERT and G. S. WHITT, *Experientia* **24**, 977 (1968).

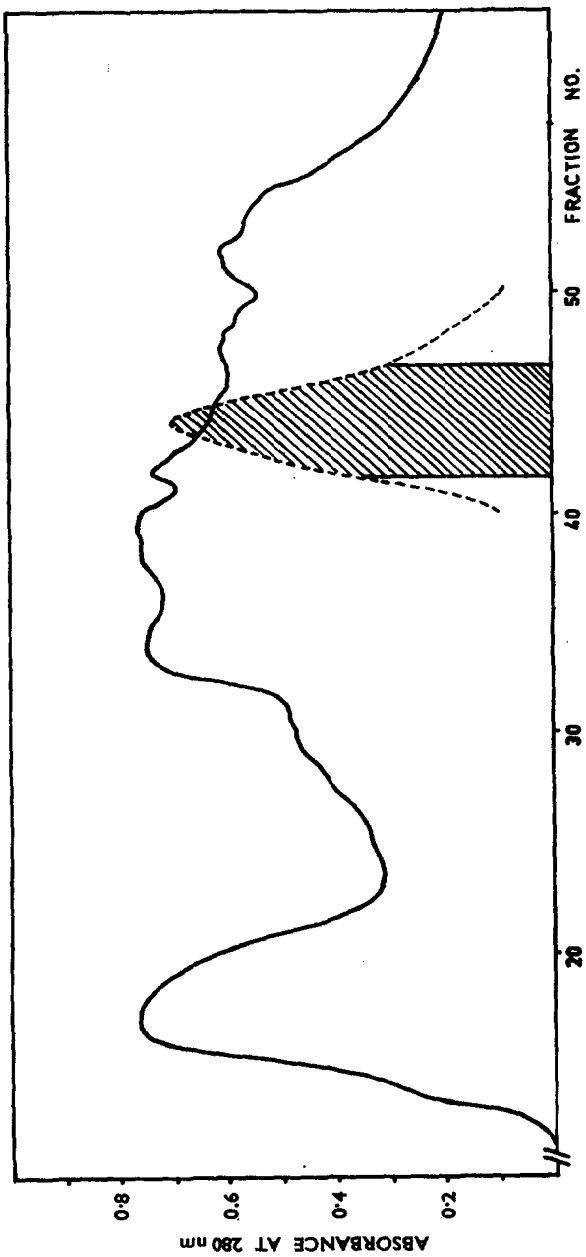


FIG. 1. DEAE CELLULOSE CHROMATOGRAPHY OF THE PROTEIN FROM MUNG BEAN AFTER AMMONIUM SULFATE FRACTIONATION.
– – – Enzyme activity.

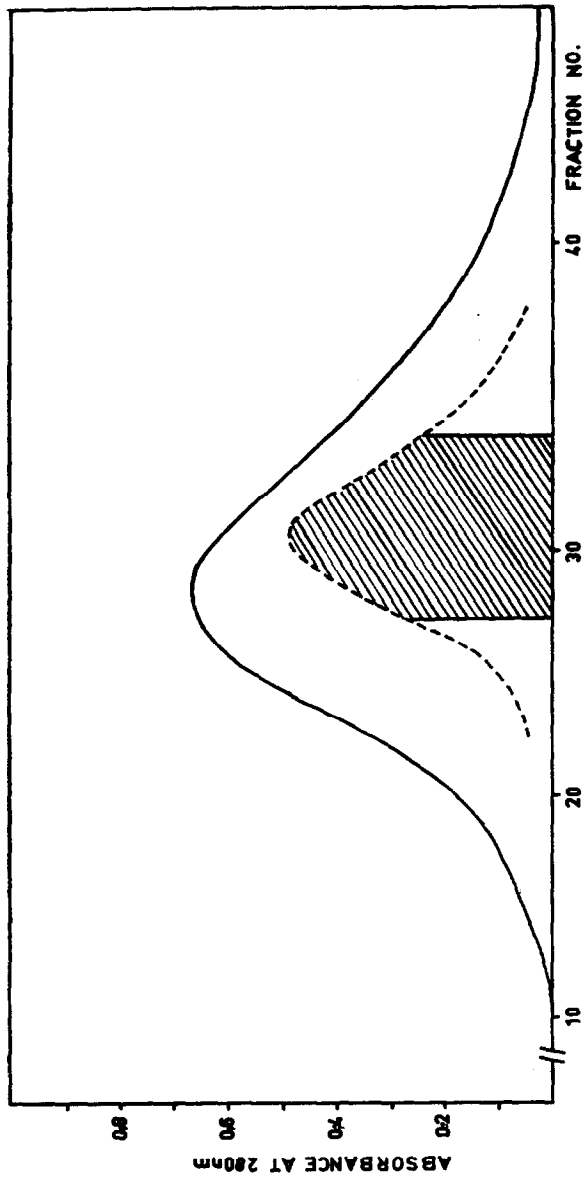


FIG. 2. SECOND SEPHADEX G-200 CHROMATOGRAPHY OF MUNG BEAN ISOMERASES.
– – – Enzyme activity.

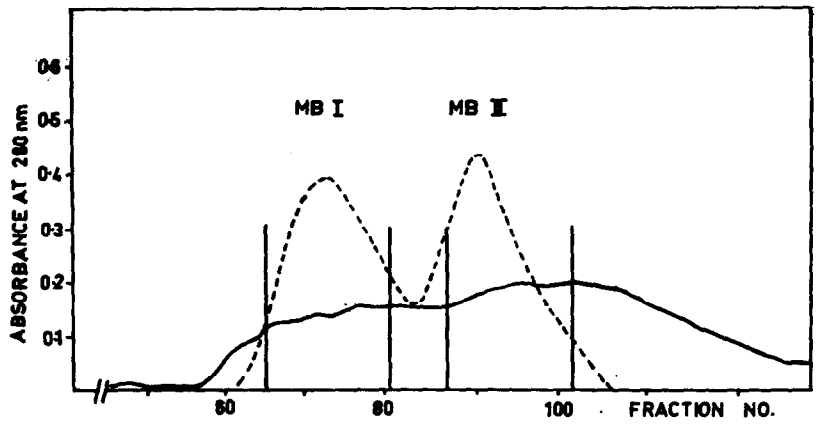


FIG. 3. PREPARATIVE POLYACRYLAMIDE GEL ELECTROPHORESIS OF MUNG BEAN ISOMERASES.
– – – Enzyme activity.

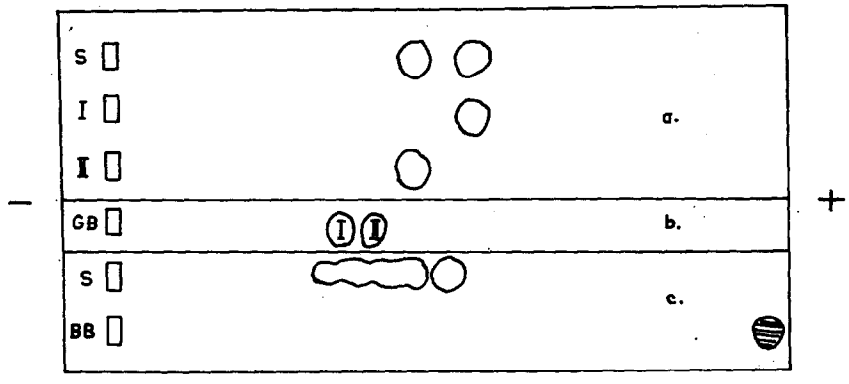


FIG. 4. ELECTROPHORESIS OF CHALCONE–FLAVANONE ISOMERASES ON POLYACRYLAMIDE GEL PLATES.
a. Mung bean isomerases: S = after second Sephadex G-200 fractionation, I = isoenzyme MB I, II = isoenzyme MB II; *b.* garbanzo bean isomerases GB I and GB II; *c.* parsley isomerases: S = after Sephadex G-200 fractionation. BB = bromophenol blue.

Reaction Product

The two mung bean isoenzymes and the isomerase from garbanzo bean were incubated with 2',4,4'-trihydroxychalcone-[β-^{14}C] and the labelled products investigated by paper and thin-layer chromatography. In three different solvent systems radioactivity was found only in 4',7-dihydroxyflavanone and in the small amounts of non-converted chalcone. Analogous experiments with the parsley enzyme were not carried out, since the only substrate known thus far, 2',4,4',6'-tetrahydroxychalcone, rapidly isomerizes to the flavanone even in the absence of enzyme in the pH range studied.

K_m Values

The results of kinetic studies with seven different chalcones and chalcone glucosides are summarized in Table 2. None of the glucosides tested was isomerized by any of the four enzymes at any detectable rate. The two mung bean enzymes and the isomerase from garbanzo bean have Michaelis constants in the range from 7×10^{-6} to $2{\cdot}6 \times 10^{-4}$ mole/l. for

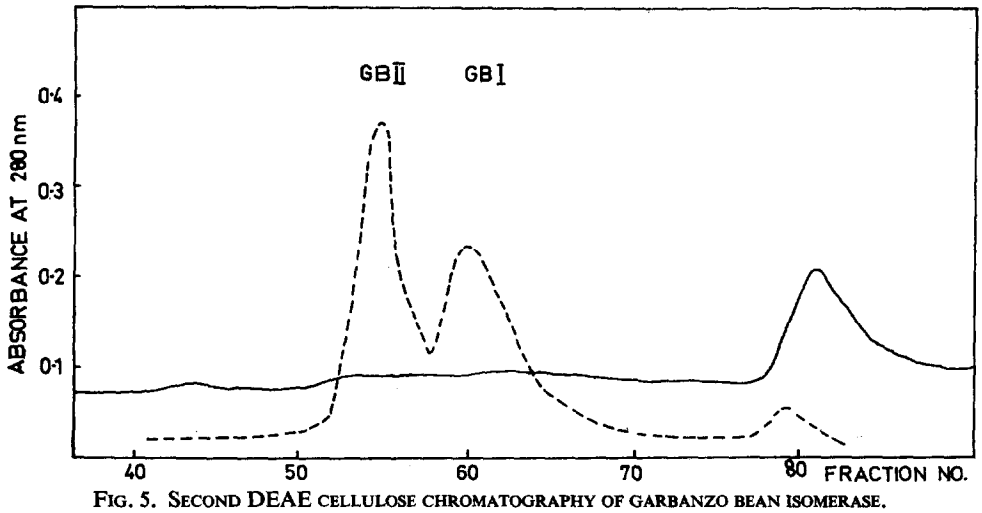


FIG. 5. SECOND DEAE CELLULOSE CHROMATOGRAPHY OF GARBANZO BEAN ISOMERASE.
– – – Enzyme activity.

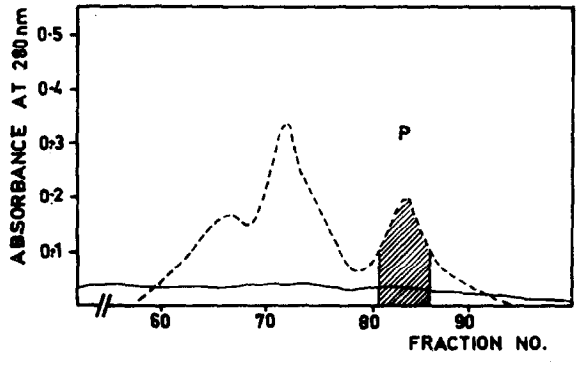


FIG. 6. PREPARATIVE POLYACRYLAMIDE GEL ELECTROPHORESIS OF PARSLEY ISOMERASES.
– – – Enzyme activity.

the chalcones investigated. Maximum velocities are roughly of the same order of magnitude for all of these substrates. These three enzymes therefore seem to have a rather low specificity. In contrast, the parsley isomerase reacts only with the chalcone possessing a 6′-hydroxyl group.

DISCUSSION

The experimental data presented in this paper clearly demonstrate that the chalcone–flavanone isomerases isolated from a number of plants differ with regard to some of their properties. In all plants so far examined chalcone–flavanone isomerase exists as a varying number of isoenzymes. The properties of MB I and MB II show that isomerases from the same plant can differ quite appreciatively. Preliminary studies also show that the number and properties of mung bean isoenzymes vary with differentiation and age of the plant.[3]

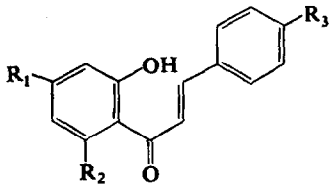
During the course of mung bean isomerase purification no enzyme activity other than that of isoenzymes I and II was detected on gel plates at any stage of increasing purity. The two

[3] K. HAHLBROCK and H. GRISEBACH, unpublished results.

TABLE 2. K_m VALUES (mole/l.) FOR VARIOUS SUBSTRATES OF CHALCONE–FLAVANONE ISOMERASES

Chalcone substitution pattern*	Enzyme			
	MB I	MB II	GB II	P
2′,4′-Dihydroxy (A)	26×10^{-5}	$4{\cdot}1 \times 10^{-5}$	$1{\cdot}8 \times 10^{-5}$	—
2′,4-Dihydroxy (B)	19×10^{-5}	$3{\cdot}7 \times 10^{-5}$	$0{\cdot}7 \times 10^{-5}$	—
2′,4,4′-Trihydroxy (C)	$5{\cdot}7 \times 10^{-5}$	$1{\cdot}4 \times 10^{-5}$	$1{\cdot}6 \times 10^{-5}$	—
2′,4,4′,6′-Tetrahydroxy (D)	$1{\cdot}8 \times 10^{-5}$	$4{\cdot}4 \times 10^{-5}$	$4{\cdot}4 \times 10^{-5}$	$1{\cdot}6 \times 10^{-5}$

* The C-4′-glucoside (E), D-4′-glucoside (F), and D-6′-glucoside (G), show no activity with either of the four enzymes examined.



(A) $R_1 = OH$; $R_2 = R_3 = H$
(B) $R_1 = R_2 = H$; $R_3 = OH$
(C) $R_1 = R_3 = OH$; $R_2 = H$
(D) $R_1 = R_2 = R_3 = OH$
(E) $R_1 = OGlc$; $R_2 = H$, $R_3 = OH$
(F) $R_1 = OGlc$; $R_2 = R_3 = OH$
(G) $R_1 = R_3 = OH$; $R_2 = OGlc$

purified enzymes therefore probably represent the total number of chalcone–flavanone isomerases extracted from 1-day-old mung bean seedlings. Neither enzyme catalyses the isomerization of the three chalcone glucosides studied (Table 2). Since a crude extract did not exhibit chalcone glucoside isomerizing activity either, it must be assumed that chalcones and not chalcone glucosides are the natural precursors of flavanones and flavanone glucosides in mung bean seedlings.

Both mung bean enzymes I and II isomerize chalcones with or without a 6′-hydroxy group (5-hydroxy group of the corresponding flavanones). Tracer studies support the hypothesis that removal of this hydroxy group takes place at a stage prior to chalcone formation.[4]

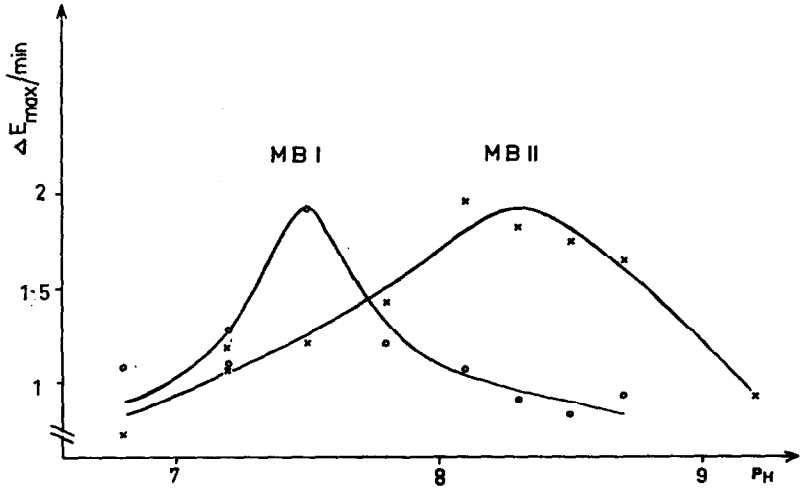


FIG. 7. pH-DEPENDENCE OF ENZYME ACTIVITY FROM MUNG BEAN ISOMERASE ISOENZYMES I AND II.

[4] H. GRISEBACH and G. BRANDNER, *Z. Naturforsch.* **16b**, 2 (1961).

Since both types of flavanones and other flavonoid compounds occur in mung bean, isomerase activity therefore would be expected for chalcones with and without 6'-hydroxy substitution. The experimental data presented in Table 2 demonstrate that in 1-day-old mung bean seedlings there are two enzymes with different but overlapping specificities towards chalcones.

Although the two mung bean enzymes are similar in their substrate specificity and could not be separated by DEAE cellulose or Sephadex G-200 column chromatography, the pH optima for the enzymic reaction and the Michaelis constants for a number of substrates (Table 2) differ distinctly. Based on these results mung bean isomerases I and II must be regarded as isoenzymes* and not as "conformers", that is not as enzymes differing in protein conformation only.[5] Furthermore, there is some indication that these isoenzymes also differ in molecular weight.[3]

The chalcone–flavanone isomerase from garbanzo seedlings resembles very much the mung bean isoenzyme MB II in general substrate specificity and pH effect. Electrophoretic mobilities for these two enzymes, however, differ considerably.

So far the only example for a chalcone–flavanone isomerase with apparent high substrate specificity is the parsley enzyme P (Fig. 6). Among the seven chalcones tested only 2'4,4',6'-tetrahydroxychalcone was found to serve as a substrate (Table 2). This observation is interesting in view of the fact that only flavonoids of this substitution pattern in ring A have been found in parsley, in contrast to the flavonoid constituents of mung bean and garbanzo bean. Since purified enzyme "P" is only one out of at least five isomerases isolated from parsley leaves (Fig. 4), the Sephadex G-200 fraction containing all of these enzymes was incubated with 2',4,4'-trihydroxychalcone. Within the limits of experimental error there also was no enzyme-catalysed reaction detectable.

The results obtained from these experiments suggest that there is a correlation between substrate specificity of chalcone–flavanone isomerases and substitution patterns of flavonoid compounds in a particular plant. Further experiments are in progress to test this interesting point.

EXPERIMENTAL

Plants. Seeds of mung bean (*Phaseolus aureus* Roxb.), garbanzo bean (*Cicer arietinum* L.), and parsley (*Petroselinum hortense* Hoffm.) were obtained from commercial sources.

Mung Bean Isomerases I, II

650 g of mung bean seed were soaked in tap water for 24 hr. The germinated seeds were homogenized in a Waring blendor with 750 ml of 0·05 M Tris-HCl buffer, pH 7·6, containing 0·025 M EtSH and crushed ice. The resulting suspension was filtered through cheese-cloth. All of the subsequent operations were carried out at 4°.

The mixture was acidified to pH 5·0 with 0·5 N HCl, centrifuged for 5 min at 10·000 *g*, and quickly titrated back to pH 7·5 using 0·5 N KOH. The precipitate was spun down for 2 hr at 16·000 *g*. The supernatant was fractionated with solid $(NH_4)_2SO_4$ and an enzymatically active protein fraction was obtained between 30 and 80% saturation. The precipitate was dissolved in 50 ml of 0·05 M Tris-HCl buffer, pH 7·6, 0·025 M EtSH, and dialysed against distilled water for 6 hr and then against 0·002 M Tris-HCl buffer, pH 7·6 (0·025 M mercaptoethanol), for another 12 hr.

The mixture was cleared by centrifugation and divided into three equal parts which were passed separately through a DEAE cellulose column (3 × 30 cm). The proteins were eluted with a linear gradient of 0·002 M to 1 M Tris-HCl buffer, pH 7·6 (500 g, each), and collected in 10-ml portions. The fractions highest in isomerase activity (Fig. 1) from the three runs were combined. The protein was precipitated with $(NH_4)_2SO_4$ (80% saturation), and dissolved in 25 ml 0·05 M Tris-HCl buffer, pH 7·6 (0·025 M EtSH).

* For definition of the terms "isoenzyme" and "heteroenzyme" cf. Ref. 6.

[5] G. B. KITTO, P. M. WASSARMAN and N. O. KAPLAN, *Proc. Nat. Acad. Sci.* **56**, 578 (1966).

[6] L. M. SHANNON, *Ann. Rev. Plant Physiol.* **19**, 187 (1968).

The pale-yellow solution was chromatographed on a Sephadex G-200 column (3 × 30 cm) with 0·02 M Tris-HCl buffer, pH 7·6. The fractions containing the enzyme were combined (Fig. 2), concentrated by $(NH_4)_2SO_4$ precipitation, and dissolved in Tris-HCl buffer (6·5 ml) as described above. The solution was passed through the same column again, and the enzyme fraction was concentrated in a "Diaflo" concentrator (AMICON, Model 50, ultrafiltration cell).

Preparative gel electrophoresis was carried out on a polyacrylamide gel column in a Shandon apparatus with 0·4 M Tris-glycine buffer, pH 8·7. A gel column *ca.* 55 mm in length was prepared with the gel described below for the analytical gel electrophoresis.

In a preliminary run with bromophenol blue as a marker dye the gel was washed before the protein was applied to the column. The apparatus was run at a constant voltage of 300 V in a current of 55–70 mA. The proteins were eluted from the column with 0·34 M Tris-acetate buffer, pH 8·4, and collected in fractions of 2·5 ml. Two isomerases were obtained in fractions 66–80 and 87–101, respectively (Fig. 3). They were dialysed against two changes of 2 l. of 0·05 M phosphate buffer, pH 7·6, and used as purified mung bean isomerases "MB I" and "MB II".

Parsley Isomerase

360 g of leaves and stems were harvested from 4-week-old parsley plants grown under standardized conditions in growth chambers. The chalcone–flavanone isomerases were extracted and purified according to the method described above for the isomerases from mung bean. Exceptions were:

a. A treatment for 2 hr with 10 g Dowex 1 (equilibrated with 0·2 M Tris-HCl buffer, pH 7·6) of the protein solution before DEAE cellulose chromatography in order to remove phenolic compounds and anionic material. After this treatment with the anion exchanger, the suspension was filtered through a small-pore glass filter.

b. The second Sephadex G-200 chromatography was omitted. Fractions 81–85 from the preparative gel electrophoresis column were combined (Fig. 6), dialysed against 0·05 M Tris-HCl buffer, pH 7·6, and concentrated to 1 ml in the "Diaflo" apparatus. This enzyme preparation was called isomerase "P".

Garbanzo Bean Isomerase

540 g of 4-day-old garbanzo seedlings were extracted with Tris-HCl buffer and the enzyme extract was partially purified via acid treatment, $(NH_4)_2SO_4$ precipitation (45–60% saturation) and chromatography through DEAE cellulose, essentially as described above for the mung bean isomerase.

The enzyme activity/column fraction plot for the DEAE cellulose chromatographic step indicated that two overlapping peaks were present. Two active fractions, D_1 (tube 43–45) and D_2 (tube 46–49), were therefore collected. These fractions were separately passed through a Sephadex G-200 column (see Mung Bean isomerase). In each case a single peak of activity only was eluted. The active fractions from D_1 and D_2, after the Sephadex step, were designated D_1S and D_2S, respectively. The purification at this stage was about 45-fold that of the $(NH_4)_2SO_4$ stage.

D_1S and D_2S, on analysis by gel electrophoresis, was each shown to be a mixture of two isoenzymes, designated GB I and GB II (Fig. 4*b*), with D_1S giving more GB II and D_2S more GB I.

Fraction D_1S was chromatographed through a second DEAE cellulose column. A concave gradient of 0·005 M–0·7 M Tris-HCl buffer was used for elution. This resulted in the separation of two peaks of activity corresponding to GB I and GB II, respectively, on electrophoresis (Fig. 5). The enzyme solution in tube 55, consisting of GB II only, was used for the study of enzyme properties.

In a parallel experiment, fraction D_2S was subjected to preparative gel electrophoresis, as described above for the mung bean enzyme. Two distinct peaks were again obtained, corresponding to GB I and GB II, respectively.

GB I and GB II, separated from each other by either the second DEAE chromatographic step or by preparative gel electrophoresis, reconverted to a mixture of both forms (as revealed by gel electrophoresis) on standing for several weeks at 4°.

Enzyme Assay

The incubation mixture contained 10 μg of chalcone (dissolved in 10 μl of ethylene glycol monomethylether) in a total volume of 3 ml of 0·05 M Tris-HCl buffer, pH 7·6. The reaction was started by addition of the enzyme. Incubation was carried out at 30°. The decrease in absorptivity at the absorption maximum was plotted against time and used as a measure for the enzymatic activity. One enzyme unit was defined as the amount of enzyme catalysing the conversion of 1 μmole of chalcone per min. All of the results were corrected for the non-enzymatic rate of isomerization.

Determination of pH Optima

The buffers used were 0·2 M sodium potassium phosphate (pH 6·0–7·8), 0·2 M Tris-HCl (pH 7·1–8·7), and 0·2 M glycine–NaOH (pH 8·7–9·2). The reaction rates were measured at the absorption maxima of the chalcones at the respective pH values using an Unicam SP 800 Spectrophotometer. Substrates were 2′,4,4′-trihydroxychalcone for MB I, MB II, and GB II, and 2′,4,4′,6′-tetrahydroxychalcone for P.

Determination of K_m Values

The K_m values were measured at the respective pH optima for the enzymes. Lineweaver–Burk plots were used for the determination of K_m values.

Determination of Protein

Up to the DEAE cellulose purification step all proteins were determined by the Biuret method. After further purification the method of Warburg and Christian was used.[7]

Preparation of Chalcones

All of the chalcones were synthesized and purified as described by Moustafa and Wong.[1]

Analytical PAA Gel Electrophoresis[8]

Glass trays (30 × 15 × 0·2 cm) were filled with an aqueous solution containing 50 ml of 0·06 M Tris-HCl buffer, pH 8·5, 5 g acrylamide, 250 mg N,N′-methylenebisacrylamide, 0·7 ml dimethylaminopropionitrile, and 50 mg ammonium peroxodisulfate in a total volume of 100 ml. After polymerization of the gel the plates were washed for 12 hr in 1 l. of 0·03 M Tris-HCl buffer, pH 8·5, to remove low molecular weight contaminants from the gel. Four to six rectangular holes (5 × 10 cm) were cut out of the gel with a razor blade and filled with protein solutions. The current applied was 20–30 mA at 1500 V for each plate. When a sample of bromophenol blue as a suitable marker dye had migrated over the whole distance of the plate, the gel was sprayed with a solution of 0·25% 2′, 4,4′-trihydroxychalcone in 0·02 N NaOH and developed at room temperature. Within 10–60 min the yellow colour of the chalcone disappeared from the spots where isomerase activity was located. Parsley isomerases were sprayed with 2′,4,4′,6′-tetrahydroxychalcone solutions of the same concentration.

Identification of the Reaction Product

The mung bean enzymes, MB I (27 μg) and MB II (510 μg), and the garbanzo bean enzyme, GB II (42 μg), were each incubated with 0·1 μmole of 2′4,4′-trihydroxychalcone-[β-^{14}C][9] in 2 ml of 0·2 M Tris-HCl buffer, pH 7·5, for 5 min at 30°. The specific radioactivity of the chalcone was 0·21 mc/mmole. The reaction was stopped by addition of one drop of 6 N HCl and the product extracted with 2 × 1 ml of ether. After evaporation of the solvent, the residue was dried in a desiccator over P_2O_5 and NaOH. The product was purified by the following procedure: Paper chromatography on Whatman 3 MM with the solvent systems 30% acetic acid and 20% isopropanol, followed by TLC on silica gel with benzene/ethyl acetate/methanol/water (6:4:1:3).

Determination of Radioactivity

C^{14} and T were measured in a Beckman CPM-100 scintillation spectrometer.

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[7] E. LAYNE, in *Methods in Enzymology* (edited by S. P. COLOWICK and N. O. KAPLAN), Vol. III, p. 450, Academic Press, New York (1957).
[8] M. BIEDERMANN and G. DREWS, *Arch. Mikrobiol.* **61**, 48 (1968).
[9] H. GRISEBACH and L. PATSCHKE, *Chem. Ber.* **93**, 2326 (1960); H. ZILG, K. HAHLBROCK and H. GRISEBACH, in preparation.