myo-Inositol Dehydrogenase(s) from *Acetomonas oxydans*

OPTIMIZATION OF CONDITIONS FOR SOLUBILIZATION OF MEMBRANE-BOUND ENZYME

By W. J. CRIDDLE, J. C. FRY and MAUREEN M. KEANEY
*Departments of Chemistry and Applied Biology, University of Wales Institute of
Science and Technology, Cardiff CF1 3NU, U.K.*

(Received 11 June 1973)

Methods are described for the first reported successful isolation of the soluble form of the membrane-bound *myo*-inositol dehydrogenase(s) from *Acetomonas oxydans*. Conditions for optimum yields of active enzyme in a crude membrane-free protein extract were established. The relevant conditions are (a) cell rupture by solid-shearing, (b) solubilization of the complete 60000g pellet with sodium deoxycholate at pH 7.2, (c) rapid separation of the released protein from sodium deoxycholate by gel chromatography.

Initial work carried out on the oxidation of *myo*-inositol by *Acetomonas oxydans* (Shimwell & Carr, 1959; syn. *Gluconobacter oxydans* and *Acetobacter suboxydans*) showed that the main product of the fermentation was *myo*-inosose-2 (Kluyver & Boezaardt, 1939; Posternak, 1941; Carter *et al.*, 1948). Later work (Franzl & Chargaff, 1951; Rapin *et al.*, 1967) centred on the attempts to obtain cell-free extracts. Results showed that the inositol dehydrogenase(s) was membrane-bound, but attempts at the isolation of soluble enzyme were unsuccessful (Franzl, 1952).

In this study we have successfully isolated soluble enzyme and we describe the optimized conditions used.

Experimental

Culture conditions

Throughout this work *A. oxydans* N.C.I.B. 621 was used and maintained by freeze-drying in mist, desiccans (Fry & Greaves, 1951). Working cultures were made by a single subculture on glucose-yeast-extract-agar (G.Y.E.A., N.C.I.B. records). The organism was grown in inositol-yeast-extract medium having the composition *myo*-inositol (50g), yeast extract (10g; Oxoid Ltd., London E.C.4, U.K.), D-sorbitol (1g) and water (1 litre). The medium was autoclaved at 103 kPa (15 lbf/in²) for 15 min, giving the complete medium with pH 6.1.

Cells were grown at 30°C for 84 h in a New Brunswick 14-litre Microferm fermentor containing the medium (8 litres) and added anti-foam (polyethylene glycol, 5 ml). Inocula were prepared from 72 h D-sorbitol-yeast-extract cultures having D-sorbitol (100g) and yeast extract (5g) in water (1 litre). Inocula of 60 ml were used.

Preparation of broken-cell suspensions

Cells were harvested by centrifugation at 5000g for 10 min in a Mistral 6L (MSE) and the pellet was washed three times and suspended in 0.1 M-sodium phosphate buffer (pH 7.2). Methods used for cell breakage were (a) ultrasonication for 10 min at 0°C in an MSE 60 W ultrasonicator under conditions of maximum cavitation; (b) solid-shearing, by using a cell concentrate in a X-press (Biotec Ltd.), three successive pressings being made at –25°C; (c) lysozyme-EDTA treatment of the pellet (1g wet wt.), with Tris buffer (0.1 M, pH 8.0, 3 ml), EDTA solution [(0.4% (w/v), pH 8.0, 3 ml) containing egg-white lysozyme [Sigma (London) Chemical Co. Ltd., London S.W.6, U.K.; 4.8 mg] and followed by deoxyribonuclease (Sigma; 1 mg) treatment. The mixture was stirred for 30 min at 20°C.

Fractionation of broken cells

The broken-cell suspension was spun at 60000g in an MSE Superspeed 50 ultracentrifuge for 45 min at 4°C.

Membrane-solubilization technique

The 60000g pellet was resuspended in 0.1 M-sodium phosphate buffer (pH 7.2) containing a range of concentrations of sodium deoxycholate (Sigma) for different periods and the mixtures were centrifuged at 60000g for 45 min. Separation of protein from sodium deoxycholate was obtained by using a Pye LCM Mark 2 liquid chromatograph fitted with a Sephadex G-10 column (1.5 cm × 25 cm) with water as eluent. The fractions were characterized by protein assay on the one hand and precipitation of deoxycholate from solution at pH 5.5 on the other. Attempts were also made to separate sodium deoxycholate from

protein by dialysis against sodium phosphate buffer at pH 7.2. Preliminary experiments were carried out by using simple deoxycholate solutions (0.15M, 10ml) to determine the time required for complete dialysis of deoxycholate into buffer (4 litres). Deoxycholate remaining in the bag was determined with the Pye LCM 2 detector. Separations were subsequently attempted on solubilized extract-deoxycholate mixtures, the activity being determined immediately after removal of deoxycholate.

Enzyme-assay techniques

The 2,6-dichlorophenol-indophenol-reduction procedure was used for the determination of inositol dehydrogenase activity, in which the reduction of dichlorophenol-indophenol (25 μ M, 0.9ml) in 0.1M-sodium phosphate buffer (pH 6.2, 1.8ml), cell extract (0.1ml), *myo*-inositol (0.1M, 0.2ml), contained in 1cm cuvettes, was followed by measuring the decrease in E_{600} of the reagent at 20°C. Control experiments used all the reagents except *myo*-inositol. One enzyme unit was defined as the amount of enzyme that reduced 1 μ mol of dichlorophenol-indophenol/min under the conditions described above.

Total protein was assayed by using the method of Lowry *et al.* (1951) with crystallized bovine serum albumin (BDH Chemicals Ltd., Poole, Dorset, U.K.) as the standard.

Results

Cell breakage

The use of lysozyme-EDTA (method c above) was completely unsuccessful as a method of cell breakage. A comparison of sonication and solid-shearing methods, both of which resulted in cell breakage, showed that the solid-shearing was significantly superior as a method of cell breakage, because of the consistently high activities in broken cells (approx. 80% of whole-cell activity). Sonication, on the other hand, produced very erratic results (20–95% of the whole-cell activity). Subsequent fractionation indicated that the enzyme was membrane-bound. The additions of Mg^{2+} or Ca^{2+} (0.14mM) or heat-killed cells (80°C for 30min; 10mg) to the enzyme-assay mixtures did not enhance enzyme activity at any stage of the fractionation procedure and were not subsequently used.

Membrane solubilization

The property of sodium deoxycholate solutions to form precipitates below pH 6.2 and gels between pH 6.2 and 7.0 in phosphate buffers prevented efficient membrane solubilization at these pH values. The membrane-bound enzyme had optimum activity at pH 6.2 and was most stable at pH 6.8 (Fig. 1).

However, Fig. 1 indicates that enzyme stability is adequate at pH 7.2 to allow the solubilization procedure to be carried out. Enzyme activity is significantly decreased at this pH but is regained at pH 6.2.

The effect of deoxycholate concentration on protein release and released enzyme activity is shown in Table 1. Although protein release from the 60000g pellet increases with increasing concentration of deoxycholate, enzyme activity is variable, showing maximum values at deoxycholate/total 60000g-pellet-protein ratios of about 1:2. It must be noted that enzyme stability and activity are much depressed by contact with deoxycholate, which should be

Fig. 1. Effect of pH on (a) activity and (b) stability of the whole 60000g pellet

The 60000g pellet was prepared as described in the text and was subsequently used as a suspension in water. The desired pH was obtained by the addition of 0.075M-citrate-phosphate buffer (Δ), 0.1M-sodium phosphate buffer ($\bullet$), or 0.025M-Tris-HCl buffer ($\blacktriangle$) to the suspended pellet. Enzyme activity was measured by the dichlorophenol-indophenol-reduction procedure. For stability determinations the pellet, suspended in the appropriate buffer, was stored for 3h at 4°C, after which 0.1ml samples were added to 1.8ml of 0.1M-sodium phosphate buffer, pH 6.2, and the enzyme activity was determined. In the typical sets of results shown 100% activity was (a) 274munits/mg of protein and (b) 183munits/mg of protein. The average coefficients of variation were (a) 15.7% and (b) 12.5%.

Table 1. *Effect of sodium deoxycholate concentration on the release of protein and inositol dehydrogenase from the 60000g pellet of Acetomonas oxydans*

The preparation of the 60000g pellet, the membrane-solubilization procedure and the separation of protein from sodium deoxycholate are described in the text. Released protein was measured in the 60000g supernatant after treatment with sodium deoxycholate for 15 min. Released enzyme activity was determined (by the dichlorophenol-indophenol-reduction procedure) in the protein fraction after separation from sodium deoxycholate. The enzyme activity in the whole pellet was: Expt. 1, 96 munits/mg of protein; Expt. 2, 112 munits/mg of protein.

Ratio of sodium deoxycholate to total pellet protein (w/w)	Expt. no.	Protein released from whole 60000g pellet (%)	Enzyme activity released from whole 60000g pellet (%)
1:5	1	32	42
	2	23	38
1:2	1	39	69
	2	28	57
1:1	1	37	43
	2	47	27

removed immediately by the chromatographic procedure described in the Experimental section once solubilization is terminated, as all enzyme activity is otherwise lost after 40 h. Significant amounts of deoxycholate (6% of the original) remain after dialysis for 70 h.

Similarly, Table 2 shows the effect of solubilization time on protein release and released enzyme activity at the optimum deoxycholate/pellet-protein ratio. The results indicate that the optimum solubilization time for release of enzyme is 15 min, although protein continues to be released for longer periods.

Discussion

In this study, the complete 60000g pellet resulting from solid-shearing was used in the solubilization procedure to optimize yields of soluble enzyme, rather than the upper layer, which consists of intact envelopes (Hunt *et al.*, 1959) and which is usually used for membrane studies.

The removal of deoxycholate after solubilization must be carried out expeditiously or substantial losses of activity occur. Dialysis as a method has been previously tried (Franzl, 1952) and repeated as described above, but although adequate as a separation technique, the times involved for the removal of deoxycholate were excessive, and resulted in total loss of enzyme activity. The gel-chromatographic separation was quick and efficient, the time involved being of the order of 30 min for the total separation of deoxycholate from protein.

Table 2. *Effect of length of treatment with sodium deoxycholate on the release of protein and inositol dehydrogenase from the 60000g pellet of Acetomonas oxydans*

The preparation of the 60000g pellet, the membrane-solubilization procedure and the separation of protein from sodium deoxycholate are described in the text. Released protein was measured in the 60000g supernatant after treatment with sodium deoxycholate at a ratio of 1:2 (w/v) with total pellet protein. Released enzyme activity was determined (by the dichlorophenol-indophenol-reduction procedure) in the protein fraction after its separation from sodium deoxycholate. The enzyme activity in the whole pellet was: Expt. 1, 189 munits/mg of protein; Expt. 2, 141 munits/mg of protein; Expt. 3, 96 munits/mg of protein; Expt. 4, 112 munits/mg of protein.

Treatment time (min)	Expt. no.	Protein released from the whole pellet (%)	Enzyme activity released from the whole pellet (%)
5	1	50	36
	2	—	27
	2	72	42
	3	39	69
15	4	28	57
	1	58	54
	2	78	56
	2	78	56

A comparison of the behaviour and characteristics of the crude enzyme mixture obtained in this work shows some differences from that reported by other workers. It has been reported that, while still membrane-bound, the enzyme(s) requires the addition of Mg^{2+} and Ca^{2+} ions (Rapin *et al.*, 1967) or heat-killed whole cells (Franzl & Chargaff, 1950) before high activity can be obtained. The results reported here, however, show that these additions are not a requirement of enzyme activity in either the membrane-bound or solubilized enzyme. A point of similarity with previously published work, however, exists in the pH at which maximum activity is shown. Values of pH 6.4 previously reported compare favourably with the pH 6.2 reported here.

We gratefully acknowledge financial support from the Science Research Council.

References

- Carter, H. E., Belinsky, C., Clark, R. K., Jr., Flynn, E. H., Lytle, B., McCasland, G. E. & Robbins, M. (1948) *J. Biol. Chem.* **174**, 415–426
- Franzl, R. E. (1952) Ph.D. Thesis, Columbia University, New York
- Franzl, R. E. & Chargaff, E. (1950) *Fed. Proc. Fed. Amer. Soc. Exp. Biol.* **9**, 173

- Franzl, R. E. & Chargaff, E. (1951) *Nature (London)* **168**, 955-957
- Fry, R. M. & Greaves, R. I. N. (1951) *J. Hyg.* **49**, 220-246
- Hunt, A. L., Rodgers, A. & Hughes, D. E. (1959) *Biochim. Biophys. Acta* **34**, 354-372
- Kluyver, A. J. & Boezaardt, A. G. J. (1939) *Rec. Trav. Chim. Pays Bas* **58**, 956-958
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265-275
- Posternak, T. (1941) *Helv. Chim. Acta* **24**, 1045-1048
- Rapin, A., Haenni, A. L. & Posternak, T. (1967) *Helv. Chim. Acta* **50**, 1801-1810
- Shimwell, J. L. & Carr, J. G. (1959) *Antonie van Leeuwenhoek; J. Microbiol. Serol.* **25**, 353-368