Rapid Purification of Glucose-6-Phosphate Dehydrogenase by Affinity Chromatography*

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Affinity Chromatography를 이용한 Glucose-6-Phosphate Dehydrogenase의 신속한 정제방법 개발

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ABSTRACT

An improved procedure for the rapid purification of glucose-6-phosphate dehydrogenase from extracts of *Saccharomyces cerevisiae* was developed by using affinity chromatography. Among six affinty media tested, $NADP^+$-agarose and Affi-gel Blue were more effective than others (*i.e.*, Affi-gel Red, AMP-agarose, ATP-agarose, and NAD^+-agarose). Conditions to desorb the enzyme bound to the affinity media were examined to increase the purity as well as yield. The best result was obtained when the column was developed with a linear gradient of KCl (0-1.0M). In case of Affi-gel Blue, introduction of NAD^+ (15mM) washing step prior to the salt gradient was most effective to remove NAD^+-binding proteins.

For a large scale preparationof G-6-P dehydrogenase higher recovery was obtained by Affi-gel Blue than $NADP^+$-agarose, however, the purity of the enzyme was decreased by 10 times if the former was used as the affinity medium. The capacity of Affi-gel Blue for G-6-P dehydrogenase was found to be 5 times higher than that of $NADP^+$-agarose. Furthermore Affi-gel Blue could be reused repeatedly and its preparation is relatively easier and less expensive than $NADP^+$-agarose.

INTRODUCTION

Glucose-6-phosphate dehydrogenase is the enzyne which catalyzes the first reaction of pentose phosphate pathway that produces most of the reducing power, NADPH, required in several biosynthetic pathways. If this enzyme is genetically altered, hemolytic anemia is appeared since the life span of the erythrocyte deficient in this enzyme is much shorter. Glucose-6-phosphate dehydrogenase was first discovered by Warburg and Christian in 1931. Since then, great efforts have been made to purity it from a variety of sources by several investigators. However, the conventional purification techniques they used were not only very complicated and time consuming, but also resulted in poor recovery of the purified enzyme.

Affinity chromatography which has been developed as a new separation technique since the last '60s has many advantages over the conventional purification methods. Many spectacular separations have been achieved in a single step allowing immense time saving over less selective multi-stage procedures. Lowe, C.

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R. *et al.* (1972) have separated G-6-P dehydrogenase from crude yeast extract by using an affinity medium ε-aminohexanoyl-$NADP^+$-Sepharose. In addition to $NADP^+$-agarose, Brod elium, P. *et al.* (1974) repoted that N^6-immobilized adenosine 2′,5′-bis-phosphate can be used to purify G-6-P dehydrogenase in a single step. However, their results were not fully satistactory with respect to specificity and yield. In this report, we examined the effectiveness of several affinity media for the rapid purification of G-6-P dehydrogenase.

MATERIALS AND METHODS

'Materials'

Dried Baker's yeast *Saccharomyces cerevisiae* was purchased from Sigma Chem. Co., St. Louis. Affi-Gel Blue (Cibacron Blue F3GA-agarose) was purchased from Bio-Rad Lab. and $NADP^+$-agarose (ε-aminohexanoyl $NADP^+$-agarose), NAD^+-agarose (ε-aminohexanoyl NAD^+-agarose), Affi-Gel Red (Procion Red HE-3B) were from Sigma Chem. CO. AG AMP (agarose-hexane-adenosine-5′-monophosphate) was purchased from P-L Biochemical, Inc. Bio-Gel HTP (hydroxylapatite powder) and the reagents for polyacrylamide gel electrophoresis were the product of Bio-Rad Lab.

'Methods'

1. Enzyme and protein assay

The enzymatic activity was determined by following the changes in absorbancy at 340nm with Gilford Spectrophotometer (Model No. : 250). The temperature was maintained at 25°C by pumping water through the thermospacers of the spectrophotometer. One unit is the amount of enzyme that catalyzes the formation of 1μ mole $NADP^+$/min under the standard assay conditions.

The standard reaction mixture for G-6-P dehydrogenase assay contained: 0.12ml of 0.2M glycyl-glycine, pH 8.0, 0.12ml of 0.1M $MgCl_2$, 0.12ml of 2.0mM $NADP^+$, 0.12ml of 10mM glucose-6-P, and 0.72ml of diluted enzyme solution. The reaction was initiated by the addition of G-6-P and $NADP^+$. Alcohol dehydrogenase (Dickinson, F.M., 1970), glutamate dehydrogenase (Doherty, D., 1969), 6-phosphoglconate dehydrogenase (Herecker. B.L., and Smyrniotis, P.Z., 1951), glutathione reductase (Racker, E., 1955), and malate dehydrogenase (Munkres, K.D., 1965) were assayed by the previously publisbed procedures.

Protein was determined by using Bio-Rad protein assay reagent, according to Bradford, M. (1976).

2. Preparation of Cibacron Blue-Sepharose.

Affi-Gel Blue (Cibacron Blue F3GA) was prepared according to Ryan and Vestling (1974). Fifty grams of packed Sepharose 4B were washed with 1 liter of water and resuspended in 50ml of distilled water. Fifteen grams of finely curshed cyanogen bromide were added, and the pH was immediately adjusted to and maintained at 11±0.2 by dropwise addition of 10N NaOH. Temperature was maintained at 20±5°C by small additions of crushed ice. When the reaction had subsided (about 10min) the resin was immediately washed with 500ml of cold 0.1M $NaHCO_3$ in a Büchner funnel. The activated resin was immediately suspended in 50ml of 0.4M sodium carbonate buffer (pH 10.0) containing 1.0g of dissolved Cibacron Blue F3GA. The suspension was stirred slowy with rotary shaker for 18hrs. at 4°C. The derivatized Sepharose was washed with 2 liters of 1M KCl to remove unreacted materials.

3. Storage and regeneration of affinity medium

Affinity media was stored in 0.02% sodium azide soln. at 4°C. Regeneration of the medium was efficiently accomplished by washing with 10 bed volvmes of 0.5M potassium phosphate buffer, pH 7.4.

4. Polyacrylamide gel electrophoresis

Polyacrylamide gel (total acrylamide concen-

tration 7%, cross-liker concentration 2.5%) was prepared according to Davis, B. (1964). In order to test the homogeneity of the purified enzyme, enzyme samples (50μl) were mixed with 0.02% bromophenol blue (5μl) and glycerol (25μl) and the mixtures were layered on the top of the gel. The samples were then subjected to electrophoresis in Tris-glycine, pH 8.3, buffer system at 4°C. Current applied was 1.5 mA per gel. Electrophoresis was proceeded for 5 hrs. until the tracking dye had migrated to the bottom end of the gel.

RESULTS

1. Purification of G-6-P dehydrogenase by conventional purification procedures.

Autolysis: 10 grs of dried Baker's yeast was suspended in 30ml of 0.1M sodium bicarbonate and kept at 40°C for 5hrs. The clear autolyzate was obtained by centrifugation at 15.000×g for 15min. at 2°C.

Streptomycin precipitation: The nucleic acids in the autolyzate was precipitated by the addition of streptomycin sulfate (2%(w/v)), removed by centrifugation at 15,000×g for 15min. and discarded.

Fractionation with ammonium sulfate: We examined the range of $(NH_4)_2 SO_4$ precipitates that contained the maximum activity of G-6-P dehydrogenase by stepwise fractionation with $(NH_4)_2 SO_4$. Approximately 92% of the enzyme activity was recovered in 60~80% saturated $(NH_4)_2 SO_4$ fraction of the crude yeast extract.

Adsorption to hydroxylapatite gel: Small trial runs were made to determine the minimum amount of gel required for the adsorption of the enzyme. Best result was obtained with 2.68 mg (dry weight) of HAP gel per 1mg of protein in 60~80% $(NH_4)_2 SO_4$ fraction. We also examined the condition to elute maximum amount of enzyme adsorbed to HAP gel using different concentrations of phosphate buffer. Elution with 0.15M potassium phosphate buffer, pH 7.4 produced 80% recovery of enzyme activity.

2. Purification of G-6-P dehydrogenase by affinity chromatography.

1) Selection of affinity medium. The usefulness of six affinity media for the purification of G-6-P dehydrogenase were examined. Figure 1 shows that Affi-Gel Blue, $NADP^+$-agarose, and Affi-Gel Red were effective to purly G-6-P dehydrogenase. On the other hand, G-6-P dehydrogenase was not effectively adsorbed by NAD^+-agarose, AMP-agarose, and ATP-agarose column. Higher yield of active enzyme was obtained by Affi-Gel Blue than $NADP^+$-agarose. But the purity of enzyme was about 10 times higher if $NADP^+$-agarose was used as the affinity medium (Table 1). G-6-P dehydrogenase

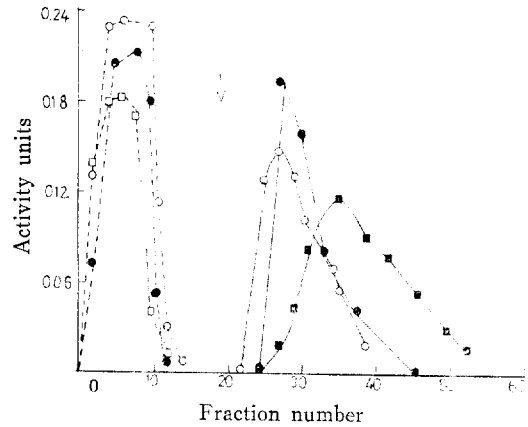


Fig. 1. Affinity chromatography of yeast G-6-P dehydrogenase on (1) Affi-gel Blue (●—●), (2) Affi-gel Red (■—■), (3) $NADP^+$-agarose (○—○), (4) NAD^+-agarose (○···○), (5) ATP-agarose (□···□), and (6) AMP-agarose (●···●) A diluted sample (10ml) of HAP gel eluate was applied to a column (0.5×10cm) containing 1ml of affinity medium equilibrated with 50mM potassium phosphate butter, pH 7.4. Non-adsorbed protein was washed off with 8ml of the same buffer containing 1mM EDTA and column was developed with 0.3M phosphate buffer, pH 7.4 (arrow denotes the position of elution). Flow rate was maintained at 5ml/h and 1ml fractions were collected. G-6-P dehydrogenase was assayed by the procedure described in "Materials and methods."

Table 1. Summary of purification of yeast G-6-P dehydrogenase by affinity chromatography

		Total volume(ml)	Total activity (units)	Recovery (%)	Protein (mg/ml)	Specific activity (units/mg protein)
Hydroxylapatite gel eluate		3.30	1.97	79.19	3.20	0.184
Affinity chromatography	Affi-Gel Blue	20	1.56	79.19	0.039	2.02
	Affi-Gel Red	28	1.51	76.65	0.040	1.34
	$NADP^+$-agarose	18	1.26	63.96	ND	—

(ND: not detectable)

purified by Affi-Gel Red column was less pure than by other two affinity columns. Moreover, since the enzyme recovered from Affi-Gel Red became more diluted than those from the others, we selected Affi-Gel Blue and $NADP^+$-agarose for the purification of G-6-P dehydrogenase.

2) Determination of the capacity of affinity medium.

We determined the capacities of the affinity columns for G-6-P dehydrogenase by 'frontal analysis' method (Lowe, C.R. *et al.* 1973). In this method HAP eluate was applied to a column of Affi-gel Blue or $NADP^+$-agarose until the activity of G-6-P dehydrogenase in the eluate reached to the activity of the applied sample. Subsequent washing was carried out with 50mM phosphate buffer, pH 7.5 and with 0.3M phosphate buffer. Total activity of eluted G-6-P dehydrogenase was 87.8 unit/ml gel for Affi-gel Blue and 17.6 unit/ml gel for $NADP^+$-agarose. From this result, it can be calculated that the capacity of Affi-gel Blue for G-6-P dehydrogenase was about 5 times higher than that of $NADP^+$-agarose.

3) Establishment of effective elution conditions.

Affinity columns were first washed with a KCl gradient (0~1.0M) as 'nonselective elution' method (Fig. 2). Table 2 Shows that the purity of the enzyme obtained by a KCl graddient was higher than the enzyme obtained by one step elution with 0.3M phosphate buffer.

In order to further increase the purity of the enzyme, we used selective elution method. Since

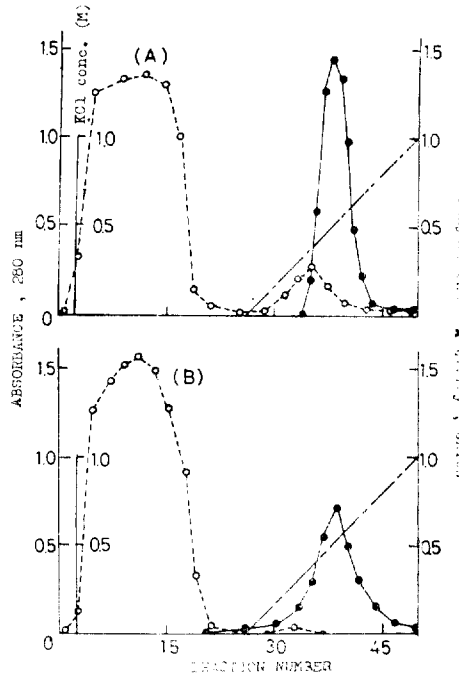


Fig. 2. Affinity Chromatography of G-6-P dehydrogenase on (A) Affi-gel Blue and (B) $NADP^+$-agarose. A diluted sample (30ml) of HAP gel eluate was applied to a column (0.7×10cm) containing 1.5ml affinity gel equilibrated with 50mM phosphate buffer, pH 7.4. Non-adsorbed proteins were washed off with 16ml of the same buffer containing 1mM EDTA. The column was developed with a linear gradient of KCl (0~1.0M) in 50mM phosphate buffer, pH 7.4. Flow rate was maintained at 4ml/h and 2ml fractions were collected. G-6-P dehydrogenase activity (●—●) and protein (○···○) were measured in the effluent.

NAD^+ is competitive to Cibacron Blue F3GA we added NAD^+ in the elution buffer to desorb enzyme from the Affi-gel Blue Column. Figure 3 shows that no enzyme was eluted by NAD^+

Table 2. Comparison of Affi-gel Blue and NADP$^+$-agarose for the purification of yeast G-6-P dehydrogenase in HAP gel eluate. Columns were developed with a linear gradient of KCl (0~1.0M) as described in Fig. 2.

		Total volume(ml)	Total activity (units)	Recovery (%)	Protein (mg/ml)	Specific activity (units/mg protein)
HAP gel eluate		10	7.64	—	3.62	0.211
Affinity chromatography	Affi-gel Blue	16	6.68	87.43	0.087	4,790
	NADP$^+$-agarose	24	4.87	63.74	ND	—

Table 3. Summary of purification of yeast G-6-P dehydrogenase by Affi-gel Blue and NADP$^+$-agarose chromatography. Columns were developed with a linear gradient of NAD$^+$ (0~15mM) followed by a KCl gradient (0~1.0M).

		Total volume (ml)	Total units	Recovery (%)	Protein (mg/ml)	Specific activity (units/mg protein)
HAP gel eluate		9.7	7,824	—	3.65	0.221
Affinity chromatography	Affigel Blue	12	6,394	81.72	0.072	7.403
	NADP$^+$-agarose	14	5.430	69.40	ND	—

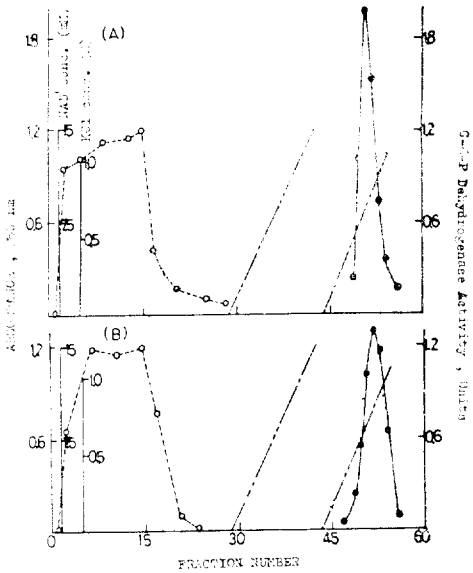


Fig. 3. Affinity chromatography of G-6-P dehydrogenase from HAP gel eluate on (A) Affi-gel Blue and (B) NADP$^+$-agarose. Chromatographic analyses were performed as described in Fig. 2 except the following elution condition; The columns were developed with a linear gradient of NAD$^+$ (— - - —) (0~15mM) followed by a KCl (— - —) gradient (0~1.0M).

gradient (0~15mM). However, the purity of the enzyme obtained by the subsequent elution with KCl gradient was much higher than the enzyme obtained by the nonselective elution method (Table 3).

We have also tried pH shift method to desorb the enzyme from the affinity column. When pH elution buffer (50mM KPi) was varied, 20~30% of active enzyme was recovered in the buffer ranging pH 10~11, whereas no enzyme was eluted under acid condition. Considering the stability of enzyme according to pH (Glaser, L. and Brown, D.H., 1955), we concluded that it is inappropriate to increase pH of the elution buffer to 10~11.

Figure 4 & 5 illustrate the behaviour of several pyridine nucleotide-dependent dehydrogenases when affinity chromafography was carried out with the above described procedures. In case of Affi-gel Blue, G-6-P dehydrogenase was completely resolved from alcohol dehydrogenase and glutamate dehydrogenase. Glutathion reductase was strongly retained and its activity somewhat overlaps with G-6-P dehydrogenase. However, malate dehydrogenase was not resolved from G-6-P dehydrogenase. When NADP$^+$-agarose was used as affinity medium, all dehy-

Table 4. Purification of Glucose-6-phosphate dehydrogenase

		Volume (ml)	Total Activity (units)	Protein (mg/ml)	Specific Activity (U/mg)	Recovery (%)	Purification
Yeast autolyzate*		21	43.13	40.5	0.051	—	—
Stremptomycin sulfate treatment		23.4	41.79	35.2	0.051	96.9	1
$(NH_4)_2 SO_4$ fractionation		22.8	38.08	15.7	0.106	88.3	2.08
HAP gel eluate		40	31.08	3.5	0.222	72.1	4.35
Affinity Chromatography	Affi-gel Blue	26	25.84	0.11	9.036	59.9	177.18
	$NADP^+$-agarose	36	22.54	6.1×10^{-3}	102.62	52.3	2012.16

* 10 grs of baker's yeast were autolyzed.

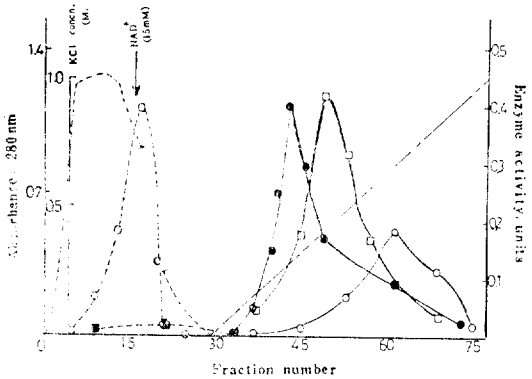


Fig. 4. Separation of NAD^+-and $NADP^+$-dependent dehydrogenases by Affi-gel Blue using a salt gradient. A diluted sample (30ml) of HAP gel eluate was applied to a column of Affi-gel Blue (1.5ml of gel) equilibrated with 50mM phosphate buffer, pH 7.4. Nonadsorbed protein was washed off with 20ml of the same buffer containing 1mM EDTA. The column was then washed with 15mM NAD^+ (8ml) and developed with a KCl gradient. The following enzyme activities as well as protein (·····) were measured: alcohol dehydrogense (○·····○); malate dehydrogenase (□·····□); glutamate dehydrgenase (■·····■); glutathione reductase (○——○); and G-6-P dehydrogenase (●——●).

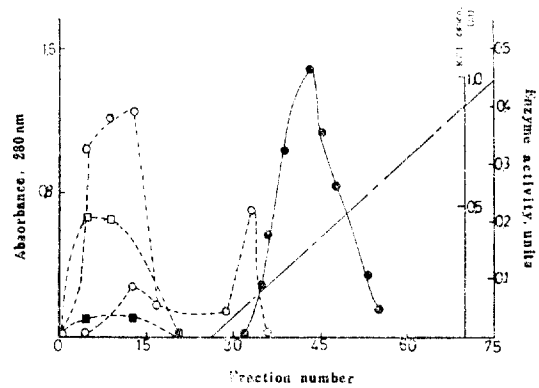


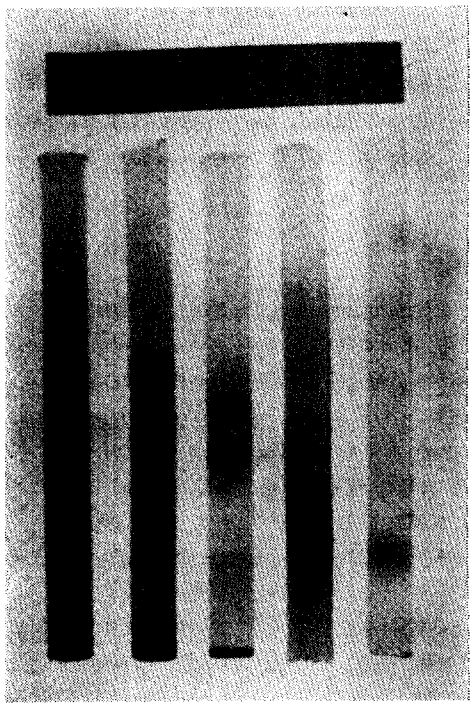
Fig. 5. Separation of NAD^+-and $NADP^+$-dependent dehydrogenases by $NADP^+$-agarose using a salt gradient. Chromatographic analyses were performed as described in Fig. 4 except no introducion of NAD^+ into column.

drogenases tested except G-6-P dehydrogenase were not retained.

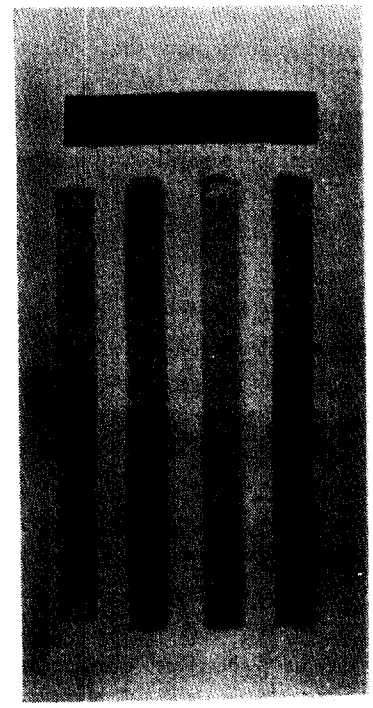
In summary, G-6-P dehydrogenase from baker's yeast was purified about 180-fold by Affi-gel Blue, and 2,000 fold by chromatography on $NADP^+$-agarose in combination with the conventional purification procedure (Table 4).

4) Test for purity

We examined the purity of the enzyme samples obtained during the purification procedure by using disc gel electrophoresis (Fig. 6). From the results shown in Fig. 6 we were able to confirm that addition of NAD^+ washing step is very effective to increase the purity of the enzyme. Two discrete protein bands shown in the middle of gel C disappeared by the NAD^+ (15mM) washing step as shown in gel D. But the enzyme purified through on Affi-gel Blue was not completely pure. Meanwhile, single protein band was detected if $NADP^+$-agarose was used as the affinity medium (gel E). The site on the gel corresponds exactly to the position that represents G-6-P dehydrogenase (Fig. 7).



(Fig. 6)



(Fig. 7)

Fig. 6. Electrophoresis on polyacrylamide gel of several enzyme samples obtained during the purification process. Electrophoresis was carried out according to the method described under "Materials and Methods". Gels were stained with Coomassie blue for protein. Samples: (A) Yeast autolyzate (B) HAP gel eluate and G-6-P dehydrogenase obtained by affiinity chromatography using Affigel Blue (C-D) and $NADP^+$-agarose (E). Samples (C)—(E) were obtained from the fractions containing G-6-P dehydrogenase shown in Fig. 2 (C), Fig. 3-A (D), and Fig. 3-B (E).

Fig. 7. Electrophoresis on polyacrylamide gel of G-6-P dehydrogenase isolated by affinity chromatography on Aff-gel Blue and $NADP^+$-agarose. Gels A and B were incubated with a dye (NBT), an electron carrier (PMS), $NADP^-$, and either with (B) or without (A) G-6-P to demonstrate enzyme activity. Gels C and D were stained with Coomassie blue for protein. Samples (A), (B), and (D) are identical to the sample (D) used in Fig. 6., and Sample (C) is identical to (E) in Fig. 6.

DISCUSSION

We have shown that Affi-gel Blue (immobilized Cibacron Blue F3GA) and $NADP^+$-agarose are convenient and useful affinity chromatographic media for the purification of glucose-6-phosphate dehydrogenase. It was reported (Watson *et al.*, 1978) that the ligand of Affi-gel Red, Procion Red HE-3B has broad specficity. Actually, our results indicate that G-6-P dehydrogenase purified by Affi-gel Red column is less pure than the enzyme purified by $NADP^+$-agarose and Affi-gel Blue column. (Table 1).

Enzyme adsorbed to affinity column was eluted by using 'nonselective' and 'selective' elution methods. The enzyme eluted by nonselective elution method such as KCl gradient was not very pure if Affi-gel Blue was used as affinity medium. By adding NAD^+ in the developing buffer prior to the KCl elution, the purity of enzyme was increased considerably.

For a large scale preparation of G-6-P dehydrogenase, higher recovery was obtained by Affi-gel Blue than $NADP^+$-agarose, however, the purity of the enzyme was lower if Affi-gel Blue was used as the affinity medium. The

capacity of Affi-gel Blue for G-6-P dehydrogenase was much higher than that of $NADP^+$-agarose.

Affi-gel Blue could be reusable manytimes and it is very stable regardless of storage time. Furthermore preparation of Affi-gel Blue is relativey easier and less expensive than $NADP^+$-agarose. These suggest that Affi-gel Blue column is potentially very useful for the rapid purification of G-6-P dehydrogenase from yeast.

摘 要

*Saccharomyces cerevisiae*로 부터 glucose-6-phosphate dehydrogenase를 신속하고 간편하게 정제하는 과정을 affinity chromatography를 이용하여 개발하였다. 이 효소를 정제하는데 적절한 affinity medium을 조사해 본 결과, $NADP^+$-agarose와 Affi-gel Blue(Cibacron Blue F3GA)가 Affi-gel Red(Procion Red HE-3B), AMP-agarose, ATP-agarose, 그리고 NAD^+-agarose보다 유용함이 밝혀졌다.

이 두가지 affinity media에 흡착된 효소를 분리하는데 가장 적합한 elution 조건을 조사하였는데 KCl gradient (0~1.0M)가 효소의 순도 및 수확률을 가장 높일 수 있는 적합한 방법이었다. 특히 Affi-gel Blue를 사용한 경우, KCl gradient로 효소를 용출시키기 전에 NAD^+(15mM)로 NAD^+에 친화력을 갖는 효소들을 제거하는 것이 enzyme의 순도를 높이는데 매우 효과적이었다. 그 결과 glucose-6-phosphate dehydrogenase를 baker's yeast로 부터 기존의 간단한 정제 과정과 affinity chromatography를 병행한 방식을 사용하여 분리하였는데, affinity medium으로 Affi-gel Blue를 사용했을 때는 180배 정도, $NADP^+$-agarose를 사용했을 때는 2,000배 정도로 정제되었다.

대량으로 glucose-6-phosphate dehydrogenase를 정제하는 경우, Affi-gel Blue를 사용하면 효소의 순도는 $NADP^+$-agarose보다 낮으나, 효소의 회수율은 훨씬 더 높았다. 또한 G-6-P dehydrogenase에 대한 affinity medium의 capacity도 Affi-gel Blue가 $NADP^+$-agarose보다 5배정도 높았으며 더우기 Affi-gel Blue는 여러번 반복적으로 사용될 수 있고, 그 제조 과정도 $NADP^+$-agarose보다 간단하며 경비도 적게 들었다.

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