Homology of Starfish and Vertebrate Hexose 6-Phosphate Dehydrogenases[1)]

By

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(With 4 Text-figures and 4 Tables)

In a previous study (Mochizuki and Hori, 1976), hexose 6-phosphate dehydrogenase (H6PD) was purified 130-fold from pyloric caeca of *Asterias amurensis* by chromatography on DEAE-Sephadex and DEAE-cellulose, and its properties were examined with respect to a variety of parameters in order to ascertain whether the enzyme would possess properties similar to vertebrate H6PD. As a result, the starfish enzyme was found to be very similar to the vertebrate enzyme in the following respects: affinity to galactose 6-phosphate (Gal6P), 2-deoxyglucose 6-phosphate (dG6P), and NAD as well as on glucose 6-phosphate (G6P) and NADP, inhibition by Mg^{++} and Ca^{++} and insensitivity to SH-inhibitors and dehydroepiandrosterone (DEA). It was found, however, that the molecular weight of partially purified enzyme was about half the molecular weight of the enzyme present in crude extracts and that the latter was converted into enzymatically active, smaller molecules by mild digestion with trypsin. It was thus assumed that degradation of the enzyme had occurred during purification by the action of contaminated proteinases. Since a similar phenomenon is also known with vertebrate H6PD (Hori and Noda, 1971), this furnishes another evidence for homology of starfish and vertebrate H6PD. It is nevertheless desirable to isolate the starfish enzyme in a native form for detailed comparison with vertebrate enzymes, which is dealt with in this report.

Materials and Methods

Pyloric caeca were isolated from the starfish, *Asterias amurensis*, collected at Akkeshi Bay, Hokkaido, washed with cold 0.25 M sucrose and stored at –70°C until use.

G6P, Gal6P, dG6P, glucose, soybean trypsin inhibitor (Type I-S) and bovine serum albumin were obtained from Sigma Chemical Co., DEAE-Sephadex (A-50),

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Sephadex G–25 (coarce) and CNBr-activated Sepharose 4B from Pharmacia Fine Chemicals and Ultrogel AcA 34 from LKB Produkter Ab. DEA was donated by Teikokuzoki Co. All other reagents were commercial products of the highest grade available.

Enzyme activity was assayed spectrophotometrically by following NAD or NADP reduction at 340 nm. The reaction mixture contained in a total volume of 1 ml; 0.7 ml glycine-NaOH buffer (I=0.2, pH 9.0–11.0) or Tris-HCl buffer (I=0.1, pH 7.5–8.5), 0.05 ml 120 mM G6P, Ga16P or dG6P or 0.1 ml 2 M glucose, 0.05 ml 12 mM NAD or NADP, 0.1 ml enzyme and water as required.

Protein assays, molecular weight estimation and examinations of the effects of *p*-chloromercuribenzoate (PCMB), DEA, $MgCl_2$, $CaCl_2$ and heat on enzyme activity were carried out by the methods described previously (Hori and Sado, 1974; Mochizuki and Hori, 1976). The specific activity of enzyme was expressed as units/g protein.

Km and *V* values were determined from Lineweaver-Burk plots of $1/v$ *versus* $1/s$ and Hofstee plots of s/v *versus* s.

Casein-hydrolyzing activity was assayed by the method of Kunitz (1947) as described previously (Hori, Tanahashi and Matsuoka, 1977).

Preparation of hemoglobin-substituted Sepharose

Five g of CNBr-activated Sepharose 4B was swollen and washed on a glass filter with 1 liter of 1 mM HCl solution. The washed gel was suspended in an appropriate volume of 0.1 M sodium bicarbonate buffer, pH 8.3, containing 0.5 M NaCl and was quickly mixed with 0.6 g hemoglobin. The mixture was stirred gently overnight at 4°C and was poured into a column. Unbounded material was washed away with the coupling buffer, and any remaining active groups were reacted with 1 M Tris at pH 8.0 for 2 hr at room temparature. Three washing cycles were used to remove noncovalently adsorbed protein, each cycle consisting of a wash at pH 4.0 (0.1 M acetate buffer containing 0.5 M NaCl) followed by a wash at pH 8.3 (0.1 M sodium bicarbonate buffer containing 0.5 M NaCl). Finally, it was equilibrated with 20 mM Tris-HCl buffer, pH 8.5, containing 50 mM ammonium sulfate and 1 mM EDTA.

Partial purification of H6PD

All procedures were carried out at 2–4°C. Pyloric caeca (100 g) were homogenized with 400 ml of 20 mM Tris-HCl buffer, pH 7.4, containing 10 mM EDTA and soybean trypsin inhibitor (0.1 mg/g tissue) in a Waring blender. The homogenate was centrifuged at 34,000 × g for 20 min, and the supernatant was filtered through a sheet of filter paper. To the filtrate (crude extract) was added cold 99% ethanol at a final concentration of 10%, and the precipitate was removed by centrifugation at 27,000 ×g for 5 min. The supernatant was again treated with cold 99% ethanol to bring about a final concentration of 20%. The precipitate was

collected by centrifugation and dissolved in 2 volumes of Tris-HCl buffer, pH 7.4, containing 10 mg% soybean trypsin inhibitor. The solution was treated with ammonium sulfate and the 25–50% (w/v) fraction was dissolved in Tris buffer, pH 7.4. The enzyme solution was then passed through a 5.0 × 26 cm column of Sephadex G–25 which was equilibrated with 20 mM Tris-HCl buffer, pH 8.5, containing 1 mM EDTA and 50 mM ammonium sulfate, and applied to a 2.7 × 3.1 cm column of hemoglobin-substituted Sepharose 4B equilibrated with the same buffer. H6PD activity emerged in the breakthrough fraction of protein, while proteolytic activity was mostly retained in the column. The breakthrough fraction was then applied to a 5 × 6 cm column of DEAE-Sephadex pre-equilibrated with the buffer. After washing with the buffer, the enzyme was eluted using a linear gradient formed from 300 ml each of the buffer with 50 mM and 0.2 M ammonium sulfate at a flow rate of 240 ml/hr. Fractions of 20 ml were collected, and those with high enzyme activity were pooled. The solution was then made to about 0.5 M with respect to ammonium sulfate, concentrated to 5 ml in an Amicon ultrafiltration cell equipped with an XM–50 membrane, and applied to a 2.5 × 96 cm column of Ultrogel AcA 34 which was equilibrated with 20 mM Tris-HCl buffer, pH 7.4, containing 1 mM EDTA and 0.5 M ammonium sulfate. The flow rate was adjusted to 30 ml/hr and 10 ml-fractions were collected. H6PD activity emerged in two peaks (Fig. 1, peaks A and B). Electro-

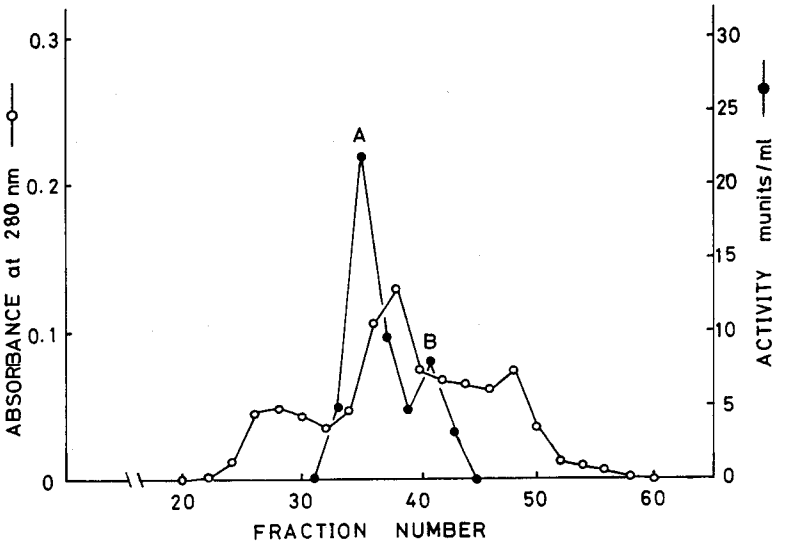

Fig. 1. Gel filtration on Ultrogel AcA 34. Samples eluted from a DEAE-Sephadex column were concentrated, placed on a 2.5 × 96 cm column, and eluted with 20 mM Tris-HCl buffer, pH 7.4, containing 1 mM EDTA and 0.5 M ammonium sulfate at a flow rate of 30 ml per hr. Peaks A and B contained the native and native plus degraded forms, respectively, as shown in Fig. 2.

phoresis of these fractions showed that peak A contained the native form of H6PD, while peak B contained both native and degraded forms of the enzyme (Fig. 2).

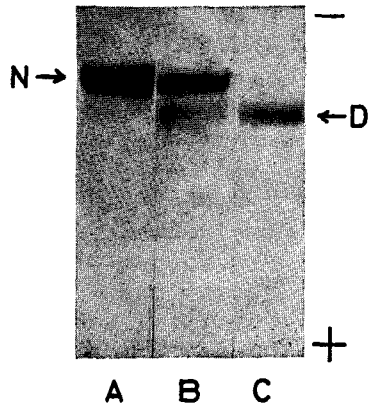

Fig. 2. Electrophoretic patterns of native (N) and degraded (D) H6PD derived from the gel filtration on Ultrogel AcA 34. A, peak A fraction; B, peak B fraction; C, fraction prepared without trypsin inhibitor and affinity chromatography step.

Therefore, fractions of peak A were pooled, concentrated as above and used for kinetic studies. This sample had neither G6P dehydrogenase nor 6-phosphogluconate dehydrogenase activity.

Results and Conclusion

The yields and specific activities of the various fractions obtained during one purification procedure is summarized in Table 1.

Table 1. Purification of starfish H6PD.

Step	Total activity[a] (units)	Total protein (mg)	Specific activity (units/g protein)	Yield (%)
Crude extract	10.1	3226	3.1	100
Ethanol	8.2	1344	6.1	81.0
Ammonium sulfate	7.6	301	25.3	75.3
DEAE-Sephadex	2.2	30.2	72.9	21.8
Ultrogel AcA 34	0.9	2.8	321.4 (500)[b]	8.9

a) Activity was assayed with Ga16P and NAD in order to neglect the activity of glucose 6-phosphate dehydrognease present in crude samples.
b) Activity on G6P+NADP at pH 10.8.

Our previous studies (Mochizuki and Hori, 1976) suggested that it was necessary to remove or to inactivate proteinases as completely as possible at early steps of purification in order to isolate H6PD in a native form. In preliminary experiments, we have first tried to separate secretory granules from pyloric caeca prior to H6PD purification, since proteinases have been known to be contained in

the granules in an inactive form (Camacho, Brown and Kitto, 1970; Bundy and Gustafson, 1973; Peng and Williams, 1973; Tillinghast and Levasseur, 1975). However, this attempt was unsuccessful because of the extreme fragility of the granules (Hori, Tanahashi and Matsuoka, 1977). On the other hand, fractionation of homogenates with ethanol was found to be very effective for removal of proteinases; i.e., more than 95% of total casein-hydrolyzing activity could be removed with 20% ethanol, which precipitated H6PD, but not proteinases. Furthermore, homogenization of pyloric caeca in the presence of soybean trypsin inhibitor caused a slight decrease in the amount of proteinases contaminated in the ethanol precipitates.

Treatments with trypsin inhibitor and ethanol were, however, still insufficient to remove proteinases completely. The sample was, therefore, subjected to affinity chromatography on hemoglobin-substituted Sepharose 4B. The sample thus prepared did not show any casein-hydrolyzing activity, but upon standing at 4°C, H6PD was gradually degraded, suggesting that a small, but significant amount of proteinases were still present.

It has been previously found that proteinases from starfish pyloric caeca exist in several distinct forms (Hori, Tanahashi and Matsuoka, 1977) and that the largest form had a molecular weight of no more than 90,000 (unpublished data). This suggests that H6PD might be separated from proteinases by gel filtlation, since H6PD is about twice as large a molecule as the largest proteinase.

Based on this assumption, the sample from affinity chromatography was subjected at the next step to gel filtration on Ultrogel. As a result, H6PD activity emerged in two peaks, one containing a native form and the other, a degraded form (Fig. 1 & 2). The native form thus prepared appeared to be stable for one day at 4°C, but underwent degradation upon standing for a few days. In the procedure finally adopted, the ion exchange chromatography on DEAE-Sephadex was performed after affinity chromatography on hemoglobin-Sepharose 4B, and the sample obtained was then subjected to gel filtration. The DEAE-Sephadex chromatography was effective for increasing the specific activity of H6PD, but not for removal of a trace of comtaminated proteinases. The Ultrogel fractions containing the native form of H6PD were immediately frozen in vials in 2 ml lots and used for enzyme characterization. Freezing had no effect on enzyme activity.

Examination of the stability of purified H6PD has revealed that difficulties in purifying starfish H6PD lie not only in the contamination of hardly separable proteinases, but also in the extreme instability of the enzyme itself in the absence of NH_4^+. This is shown in Table 2.

The partially purified enzyme lost about 50% of its activity when freed from ammonium sulfate by overnight dialysis. Upon standing for 1 day, the enzyme further lost 70% of the residual activity. Loss of activity was, however, prevented by the addition of ammonium sulfate or ammonium acetate, but not with sodium sulfate or KCl. It was thus apparent that NH_4^+ was a potent stabilizer of H6PD, if present in a sufficiently high concentration. The enzyme dissolved in 0.5 M ammonium sulfate retained its activity at least for a month at 4°C. Such depen-

dence of H6PD on a high concentration of NH_4^+ made its further purification difficult. NH_4^+ had no stimulative effect on enzyme activity, but doubled the *Km* values for Ga16P at a concentration of 50 mM (Table 3).

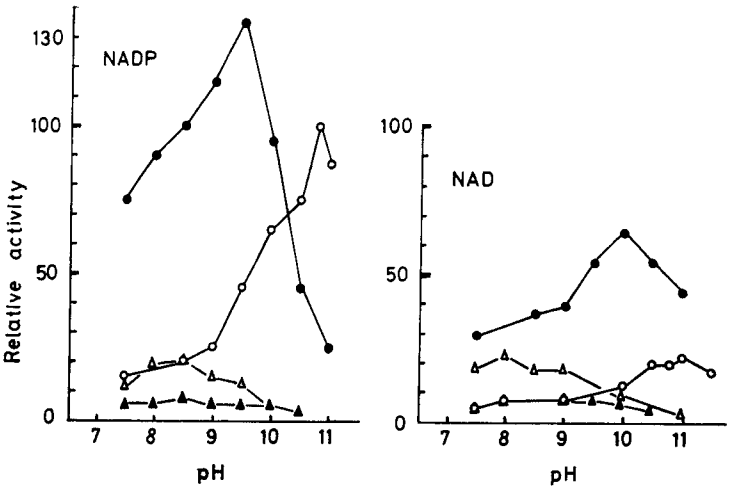

Fig. 3. Effect of pH on starfish H6PD. Left, activity with NADP; right, activity with NAD. Concentrations of substrates: G6P, Ga16P and dG6P, 6.0 mM; glucose, 0.2 M; NADP and NAD, 0.6 mM. Activity was expressed as percentage of the peak activity with G6P and NADP. ○——○, G6P; ●——●, Ga16P; △——△, dG6P; ▲——▲, glucose.

Table 2. Effects of salts on the stability of starfish H6PD.[a]

Salts	Concentration (M)	Remaining activity (%)		
		1 day	3 days	11 days
None		31	21	10
$(NH_4)_2SO_4$	1.0	117	114	110
	0.5	110	110	110
	0.1	79	72	72
	0.05	66	62	55
CH_3COONH_4	2.0	114	103	114
	1.0	100	97	103
Na_2SO_4	1.0	35	41	45
	0.5	48	45	55
KCl	1.5	66	38	34

a) Partially purified H6PD was dialyzed overnight against 20 mM Tris-HCl buffer, pH 7.4, containing 1 mM EDTA and allowed to stand at 4°C with or without indicated salts. During the dialysis, the enzyme lost 52% of the activity. Experimental values are expressed as percentage of the residual activity. The enzyme activity was assayed with Ga16P+NAD at pH 10.0.

Table 3. Kinetic constants of starfish H6PD.[a]

Substrate	Native form		Degraded form[b]	
	Km (mM)	*V* (%)	*Km* (mM)	*V* (%)
G6P (NADP)	0.008	100	0.008	100
Ga16P (NADP)	0.43 (0.23)[c]	516	0.071	243
dG6P (NADP)	0.49	108	0.94	63
Glucose (NAD)	1600	196	1400	211
NADP (G6P)	0.001	—	—	—
NAD (G6P)	0.001	—	—	—

a) Assays were performed at pH 7.5 in the presence of 50 mM ammonium sulfate (the reaction mixture contained in a total volume of 1 ml, 0.1 ml of enzyme containing 0.5 M ammonium sulfate as stabilizer).
b) Data of Mochizuki and Hori (1976).
c) Assayed in the absence of ammonium sulfate.

Effects of pH on H6PD activity are shown in Fig. 3. The profiles of pH-activity curves obtained with the four substrates in the presence of NADP were essentially the same as those reported by Mochizuki and Hori (1976) with the degraded enzyme. In the presence of NAD, the enzyme was most active on Ga16P in the whole range of pH tested. The activity on dG6P or glucose was about the same as that in the presence of NADP, but the activity on G6P or Ga16P was much lower with NAD as coenzyme than with NADP. The pH optima for activities on G6P and Ga16P shifted to higher levels when NAD was replaced for NADP, as in the case of vertebrate H6PD (Stegeman and Goldberg, 1972; Sado and Hori, 1976).

The *Km* and *V* values for six substrates are shown in Table 3 which also includes for comparison the values reported by Mochizuki and Hori (1976) with the degraded enzyme. The values were substantially the same between the native and degraded enzymes, except that the *Km* and *V* for Ga16P were higher with the native than the degraded form.

Effects of PCMB, DEA, $MgCl_2$, $CaCl_2$ and heating on H6PD are shown in Table 4 and Fig. 4. PCMB and DEA had no effect, while $MgCl_2$ and $CaCl_2$ had multiple effects on enzyme activity; they inhibited the activities on G6P and Ga16P, but stimulated the activity on dG6P. On the other hand, heating at 50°C caused an almost complete loss of activity within 10 min. In this case, no differential effects on the activities against different substrates were found. All these results were comparable with those reported with the degraded enzyme.

The molecular weights of the native and degraded enzymes were 198,000± 6,000 (n=5) and 108,000±6,000 (n=3), respectively. These are about 10% greater than the values previously reported with crude enzyme samples, but the present data with the partially purified enzyme would be more reliable. It is thus evident that H6PD from rat (Hori and Sado, 1974), crucian carp (Sado and Hori, 1976) and starfish are all of the same order of magnitude in size.

Table 4. Effects of chemicals on starfish H6PD.[a]

Chemical substances	Substrate	Native form	Degraded form[b]
PCMB (1 mM)	G6P	100	93.2
DEA (70 μM)	G6P	100	93.8
$MgCl_2$ (10 mM)[c]	G6P	78.6	52.5
	Ga16P	73.8	56.7
	dG6P	127.3	150.7
$CaCl_2$ (10 mM)[c]	G6P	78.6	55.4
	Ga16P	90.8	73.0
	dG6P	127.3	142.7

a) Assays were performed at pH 7.5 with NADP as coenzyme, and the results are expressed as percentage of control (averages of three determinations).
b) Data of Mochizuki and Hori (1976).
c) H6PD was freed of EDTA by passing through a short column of Sephadex G-25 prior to assay.

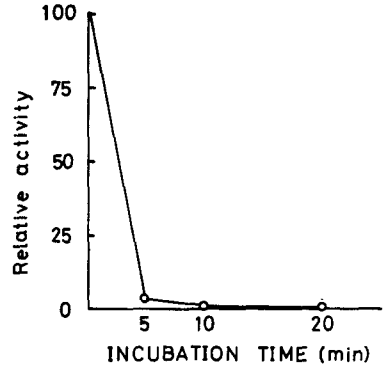

Fig. 4. Effect of heat on starfish H6PD. The partially purified enzyme (native form) was heated at 50°C for 5, 10 and 20 min, cooled and assayed with G6P and NADP at pH 10.8. The results are expressed in percentage of the untreated control. Averages of three determinations.

In conclusion, the native form of starfish H6PD appears to have properties quite similar to those of the degraded form, and of vertebrate H6PD. This lends support to the previous conclusion that starfish H6PD might be homologous with vertebrate H6PD (Mochizuki and Hori, 1976).

Summary

Hexose 6-phosphate dehydrogenase was partially purified from *Asterias amurensis* by fractionation with ethanol, ammonium sulfate, affinity chromatography on hemoglobin-Sepharose 4B, DEAE-Sephadex column chromatography and gel filtration on Ultrogel AcA 34.

The molecular weight of purified enzyme was estimated to be about 200,000 by polyacrylamide gel electrophoresis. Kinetic properties of the purified enzyme were essentially the same as those reported previously with the partially degraded

enzymes. The previous conclusion was thus supported that starfish hexose 6-phosphate dehydrogenase might be homologous to vertebrate hexose 6-phosphate dehydrogenase.

Unlike the vertebrate enzymes, the starfish enzyme was extremely labile and lost its activity rapidly in 20 mM Tris buffer, pH 7.4. The enzyme was, however, stabilized with NH_4^+ at a concentration of 0.5 M or above.

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