Detection of D-Xylose Isomerase on Disc Electrophoresis

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Gabriel and Wang[1] described the method of detecting enzymatic activity catalyzing the conversion of nonreducing substrates to reducing products. We applied the method to the detection of D-xylose isomerase (EC 5. 3. 1. 5), which was crystallized by Yamanaka[2] from *Lactobacillus brevis* cultured in a medium containing D-xylose. The crystalline preparation proved to be pure by various methods. We examined the purification steps using disc electrophoresis and found that the enzyme was produced in the cells to occupy the considerable part of the total extractable proteins and that the final crystalline enzyme preparation was pure by disc elctrophoresis.

MATERIALS AND METHODS

The crystalls of D-xylose isomerase were obtained from *L. brevis* cultured in the medium containing D-xylose, according to the procedure of Yamanaka[2]. The outline of the purification steps is as follows (see Table 1.) : ca. 25 g of wet cells obtained were ground and extracted with a 50 mM Tris-HCl buffer (pH 7.4) containing 5 mM $MnCl_2$ (A : crude extract). The extract was cooled to 5° and about 1/20 of its volume of 1 M $MnCl_2$ was added and centrifuged after 24 hr (B). The supernatant was fractionated using ammonium sulfate between 45 and 95 % saturation (C). The precipitate obtained by centrifugation was dialyzed against the Tris-HCl buffer and heated to 50° for 10 min., cooled with ice water and centrifuged (D). The supernatant was fractionated through DEAE-cellulose column (E) and precipitated by ammonium sulfate at 90 % saturation. The centrifuged precipitate was dialyzed against the buffer and again fractionated with ammonium sulfate at 64 % saturation (F). After centrifugation the precipitate was discarded. From the supernatant, crystals of the enzyme were obtained (G_1, G_2).

Disc electrophoresis was carried out by the method of Ornstein[3] and Davis[4], except that the gels were formed in the upright position without reversing the gel tubes. Proteins were stained with 1 % Amido Black 10 B. The D-xylose

Table I. Purification steps of D-xylose isomerase

Step	Total vol.	Total activity	Specific activity
A: Crude extract	357 ml	15,350 U	2.9 U/mg protein
B: Mn Treatment	355	11,610	7.6
C: 1st Ammonium sulfate fractionation	36	9,880	10.4
D: Heat treatment	29	9,900	20.6
E: DEAE-Cellulose fractionation	35	8,870	43.7
F: 2nd Am. sulf. fractionation	2.8	5,500	50.0
G_1: 1st Crystal isation		4,160	52.0
G_2: 2nd Crystal isation		2,530	51.6

U: Activity unit according to Yamanaka[2].

isomerase activity in the gel was detected according to the method of Gabriel and Wang[1]. When the gel was incubated in the solution used for the activity measurement[2] and stained with triphenyltetrazolium chloride, an artifact appeared at the BPB band. This could be avoided using a less Mn-ion concentration. As to the concentration of the substrate, D-xylose of 0.6 M in place of 0.1 M was used to shorten the reaction time and thus to reduce further the appearance of artifact at the BPB band. For other sugars, 2 M solutions were used. The final procedure of the D-xylose isomerase activity staining in the gel was as follows : To 8 ml of 50 mM maleate buffer (pH 6.1) was added 1 ml of 0.6 M D-xylose solution and 1 ml of $MnCl_2$ solution. The acrylamide gels after disc electrophoresis were incubated in the above solution at 35° (50° for other sugars than D-xylose) for 10 min. The gels were then rinsed with water and incubated in a solution of 0.1 % triphenyltetrazolium chloride (TTC) in 1 N NaOH at room temperature in the dark. When the gels were stained to a faint pink, a red band of the stained enzyme appeared, they were withdrawn from the staining solution, washed with water, and dipped into a 2 N HCl solution to stop the reaction. The time required for the staining was usually 2 to 3 min. After washing with water, the gels were preserved in a 7 % acetic acid solution. The staining method used depends on the reaction of ketose with tetrazolium salt to form formazan at room temperature, while aldose at 100°.

RESULTS AND DISCUSSION

In Fig. 1 are shown the disc electrophoresis of the fractions obtained in the various steps of the purification of D-xylose isomerase from the extract of *L. brevis*

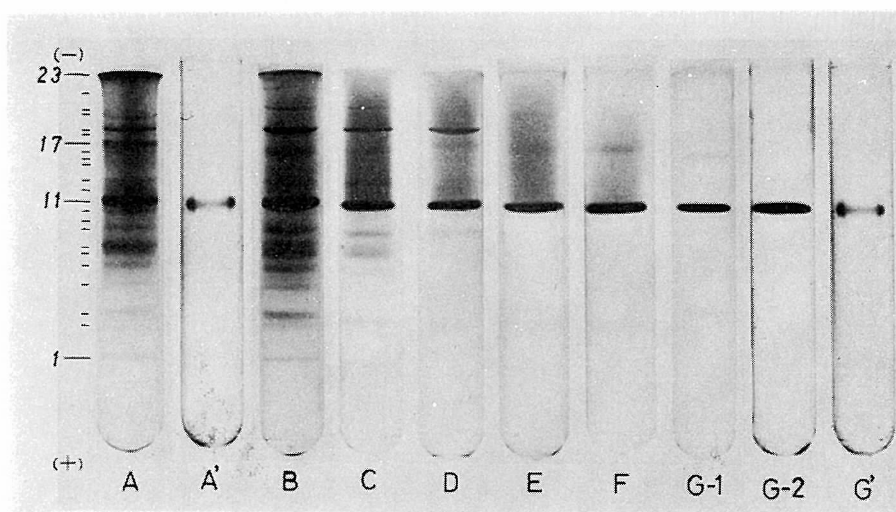

Fig. 1. Disc electrophoresis of protein fractions obtained from the extract of *L. brevis* at various steps of crystallization of D-xylose isomerase.

Electrophoresis gel contained 7 % acrylamide. Electrode buffer, Tris-glycine, pH 8.9. Electrophoresis, at 150 v., for 2.5 hr. Protein fractions were stained with Amido Black 10B. D-Xylose isomerase activity was stained with TTC, as described in the text.
A: crude extract of *L. brevis*, protein stain. A': crude extract, activity stain. B: $MnCl_2$ treated extract. C: first ammonium sulfate fractionation. D: heat treatment. E: DEAE-cellulose fractionation. F: second ammonium sulfate fractionation. G-1: crude crystalline preparation. G-2: recrystallized preparation. G': crystalline preparation, activity stain.

cells. As can be seen from the figure, the crude extract of *L. brevis* contained over 20 fractions of protein. The most abundant fraction was the 11th in the order of electrophoretic mobility. D-Xylose isomerase activity stain corresponded to this protein band. As the purification of the enzyme proceeded, this fraction increased in relation to the other fractions and finally remained as the sole protein fraction in the crystalline preparation of the enzyme. The activity stain also corresponded to this fraction up to the final step of crystallization. — From this result it is obvious that D-xylose isomerase was present in the initial extract of the bacterial cells already in a considerable concentration, to form the most abundant protein fraction. And this may have been favorable to the further purification and crystallization of the enzyme.

In Fig. 2 are shown the results of staining of the enzyme activity in the crude extract with other sugars, L-arabinose, D-arabinose, D-ribose, D-glucose, D-mannose, and D-galactose, as substrates. In these cases, the gels were incubated in the solution containing 2 M of each sugar, and at 50° for 10 min. As can be seen from the figure, when D-sugars were used, only one band of formazan

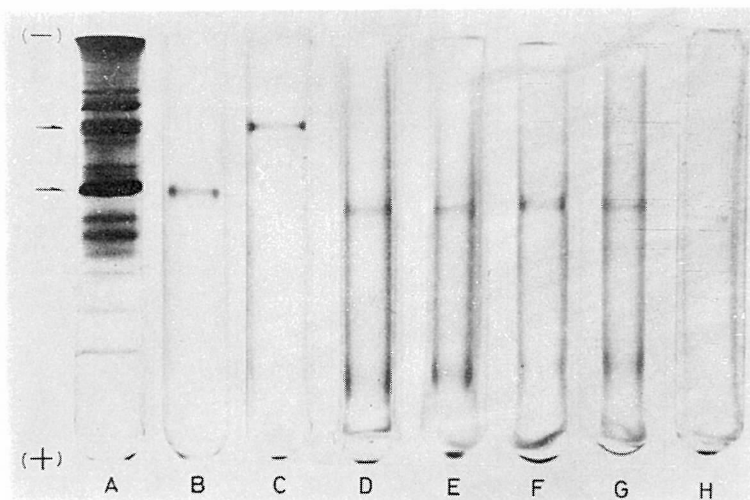

Fig. 2. Disc electrophoresis of the crude extract of *L. brevis* stained by isomerase activity using various sugars.

Conditions of electrophoresis, as in Fig. 1. For isomerase activity staining, see the text.
A: crude extract, stained with Amido Black 10B. B: stained for isomerase activity, using D-xylose as substrate. C: L-arabinose as substrate. D: D-arabinose as substrate. E: D-ribose as substrate. F: D-glucose as substrate. G: D-galactose as substrate. H: D-mannose as substrate.

stain was formed in the crude extract. The bands appeared using these sugars coincided with the band of D-xylose isomerase. This finding could also be confirmed with the crystalline preparation of D-xylose isomerase. These results may suggest that the D-monosaccharides were transformed into the corresponding ketoses, so that D-xylose isomerase may also act on these D-pentoses and D-hexoses. On the other hand, Yamanaka[2] found by the enzymatic measurements that the crystalline preparation of D-xylose isomerase acted on D-xylose, D-ribose, and D-glucose, but not on D-arabinose, D-mannose, and D-galactose. Hence, the above conclusion based on the ketose staining in disc gels coincides only partially with the results of enzymatic measurements. The reason might be ascribed to the higher sensitivity of the staining method.

On the other hand, when L-arabinose was the substrate in the gel developed from crude extract, one formazan band distant from that of D-xylose isomerase appeared (Fig. 2). The stained band corresponded to a protein band no. 17 which moved slower than D-xylose isomerase. Thus the band produced by L-arabinose may have been formed by L-arabinose isomerase, which could be proved also enzymatically in the crude extract[5]. From this result, it is supposed that L-arabinose isomerase may also be purified from the same cells.

SUMMARY

D-Xylose isomerase was crystallized from *Lactobacillus brevis* cultured in a medium containing D-xylose. Protein fractions obtained at various steps of the purification were analyzed by disc electrophoresis. D-Xylose isomerase activity was detected in the disc gels utilizing the formation of formazan dye from triphenyltetrazolium chloride by ketose. The crystalline preparation of the enzyme was pure on disc electrophoresis and found to catalyze the isomerization of other D-sugars: D-arabinose, D-ribose, D-glucose, D-mannose, and D-galactose. L-Arabinose was found to be isomerized by another fraction than D-xylose isomerase.

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