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Partial purification of L-glutamine:D-fructose 6-phosphate aminotransferase from zoospores of *Blastocladiella emersonii*

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SUMMARY

L-Glutamine:D-fructose 6-phosphate aminotransferase (EC 2.6.1.16) was purified about 88-fold from zoospores of *Blastocladiella emersonii* by centrifugation and fractionation on DEAE-cellulose. Specific activities up to 4.85 μmoles glucosamine 6-phosphate$\cdot^{-1}\cdot$mg protein^{-1} were achieved.

Encystment of *Blastocladiella emersonii* zoospores involves rapid formation of a chitinous cell wall indicating a highly active pathway for chitin synthesis[1–3]. The first step is catalyzed by L-glutamine:D-fructose 6-phosphate aminotransferase (EC 2.6.1.16), which has been partially purified from plant[4–10] and animal[7,8,11–20] sources. As part of our study of zoospore encystment in *B. emersonii*, we are attempting to purify the aminotransferase in these motile cells. Two favorable facts were previously established in this laboratory[5,6]: not only does this fungus contain a very active aminotransferase, but the highest specific activity for it reported to date from any source still remains the one (1.2 μmoles glucosamine 6-phosphate$\cdot^{-1}\cdot$ mg protein^{-1}) established 12 years ago for the partially purified enzyme from *B. emersonii*. In this communication, we provide new methods for its further purification.

The original strain of *Blastocladiella emersonii* Cantino and Hyatt was grown on Difco PYG agar (100 petri plates/experiment) at 22 °C in the dark. Each plate was inoculated with $3\cdot10^5$–$5\cdot10^5$ spores and flooded with 6 ml water 24 h later. The spores released ($2.5\cdot10^9$–$5.7\cdot10^9$) were filtered to remove dislodged plants, counted (Coulter Counter), and extracted with the procedure used by Myers and Cantino[21] to isolate gamma particles. This involved centrifugation (1000 × *g*, 8 min) of spores, their resuspension in 8–12 ml 1 M sucrose

in a buffer containing 25 mM potassium phosphate (pH 6.8), 1 mM ethyleneglycol-bis-(β-amino ethylether)-*N,N'*-tetraacetic acid (EGTA) and 1 mM KCl for 10 min, recentrifugation, a second resuspension in 9 ml 0.4 M sucrose in the same buffer for 5 min to rupture the spores by osmotic shock, and eventual centrifugation of the lysate (110 000 × *g*, 3 h) to yield a high speed supernatant. The high speed supernatant was either used immediately as the enzyme preparation or stored at −20 °C.

Assay mixtures for aminotransferase contained 25 mM potassium phosphate (pH 6.8), 10 mM L-glutamine, 15 mM D-fructose 6-phosphate (Fru-6-*P*), 1 mM EGTA, and 1 mM KCl, in a final volume of 1 ml. Incubations were started by addition of enzyme and terminated after 30 min at 30 °C by immersing the assay tubes in boiling water for 1 min. D-Glucosamine 6-phosphate (GlcN-6-*P*) was determined according to Good and Bessman[22], the extinction being measured at 540 nm. Protein was determined according to Lowry *et al.*[23] whenever possible. However, since dithiothreitol can interfere in the procedure, we also employeda tannin turbidimetric technique[24] whenever dithiothreitol was present. Low amounts (0–40 μg) of protein were determined accurately with this method. Bovine serum albumin was used as a standard. 1 I.U. of enzyme was defined as the amount which catalyzed the formation of 1 μmole of GlcN-6-*P* per min. Specific activity (spec.act.) was expressed as I.U. per mg protein.

DEAE-cellulose (Whatman powder DE 50) was washed according to Peterson and Sober[25]. Columns (1.5 cm × 24 cm and 1.5 cm × 29.5 cm) were equilibrated with 1–6 bed volumes of the following buffered solution: 25 mM potassium phosphate (pH 6.8), 1 mM EGTA, 1 mM KCl, 4 mM dithiothreitol, 0.05 mM D-glucose 6-phosphate (Glc-6-*P*), 10 mM L-glutamine, and 600 mM sucrose. 10–18 ml high speed supernatant containing 27–49 mg protein were applied, and proteins were eluted stepwise at 0–4 °C with a high flow rate (66–135 ml/h) as follows: 50–60 ml solution, and then (all in the same solution) 50–60 ml 10 mM KCl, 60–100 ml 100 mM KCl, 50–100 ml 500 mM KCl, and 50–70 ml 1 M KCl. Eluate fractions (14 ml) were assayed for enzyme activity the same day.

For electrophoresis, 10 % polyacrylamide gels with 2.5 % cross-linking were prepared in 5 mm × 127 mm glass tubes without stacking gel. Gels were prepared with a Tris (3 g/l)–glycine (14.4 g/l) buffer (pH 8.3) and prerun for at least 1 h (1–2 mA/gel) to remove persulfate. Enzyme preparations contained sucrose and were applied to the gels after addition of tracking dye (bromophenol blue). Tris–glycine buffer (above) was used for the electrophoresis at 1 mA/tube at 4 °C. Gels were fixed and stained with 1 % amido black in 7.5 % acetic acid and destained by agitation in 7.5 % acetic acid. To locate the aminotransferase, gels were sliced immediately after electrophoresis in an ice-cooled dish. Each slice was transferred to a normal assay mixture and incubated as usual.

Results of our purification efforts are summarized in Table I. The high speed supernatant contained a very active enzyme; in comparison with homogenates of washed sonicated zoospores (spec.act. 0.04), it was already significantly enriched (3–5-fold). Fractionation of high speed supernatant on DEAE-cellulose yielded an additional 22-fold purification. The spec.act. achieved is much higher than any thus far reported in the literature for this enzyme from any source. A representative profile for a DEAE-cellulose frac-

TABLE I

PARTIAL PURIFICATION OF L-GLUTAMINE:D-FRUCTOSE 6-PHOSPHATE AMINOTRANSFERASE FROM ZOOSPORES OF *B. EMERSONII*

Expt No.	*High speed supernatant (110 000 × g)*	*High speed supernatant fractionated on DEAE-cellulose*		
	spec. act. (I.U.)	*Spec. act. (I.U.)*	*Purification*	*% Recovery*
1	0.11	2.13★	19.4	80★★★
2	0.22	3.53	16.1	57
3	0.15	4.85	32.3	48
Av.	0.16	3.50	22.6★★	62

★ Values for the most active fraction from the column

★★ This represents a purification factor of 88 relative to starting levels in the whole spore homogenates (spec. act. 0.04) from which high speed supernatant was derived.

★★★ Values are based on combined total activities recovered in all active fractions per experiment.

tionation is illustrated in Fig.1. Protein was resolved into 5 major fractions, one of which contained most of the enzyme; profiles for specific and total aminotransferase activities are also shown. The relative purity of the active fractions was estimated by polyacrylamide gel electrophoresis. The high speed supernatant contained at least 25 separable protein bands. The presence of aminotransferase in the uppermost (7 mm) region of the gel was confirmed by slicing and assaying the gels.

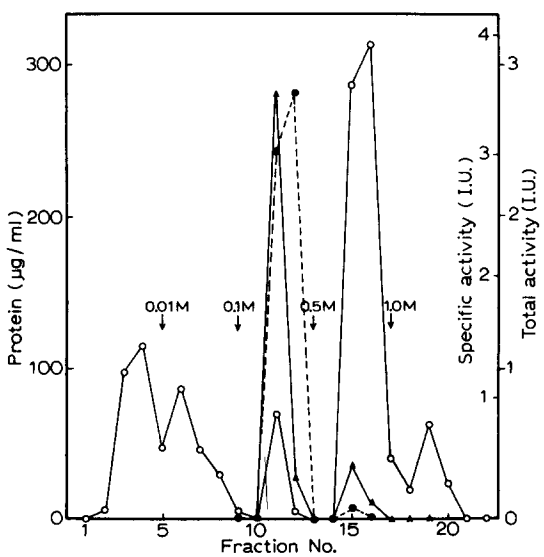

Fig.1. Fractionation of high speed supernatant (27 mg) on a DEAE-cellulose column (1.5 cm × 24 cm). The KCl eluant was added as indicated at arrows. Protein (µg/ml) (o——o); spec. act. (•– – –•); total activity (▲——▲).

In conclusion, we have developed a useful procedure for extracting and partially purifying L-glutamine:D-fructose 6-phosphate aminotransferase. This enzyme has a notorious reputation for being difficult to work with [5,11,14,17–20], and stabilizing agents have generally been needed, *e.g.* Glc-6-*P* [4,8,11,15,16,18,20,26], L-glutamine[4,7,10,11,15], Fru-6-*P* [5,10,11,19,27], glycerol[16], cysteine[12], GSH[5,7,12,19], dithioerythritol[17], mercaptoethanol[7,8,14,15,17,28,29], dithiothreitol[8,16,18,20,29] and UDP-*N*-acetylglucosamine[30]. To this list, sucrose can now be added; our high speed supernatant, which contained 0.6 M sucrose, was more stable than high speed supernatant prepared without sugar. High speed supernatant stored frozen at −20 °C lost activity only slowly, *e.g.* 21 % over a 48-day period. The column-purified aminotransferase was also relatively stable, its spec.act. decreasing about 60 % during 45 days of storage at −20 °C.

The high specific activity of our starting material is especially noteworthy. Both plants and zoospores of *B. emersonii* are attractive sources of the aminotransferase and should facilitate future attempts to purify this enzyme. Even though the high spec. act. we report here exceeds, by far, all of those previously reported for the aminotransferase extracted from other sources, our best enzyme preparations are still not free of contaminating proteins; efforts to further purify the enzyme are continuing.

UDP-*N*-acetyl-D-glucosamine is a feedback inhibitor of the aminotransferase in both animal cells[8,13,15,16,18–20,26–31] and higher plants[9,10]. Our kinetic studies in progress indicate that the same mechanism is also operative in *B. emersonii.* Such inhibition may be correlated with a high molecular weight enzyme containing subunits[8]. Our migration patterns for the *B. emersonii* enzyme in polyacrylamide gel suggest that its molecular weight is also high. Feedback inhibition is probably an important control mechanism for chitin synthesis in *B. emersonii.* The aminotransferase occurs *in vivo* in the "soluble pool" of a zoospore, although we cannot yet be completely certain that it is not released from some internal compartment when spores are ruptured. Most if not all of the zoospore's chitin synthetase[32,33] is contained within cytoplasmic organelles, the gamma particles. Hence the apparent presence of the aminotransferase outside the gamma particles, and of chitin synthetase inside these organelles, provides a possible control mechanism for initiation of chitin synthesis during zoospore encystment; this possibility is being investigated.

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REFERENCES

1 Camargo, E.P., Dietrich, C.P., Sonneborn, D. and Strominger, J.L. (1967) *J. Biol. Chem.* 242, 3121–3128
2 Truesdell, L.C. and Cantino, E.C. (1971) in *Current Topics in Developmental Biology* (Moscona, A.A. and Monroy, A., eds), Vol. 6, pp. 1–44, Academic Press, New York
3 Soll, D.R. and Sonneborn, D.R. (1971) *Proc. Natl. Acad. Sci. U.S.* 68, 459–463
4 Leloir, L.F. and Cardini, C.E. (1953) *Biochim. Biophys. Acta* 12, 15–22
5 Lovett, J.S. and Cantino, E.C. (1960) *Am. J. Bot.* 47, 499–505
6 Lovett, J.S. and Cantino, E.C. (1960) *Am. J. Bot.* 47, 550–560

7 Ghosh, S., Blumenthal, H.J., Davidson, E. and Roseman, S. (1960) *J. Biol. Chem.*, 235, 1265–1273
8 Kornfeld, R. (1967) *J. Biol. Chem.* 242, 3135–3141
9 Mayer, F.C., Bikel, J. and Hassid, W.Z. (1968) *Plant Physiol.* 43, 1097–1107
10 Vessal, M. and Hassid, W.Z. (1972) *Plant Physiol.* 49, 977–981
11 Pogell, B.M. and Gryder, R.M. (1957) *J. Biol. Chem.* 228, 701–712
12 Gryder, R.M. and Pogell, B.M. (1960) *J. Biol. Chem.* 235, 558–562
13 Kornfeld, S., Kornfeld, R., Neufeld, E.F. and O'Brien, P.J. (1964) *Proc. Natl. Acad. Sci. U.S.* 52, 371–379
14 Danishefsky, I. and Deutsch, L. (1968) *Biochim. Biophys. Acta* 151, 529–531
15 Bates, C.J. and Handschumacher, R.E. (1969) *Adv. Enzyme Regul.* 7, 183–204
16 Akamatsu, N. and Maeda, H.R. (1971) *Biochim. Biophys. Acta* 244, 311–317
17 Calcagno, M., Levy, J.A., Arrambide, E. and Mizraji, E. (1971) *Enzymologia* 41, 175–182
18 Kikuchi, H., Kobayashi, Y. and Tsuiki, S. (1971) *Biochim. Biophys. Acta* 237, 412–421
19 Winterburn, P.J. and Phelps, C.F. (1971) *Biochem. J.* 121, 701–709
20 Miyagi, T. and Tsuiki, S. (1971) *Biochim. Biophys. Acta* 250, 51–62
21 Myers, R.B. and Cantino, E.C. (1971) *Arch. Mikrobiol.* 78, 252–267
22 Good, T.A. and Bassman, S.P. (1964) *Anal. Biochem.* 9, 253–262
23 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) *J. Biol. Chem.* 193, 265–275
24 Mejbaum-Katzenellenbogen, W. and Dobryszycka, W. (1959) *Clin. Chim. Acta* 4, 515–522
25 Peterson, E.A. and Sober, H.A. (1962) in *Methods Enzymol.* (Colowick, S.P. and Kaplan, N.O., eds), Vol. 5, pp. 3–27, Academic Press, New York
26 Ellis, D.B. and Sommar, K.M. (1971) *Biochim. Biophys. Acta* 230, 531–534
27 Winterburn, P.J. and Phelps, C.F. (1971) *Biochem. J.* 121, 711–720
28 Mazlen, R.G., Muellenberg, C.G. and O'Brien, P.J. (1969) *Biochim. Biophys. Acta* 171, 352–354
29 Trujillo, J.L., Horng, W.J. and Gan, J.C. (1971) *Biochim. Biophys. Acta* 252, 443–452
30 Winterburn, P.J. and Phelps, C.F. (1971) *Biochem. J.* 121, 721–730
31 Ellis, D.B. and Sommar, K.M. (1972) *Biochim. Biophys. Acta* 276, 105–112
32 Cantino, E.C. and Myers, R.B. (1972) *Arch. Mikrobiol.* 83, 203–215
33 Myers, R.B. and Cantino, E.C. (1972) *72nd Ann. Meet., Philadelphia, Pa. 23–28 April*, p. 40, Am. Soc Microbiol., N.W., Washington, D.C.