Three Forms of *Candida utilis* Ribose 5-Phosphate Ketol Isomerase

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Three forms (I, II, and III) of R 5-P ketol isomerase (EC 5.3.1.6) were separated and purified from *Candida utilis*. The *K_m*s for R 5-P by I, II, and III were 0.77 mM, 0.67 mM and 0.16 mM, respectively. The isoelectric points of the forms, I, II, and III were 3.79, 3.87 and 3.99, respectively.

The activation energies of the forms, I, II, and III were 10,800, 12,000 and 6360 cal/mole, respectively. The forms, I, II, and III were all inhibited with chelating reagent, EDTA (1 mM), but not affected with thiol reagents such as *p*-CMB, ICH₂COOH, NEM, and DTNB. These properties are quite different from the other known R 5-P ketol isomerases, all of those are not affected with EDTA, but inhibited with the thiol reagents as described above.

There have been numerous papers on R 5-P ketol isomerases of photosynthetic organisms,^{1~5)} animal tissues,^{6~8)} bacteria^{9~12)} and yeast.^{13~15)}

However, the complete purification of R 5-P ketol isomerase is so difficult that among these R 5-P ketol isomerases, pure enzyme preparations have been obtained only from spinach⁴⁾ and *Candida* yeast.^{14,15)}

Therefore, most of data for the enzyme have been described using partially purified preparations except only a few reports in which some physicochemical properties and structure of the enzyme^{14,15)} were described.

For the presence of R 5-P ketol isomerase isoenzymes, from the side of isoenzyme-absence, Rutner⁵⁾ first concluded from his data that the spinach enzyme isolated by his procedure represents only a single protein species and, furthermore, that spinach leaves seem to have only a R 5-P ketol isomerase. Secondly, Domagk *et al.*¹⁴⁾ also described that isoenzymes of R 5-P ketol isomerase are not present in *C. utilis*.

On the other hand, from the side of iso-

enzyme-presence, Pon⁴⁾ first reported that there are two isoenzymic forms in spinach leaves basing upon solubility properties and behaviors on ion exchange columns.

Secondly, Anderson³⁾ basing upon the observation of two enzymes with different isoelectric points, reported that there were two isoenzymic forms in chloroplast and cytoplasmic fractions of pea leaves, respectively. Thirdly, David and Wiesmeyer¹¹⁾ also basing upon difference in heat stability and no linearity of Lineweaver-Burk plots, reported that there were two isoenzymic forms in *E. coli* × 289. In addition, fourthly, Skinner and Cooper¹²⁾ reported that there were two isoenzymic forms in *E. coli* K 12 since they found the presence of two different *K_m* values. So, it is not certain whether isoenzyme of R 5-P ketol isomerase is present or not. But, the presence of R 5-P ketol isomerase isoenzymes in *C. utilis* has also been suggested by the present authors.¹⁵⁾

A strange behavior which is unexplainable with the presence of only a single enzyme protein, was observed during purification of R 5-P ketol isomerase on DEAE-cellulose column chromatography by gradient method and also on Bio-Gel P-200 gel filtration.

This paper describes the separation and purification of three forms of R 5-P ketol

The following abbreviations are used: R 5-P, ribose 5-phosphate; EDTA, ethylenediamine tetraacetic acid; *p*-CMB, *p*-chloromercuric benzoic acid; NEM, N-ethylmaleimide; DTNB, 5,5'-dithio bis(2-nitrobenzoic acid).

isomerase and also difference and similarity in properties of the respective isoenzymic forms.

MATERIALS AND METHODS

Chemicals. R 5-P and Ampholine (pH 3.5~5.0) were purchased from Sigma Company, U.S.A. and LKB, Produkt, Sweden, respectively.

Material. Frozen cells of *C. utilis* were cordially given by Jujo Paper Manufacturing Company.

Enzyme assay. R 5-P ketol isomerase activity was assayed by the same method as described in the previous paper.¹⁶⁾

Separation and purification of R 5-P ketol isomerase forms, I, II, and III. For separation and purification of R 5-P ketol isomerase forms, I, II, and III, the same method as described in the previous paper except addition of second preparative polyacrylamide gel electrophoresis procedure after the first preparative polyacrylamide gel electrophoresis procedure, was used. That is, 170 g of frozen cells of *C. utilis* were treated in 17 ml of butyl acetate at 37°C for 1 hr. After treatment, the treated mixture was adjusted to pH 7.5 with 5 N-NH₄OH and then, centrifuged at 10,000 rpm for 15 min. To the cell-free extract (200 ml) was added solid ammonium sulfate to bring to 30% saturation.

The mixture was centrifuged at 10,000 rpm for 15 min and the precipitate was discarded. The supernatant solution was brought to 80% saturation by further addition of solid ammonium sulfate. The precipitate was collected by centrifugation at 10,000 rpm for 15 min and dissolved in distilled water.

The solution was dialyzed against deionized water overnight and then subjected to chromatography on a DEAE-cellulose column (2.8 × 45 cm) equilibrated with 12.5 mM Tris-HCl (pH 7.4, hereafter referred to as column buffer). After the column was washed with the column buffer, the column was eluted stepwise with the column buffer containing 0.15 M KCl, followed with the column buffer containing 0.35 M KCl. Ten gram fractions were collected. The second eluate (250 ml) was concentrated with a Diafilter to about 10 ml and then followed with a collodion bag to about 3 ml. The concentrate was applied to a column of Bio-Gel P-200 (2.5 × 90 cm) equilibrated with the column buffer. Five gram fractions were collected. The fractions having the enzyme activity were combined. The Bio-Gel P-200 eluate was concentrated with a collodion bag to about 5 ml and subjected to preparative polyacrylamide gel electrophoresis (Toyo Sangyo Company). The size of gel was 4 cm-height separating gel with 1 cm-height concentrating gel. Five ml of a solution containing 30~50 mg protein, 10% glycerol and 1

drop of 0.02% bromophenol blue, was layered on the concentrating gel through a small tube. The buffer system comprised 50 mM Tris-glycine (pH 8.6) as an upper electrode solution and 0.1 M Tris-HCl (pH 8.1) as lower electrode- and eluting solutions.

The current for electrophoresis was gradually increased to 3.8 mA/cm².

Five gram fractions were collected. Each of three respective fractions (A, B, and C) having the enzyme activity were separated and concentrated with a collodion bag. After each of the three fractions was concentrated, the each preparation was applied to second preparative polyacrylamide gel electrophoresis by the same method as the first preparative polyacrylamide gel electrophoresis.

Isoelectric focusing. The LKB 8100 Ampholine electrofocusing equipment was used for isoelectric focusing as described in the previous paper.¹⁶⁾

The LKB Ampholine (pH 3.5~5.0) was used.

Disc electrophoresis. For testing purity of the individual forms of R 5-P ketol isomerase, analytical disc electrophoresis was performed in 7% polyacrylamide gel using 50 mM Tris-glycine (pH 8.9) as the electrode buffer by the method of Davis.¹⁶⁾ About 50 µg of the individual form preparation was layered on the gel and run at 3.0 mA per tube.

RESULTS

Designation of the forms of R 5-P ketol isomerase

An enzyme component migrating fastest to an anode was designated as form I; an enzyme component migrating second fast to an anode, as form II; and an enzyme component migrating slowest to an anode as form III, respectively. The following preliminary tests indicated the presence of three forms of R 5-P ketol isomerase in *C. utilis*. Three forms of R 5-P ketol isomerase were found in all of DEAE-cellulose eluates; in each of the preparations of *C. utilis*, one treated with 0.1 M NaHCO₃ in the presence of 10⁻³ M mercaptoethanol at 30°C for 12 hr; homogenate by Braun homogenizer; and one obtained by the treatment with butyl acetate at 37°C for 1 hr. In this paper, experiment was carried out using butyl acetate preparation because this preparation gave the highest yield of R 5-P ketol isomerase among them.

Separation and purification of R 5-P ketol isomerase forms, I, II, and III

The results of purification of R 5-P ketol isomerase on several steps are described.

Purification of R 5-P ketol isomerase on DEAE-cellulose column chromatography

For the conditions of purification of R 5-P ketol isomerase on a DEAE-cellulose column chromatography, it was described under MATERIALS AND METHODS, and elution patterns of the enzyme activity and protein are shown in Fig. 1.

FIG. 1. Elution Profile on DEAE-Cellulose Column. The flow rate was 1 ml/1 min and the effluent was collected in 10 g portions. Column size; 2.8×45 cm. R 5-P ketol isomerase activity was determined under the standard conditions. R 5-P ketol isomerase, (O—O); absorbance at 280 nm, (-----).

At this step, forms of R 5-P ketol isomerase were not separated, but some incoincidences between the enzyme activity and eluting protein are observed.

Bio-Gel P-200 gel filtration

The conditions in Bio-Gel P-200 gel filtration, are described under MATERIALS AND METHODS. Figure 2 is the elution patterns of the enzyme activity and protein. At this step, the elution gave two protein peaks and the enzyme activity in the elution pattern was also dispersed in the two protein peaks. This fact indicated that more than one form of R 5-P ketol isomerase were presumably present in this preparation.

FIG. 2. Gel Filtration on Bio-Gel P-200.

The flow rate was 5 ml per 1 hr and the effluent was collected in 5 g portions. Column size; 2.5×67 cm. R 5-P ketol isomerase activity was determined under the standard conditions. R 5-P ketol isomerase, (O—O); absorbance at 280 nm, (-----).

Resolution of the enzyme preparation into individual forms by first preparative polyacrylamide gel electrophoresis

The result of elution pattern of the enzyme activity and protein is shown in Fig. 3. At this step, presence of three forms of R 5-P ketol isomerase was suggested. Next, the individual fractions having the enzyme activity separated by the first preparative polyacrylamide gel electrophoresis, were respectively concentrated by the method described in the previous paper, and respectively applied to second preparative polyacrylamide gel electrophoresis by the same method as the first run.

The elution patterns of the enzyme activity and protein of the individual fractions on the

FIG. 3. Elution Profile on the First Preparative Polyacrylamide Gel Electrophoresis.

R 5-P ketol isomerase activity was determined under the standard conditions. A (fraction Nos., 20~30); B (fraction Nos., 35~45); C (fraction Nos., 46~53).

TABLE I. PURIFICATION OF R 5-P KETOL ISOMERASE, FORMS, I, II, AND III

Step		Total activity (units)	Total protein (mg)	S.A. (units/mg)	Yield (%)
Crude extract		6045	2418	2.5	100.00
Am ₂ SO ₄		4760	1360	3.5	78.74
DEAE-cellulose		1841	236	7.8	30.45
Bio-Gel P-200		920	91	10.1	15.22
1st prep. disc electrophoresis	I	70.2	5.80	12.1	1.16
	II	64.6	3.20	20.2	1.07
	III	30.0	1.05	28.6	0.50
2nd prep. disc electrophoresis	I	44.9	3.87	11.6	0.74
	II	37.8	2.15	17.6	0.62
	III	20.0	0.61	32.8	0.33

FIG. 4. Elution Profile on the Second Preparative Polyacrylamide Gel Electrophoresis.

Preparations, A, B, and C from the first preparative polyacrylamide gel electrophoresis, were subjected on the second preparative polyacrylamide gel electrophoreses, respectively. R 5-P ketol isomerase activity was determined under the standard conditions. R 5-P ketol isomerase activity (right-hand ordinates, R), (○—○); absorbance at 280 nm (left-hand ordinates, L), (-----).

second preparative polyacrylamide gel electrophoresis are shown in Fig. 4. The three forms are obtained free of contamination. This means that by the second preparative polyacrylamide gel electrophoresis, R 5-P ketol isomerases were resolved into three individual forms, I, II, and III, respectively.

The overall purification procedures are summarized in Table I. The reason of the low recovery of the enzyme activity indicated the enzyme is not stable, particularly in the diluted state used in Bio-Gel P-200 gel filtration and others.

Properties of the three purified forms, I, II, and III

Homogeneity of the purified forms, I, II, and III. In order to examine homogeneity of the

forms, I, II, and III, they were respectively subjected to analytical disc electrophoresis in 7% polyacrylamide gel (pH 8.9) by the method of Davis,¹⁶⁾ and it was found that three forms, I, II, and III are all homogeneous as shown in Fig. 5.

FIG. 5. Disc Electrophoreses of R 5-P Ketol Isomerase Forms, I, II, and III.

Fifty μ g of the each enzyme preparation (I, II, and III) was applied to the gel. The gels were stained with 1% Amido Black 10B, and destained with 7% AcOH. Migration was from left to right.

Effect of divalent cations. The respective preparations of the forms, I, II, and III dialyzed against 12.5 mM Tris-HCl (pH 7.0), were used. The result is shown in Table II. The result indicated that the forms, I, II, and III are all activated with Co²⁺, or Mg²⁺ in contrast with the known R 5-P ketol isomerases which were not activated by these ions. Mn²⁺ slightly inhibited the activity of I and II, and inhibited the activity of III much more than those of I and II.

Thermal stability. Thermal stability of the three forms, I, II, and III was tested. The residual activity of III decreased gradually in

TABLE II. EFFECT OF DIVALENT CATIONS ON THE ENZYME ACTIVITY OF FORMS, I, II, AND III

After each enzyme preparation in 0.8 ml of 50 mM Tris-HCl, pH 7.5 (I, II, *ca.* 10 μ g; III, 5 μ g) was preincubated at 37°C for 5 min under the concentrations of cations (0.1 ml) as indicated in the table, 0.1 ml of 5.5 mM R 5-P was added and incubated at 37°C for 30 min. After then, the enzyme activity was measured under the standard assay conditions. The activity in the absence of cations was taken as 100%.

Metal	Final conc. (mM)	Form		
		I	II	III
Co ²⁺	1	180	178	129
	5	189	187	120
	10	198	199	111
	100	215	222	106
Mg ²⁺	1	163	167	129
	5	170	167	137
	10	180	156	139
	100	165	148	143
Mn ²⁺	1	92	92	51
	5	92	91	51
	10	80	87	43
	100	65	61	29

FIG. 6. Thermal Stability of R 5-P Ketol Isomerase Forms, I, II, and III.

Each enzyme preparation in 0.9 ml of 50 mM Tris-HCl, pH 7.5 (I, II, *ca.* 10 μ g; III, *ca.* 5 μ g) and 0.1 ml of 5.5 mM R 5-P were incubated at 50°C for various periods as indicated in the figure, and aliquots were taken out from the incubation mixture and used for assay of the enzyme activity under the standard assay conditions. The enzyme activity was plotted as percent against the initial activity: (A), Form I, (O—O); B, Form II, (●—●); (C), Form III, (●—●).

parallel with the increase of incubation time, whereas the residual activity of II decreased slowly, and the residual activity of I decreased scarcely as shown in Fig. 6.

Substrate affinity. Michaelis-Menten con-

stants of the forms, I, II, and III for R 5-P were calculated by the method of Lineweaver-Burk¹⁷⁾ from Fig. 7 to be 0.77 mM, 0.67 mM and 0.16 mM, respectively.

FIG. 7. Lineweaver-Burk Plots for R 5-P by Forms, I, II, and III.

Each enzyme preparation in 0.9 ml of 50 mM Tris-HCl, pH 7.5 (I, II, *ca.* 10 μ g; III, 5 μ g) and 0.1 ml of R 5-P with different concentrations as indicated in the figure were incubated at 37°C for 30 min. After then, the enzyme activity was measured under the standard assay conditions. (A), Form I; (B), Form II; (C), Form III.

Effect of thiol reagents and EDTA. As for the effect of several thiol reagents and EDTA on the activity of the three forms, I, II, and III,

TABLE III. EFFECT OF THIOL INHIBITORS AND EDTA ON THE ENZYME ACTIVITY OF FORMS, I, II, AND III

After each enzyme preparation in 0.8 ml of 50 mM Tris-HCl, pH 7.5 (I, II, *ca.* 10 μ g; III, *ca.* 5 μ g) was preincubated at 37°C for 30 min in the presence of 0.1 ml of 10 mM inhibitors or EDTA as indicated in the table, 0.1 ml of 5.5 mM R 5-P was added and incubated at 37°C for 30 min. After then, the enzyme activity was measured under the standard assay conditions. The activity in the absence of inhibitors or EDTA was taken as 100%.

The values in the parentheses represent ones measured in the presence of 5 mM MgCl₂.

Chemicals (Final conc., 1 mM)	Remaining activity (%)		
	I	II	III
NEM	99 (91)	97 (92)	107 (94)
p-CMB	102 (96)	103 (107)	79 (100)
DTNB	97 (93)	94 (91)	90 (92)
HgCl ₂	65 (82)	58 (89)	71 (77)
ICH ₂ COOH	81 (97)	103 (102)	100 (100)
EDTA	5 (113)	5 (116)	12 (119)

EDTA inhibited strongly the activity of all three forms, but the thiol reagents used here did not inhibit all of them as shown in Table III.

Reversibility of the enzyme activity by the addition of Co^{2+} or Mg^{2+} after treatment with EDTA. As described previously, the three forms, I, II, and III were all activated with Co^{2+} or Mg^{2+} , but inhibited strongly with EDTA.

So, this experiment was achieved to examine whether the enzyme activity of the forms, I, II, and III was recovered or not by the addition of Co^{2+} or Mg^{2+} after treatment with EDTA. As the result, the activity of the forms, I, II, and III was all recovered completely by the addition of Co^{2+} or Mg^{2+} as shown in Table IV.

TABLE IV. RECOVERY OF THE ENZYME ACTIVITY OF FORMS, I, II, AND III BY ADDITION OF Co^{2+} OR Mg^{2+} AFTER TREATMENT WITH EDTA

After each enzyme preparation in 0.7 ml of 50 mM Tris-HCl, pH 7.5 (I, II, *ca.* 10 μg ; III, *ca.* 5 μg) was preincubated in the presence of 0.1 ml of 10 mM EDTA at 37°C for 30 min (at this stage, there found little activity), 0.1 ml of MgCl_2 or CoCl_2 (Mg^{2+} , Co^{2+} , 50 mM) and 0.1 ml of 5.5 mM R 5-P were added to the mixture, and incubated at 37°C for 30 min. After incubation, the enzyme activity was measured under the standard assay conditions. The enzyme activity in the case of non-treatment with EDTA was taken as 100%.

Metal (Final conc., 5 mM)	Enzyme activity (%)		
	I	II	III
Mg^{2+}	109	115	92
Co^{2+}	129	151	113

Inhibition. Two nucleotides of which R 5-P and R 3-P have as sugar phosphate moiety and gave inhibitory effect on R 5-P ketol isomerase activity in the previous paper,¹⁵⁾ were tested for their ability to inhibit the reaction of the forms, I, II, and III, respectively. Both 5'-AMP and 3'-AMP inhibited all of them as shown in Table V.

Effect of temperature on the reaction velocity of the forms, I, II, and III

Activation energies, E_s , of the three forms were calculated from the slopes plotted accord-

TABLE V. EFFECT OF TWO NUCLEOTIDES ON FORMS, I, II, AND III

After each enzyme preparation in 0.7 ml of 50 mM Tris-HCl, pH 7.5 (I, II, *ca.* 10 μg ; III, *ca.* 5 μg) was preincubated at 37°C for 10 min with the nucleotides (0.1 ml, 10 mM) as indicated in the table, 0.1 ml of 5.5 mM R 5-P was added and incubated at 37°C for 30 min. After then, the enzyme activity was measured under the standard assay conditions. Remaining activity in the case of non-addition with nucleotides was taken as 100%.

Nucleotides (Final conc., 10^{-3} M)	Remaining activity (%)		
	I	II	III
None	100	100	100
5'-AMP	46	35	9
3'-AMP	52	41	20

FIG. 8. Plots of Log K vs. $1/T$.

Each enzyme preparation in 0.9 ml of 50 mM Tris-HCl, pH 7.5 (I, II, *ca.* 10 μg ; III, *ca.* 5 μg) and 0.1 ml of 5.5 mM R 5-P were incubated at 37°C for 30 min under the different temperatures as indicated in the figure. After incubation, the enzyme activity was measured under the standard assay conditions. K , velocity constant is expressed as absorbance at 560 nm which is proportional to formation of Ru 5-P. (A), Form I; (B), Form II; (C) Form III.

ing to Arrhenius equation as shown in Fig. 8 and found to be 10,800, 12,000 and 6360 cal/mole, respectively.

DISCUSSION

Three forms of R 5-P ketol isomerase were separated and purified from *C. utilis* by the preparative polyacrylamide gel electrophoresis. The three forms purified were respectively designated as form I for component migrated fastest to anode, form II for component migrated the second one and form III for the third

one. Next, enzymic properties of the three forms, I, II, and III were compared. As the results, as for K_m values of the three forms for R 5-P, form III showed the lowest value, 0.16 mM, which was about one-fourth of forms, I, and II. This fact indicated that the role of form III *in vivo* may be different from the other forms, I, and II. This fact is also supported by the following result that the activation energy of form III (E, 6360 cal/mole) is about a half of the other two forms. As for thermal stability, it is found that form III is the most labile among them. The enzymic activity of the three forms is all inactivated with EDTA, but the inactivation was also recovered by addition of Co^{2+} or Mg^{2+} after treatment with EDTA. This means that all enzymes prepared by the present authors might be metallic enzymes. But, some differences were observed in recovery power among them, that is, form III showed the lowest recovery power among the three forms. The three forms are all not inhibited with thiol inhibitors such as *p*-CMB, NEM and DTNB. This fact is a characteristic property of our *C. utilis* R 5-P ketol isomerase, and also different from the other known R 5-P ketol isomerases. Researches for their sub-unit structure are now in progress.

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