Fungal Genetics Reports

Volume 35 Article 1

A rapid and easy method for the purification of the Neurospora crassa NADP-specific glutamate dehydrogenase

J. Aguirre

W. Hansberg

Recommended Citation

Aguirre, J., and W. Hansberg (1988) "A rapid and easy method for the purification of the Neurospora crassa NADP-specific glutamate dehydrogenase," *Fungal Genetics Reports*: Vol. 35, Article 1. https://doi.org/10.4148/1941-4765.1521

A rapid and easy method for the purification of the Neurospora crassa NADP-specific glutamate dehydrogenase

Abstract

In order to study an oxidative modification of the Neurospora crassa NADP-specific glutamate dehydrogenase (GDH[NADP]) during aerial growth, we were compelled to purify this enzyme.

Aguirre, J. and W. Hansberg

A rapid and easy method for the purification of the Neurospora crassa NADP-specific glutamate dehydrogenase

In order to study an oxidative modification of the Neurospora crassa NADP-specific glutamate dehydrogenase (GDH[NADP]) during aerial growth, we were compelled to purify this enzyme. The different purification procedures which have been described by other authors follow the first three steps of the method reported by Barratt and Strickland (1963 Arch. Biochem. Biophys. 102:66-76). Two additional purifications steps are needed to obtain a single band after SDS-polyacrylamide-gel electrophoresis, usually separation by molecular weight on Sephadex and then one of the following procedures: a) affinity chromatography with N-carboxy-methyl-L-glutamate bound to Sepharose, which causes partial inactivation of the enzyme (Blumenthal and Smith 1973 J. Biol. Chem 248:6002-6008; b) a second DEAE-Sephadex A-50 column plus $(NH_4)_2SO_4$ precipitation at 45% saturation (Ashby et al. 1974 Biochem J. 143:317-329) which gives a 1.6 fold increase in specific activity but a 48% yield loss; c) electrofocusing with some inactivation of the enzyme, 30% loss in total activity, and the inconvenience that only a few milligrams of protein can be processed (Hernandez et al. 1983 J. Bacteriol. 154:524-528).

Triazine dyes immobilized to Sepharose have bean used with good results to purify various dehydrogenases. In the case of Neurospora crassa GDH(NADP), after the usual first three steps of the purification procedure, the cell extract was applied to a column of Procion Red HE-3B immobilized to Sepharose CL-4B. The enzyme was eluted from the column with NADPH. This procedure gave a high specific activity and a very good yield (Watson et al. Biochem J. 173:591-596) (Table 1). This method is probably the best to purify a small amount of enzyme (<200 U), but, for higher amounts of enzyme the NADPH elution from the affinity column turns out to be too expensive. Instead of binding Procion Red to Sepharose, we tried the commercially available Reactive Blue 2-Sepharose (Sigma). A comparison of the different purification procedures is shown in Table 1.

A detailed description of the present purification method is as follows: Neurospora crassa hyphae were grown in Erlenmeyer flasks from 1 x 10^6 conidia per ml of Vogels minimal medium supplemented with 1.5% sucrose, agitated 13-14 h in a gyratory shaker at 230 rpm and 30° C. The hyphae, harvested by filtration and dehydrated with an excess volume of acetone, were ground with mortar and pestle and dry ice until a fine powder was obtained. Ground hyphae in 220 ml of 0.1 M phosphate buffer (pH 8.0) was homogenized at 4° C, two times 2 min. each, with a Polytron homogenizer at full power. After centrifugation at 15,000 g, 15 min, the cell extract supernatant was heated at 50° for 1 h, kept on ice for 30 min and centrifuged in the same way. Solid $(NH_4)_2SO_4$ was added to the supernatant to 30% saturation. Precipitated protein was removed by centrifugation and the supernatant was adjusted to 55% saturation of $(NH_4)_2SO_4$. The precipitate was collected by 15,000 x g centrifugation, resuspended in 16 ml of 0.01 M Tris-HCl, pH 8, 50 mM $(NH_4)_2SO_4$, 0.1 mM L-glutamate (Buffer A) and dialyzed twice against 500 volumes of the same buffer. An unexplained 18% increase in total activity was observed at this point (Table 2). Then the cell extract was loaded onto a (2.5 x 33 cm) column of DEAE-Sephadex A-50, equilibrated with Buffer A. The enzyme was eluted from the column at 22 ml/h with a linear gradient of Buffer A and Buffer A containing: 300 mM $(NH_4)_2SO_4$. The fractions with high GDH(NADP) activity were pooled and precipitated by adding $(NH_4)_2SO_4$ to 55% saturation. The precipitate was dissolved in 2 ml of 50 mM Tris-HCl, pH8, 1 mM L-glutamate (Buffer B) and dialyzed twice against 500 volumes of the same buffer. The dialyzed enzyme was applied at 4.1 ml/h to a column (0.9 x 25 cm) of Reactive Blue-Sepharose (Sigma), equilibrated with Buffer B. After extensive washing with this buffer, the enzyme was eluted at 8.3 ml/h with a linear NaCl gradient obtained with 50 ml of Buffer B and

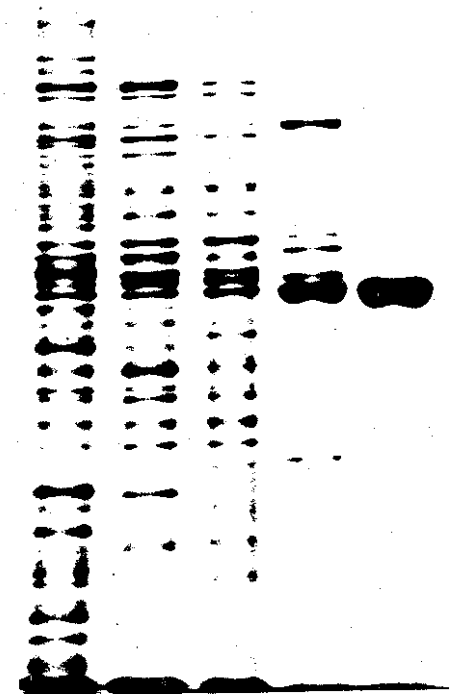

Fig. 1. **Purification of GDH(NADPH)** 1. Crude Extract; 2. After heating; 3. $(NH_4)_2SO_4$ precipitate; 4. DEAE-Sepharose eluate; 5. Reactive Blue-Sepharose eluate

50 ml of the same buffer containing 1M NaCl. The fractions with the highest enzyme activity were pooled and the enzyme precipitated with (NH4)2SO4 at 55% saturation. The precipitate was resuspended in 2 ml of Buffer B, solid (NH4)2SO4 was added to 55% saturation, and stored in aliquots at -20° C. A summary of the purification is given in Table 2. After polyacrylamide-gel electrophoresis (Laemmli 1970 Nature 227:680-685), the purified enzyme showed one major protein in overloaded gels and one or probably more minor bands, estimated to be less than 1% of the major band (Fig. 1). The most noticeable of the minor bands cross reacted with antibodies directed against the main band and its intensity increased after repeated freezing and thawing. Thus, this minor band is probably a degradation product of the enzyme. The purification procedure described here offers a combination of relative speed (4 vs 5-6 days) with both a high specific activity and a high yield.

Table 1. Specific activities (U/mg protein) and yield (% of crude lysate total activity) obtained with different purification procedures.

	Blumenthal & Smith		Ashby et al.		Hernandez et al.		Watson et al.		This report	
	U/mg	%	U/mg	%	U/mg	%	U/mg	%	U/mg	%
Heating (NH4)2So4 DEAR-Sephsrose	31.1	78	**23.2**	**46**	26.4	-	14.0	53	30.7	68
Sephadex G200 Final Step	52.2	43	68.6	23	61.0	25				
Triazine-Sepharose							66.9	43	64.2	38

* Since the hyphae were grown and harvested in the same way, these specific activities were calculated assuming our initial specific activity.

Table 2. NADP-specific Glutamate Dehydrogenase Purification

	Total Units	Protein, total mg	Units* mg prot.	Yield	Purification
Crude extract supernatant	2,484	3,380	0.73	100	
supernatant after heating	1,719	713	2.41	69.2	3.2
(NH4)2SO4 precipitation after dialysis	2,173	488.3	4.45	87.5	6.0
DEAE-Sephadex effluent	1,680	54.7	30.71	67.6	41.8
Reactive Blue-Sepharose + (NH4)2SO4 precipitation	942	14.7	64.08	37.9	87.5

* Units: umoles of NADPH oxidized/min at 25° C.

Acknowledgements. We thank A. Alagon and J. Padilla for technical advice. This work was supported by CONACYT, grant ICEXCNA-050809. - - - Centro de Investigacion sobre Fijacion de Nitrogeno, UNAM, Apartado Postal 565-A, CP 62280 Cuernavaca, Morelos, Mexico.