Use of Subzero Temperatures and Aqueous/Organic Solvent Systems to Increase the Stability of Labile Enzymes

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The loss of enzyme activity during the process of purification is a major obstacle when unstable enzymes are used. Douzou and co-workers introduced the idea of using chromatography at subzero temperature in aqueous/organic solvent systems (Balny et al., 1975).

In the present paper we describe the apparatus and a method of using an aqueous/organic solvent system to purify unstable enzymes at subzero temperatures. The nuclear enzyme poly(ADP-ribose) polymerase from pig thymus was used, which catalyses the sequential transfer of the ADP-ribose moiety of NAD+ to nuclear proteins (for reviews see Hilz & Stone (1976) and Hayaishi & Ueda (1977)). It is a very unstable enzyme, with a half-life of 15-20 h at 4°C (Brightwell et al., 1975).

A comparatively simple apparatus has been used that can achieve temperatures down to -25°C.

Experimental

A three-heated-glass-door low-temperature cabinet was used, model Verco THD 1F (Vertical Cabinet Co. Ltd.). It was thermostatically controlled between +5°C and -25°C (+3°C); lines were attached for cooling the jacketed columns. All columns, peristaltic pumps, fraction collectors and buffer containers were placed inside the cabinet.

The temperature inside the columns was further stabilized by the circulation of methanol/water (1:1, v/v). The liquid was circulated by a centrifugal pump (C25, Charles Austen Ltd.), connected to a low-temperature bath (LB-50, Grant Instr. Ltd.), thermostatically controlled from +5°C to -30°C (+1°C). The circulation rate was controlled by a ball valve (G400-20-27; Legris Ltd.), separate for each column. All lines were made by 'reinforced PVC' tubing, insulated outside the cold cabinet by Armaflex tubing (Armstrong Cork Co. Ltd.).

Two circular fraction collectors were used (Central Ignition Co.), the electrical and electronic systems placed outside the cabinet. Glass and Perspex jacketed columns were used (IEC, Whatman Labsales and ACJ Wright Scientific Ltd.); the flow rate was controlled by LKB 2120 Varioperspex II pumps.

In this work several antifreeze agents have been used; they were methanol, ethanol, glycerol, ethylene glycol, propylene glycol, dimethyl formamide and dimethyl sulphoxide. The solvent temperature was adjusted to 4°C and each solvent was added dropwise under mixing (Fink, 1976) to the aqueous 0.5m-salt nuclear extract of poly(ADP-ribose) polymerase in 20mM-Tris/HCl, pH 8.0, kept on ice, to give solvent concentrations of 0, 10, 20, 30, 40 and 50% (v/v). Each enzyme solution was separated in two parts; the first was left on ice, the second was placed at -20°C and both were left overnight. The samples were examined for protein precipitation and those with concentrations of over 20% (v/v) solvent were assayed for activity; where precipitation occurred the enzyme assay was preceded by centrifugation.

Stability studies to examine enzyme activity changes during storage and some kinetics were performed to examine the effect of ethylene glycol on the reaction rate. Poly(ADP-ribose) polymerase was isolated, purified and assayed as described by Tsopanakis et al. (1978). The purification was performed in ethylene glycol at -10°C, starting from nuclei isolation and enzyme extraction, followed by three chromatographic steps (Sepharose CL-6B, hydroxyapatite and CM-52).

Results and discussion

Ethylene glycol was the only organic solvent that did not precipitate proteins at concentrations of 40% or 50% (v/v) at 4°C overnight and did not affect the polymerase
activity. However, nuclear proteins seemed to be soluble in both 30\% (v/v) propanol and glycerol.

In Fig. 1(a) is shown a profile of the enzyme activity after storage with and without ethylene glycol at 4°C and -20°C. After 10 h at 4°C the activity of the polymerase aqueous salt extract was almost half that of the enzyme in 50\% (v/v) ethylene glycol extract, which remained unchanged for 10 h. After 36 h at -20°C the enzyme activity of aqueous extracts was lost completely; however, that containing ethylene glycol remained 100\% active for at least 15 days.

The effect of ethylene glycol concentrations on the reaction rate of the polymerase in nuclear salt extract is shown in Fig. 1(b), where enzyme and substrate concentrations were kept constant. The reciprocal of the reaction rate \([\text{munits/ml}^{-1}]\) was plotted against ethylene glycol concentrations (this plot should give a straight line for a normal

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**Fig. 1. Effect of ethylene glycol on stability (a) and activity (b) of poly(ADP-ribose) polymerase**

(a) The activity of poly(ADP-ribose) polymerase in 50\% (v/v) ethylene glycol was measured at different times after storage at 4°C or at -20°C. The enzyme activity in aqueous solution was also measured under the same conditions. The activity is expressed in munits/mg of protein (specific activity): \(\bullet\), at 4°C in ethylene glycol; \(\circ\), at -20°C in ethylene glycol; \(\triangle\), at 4°C in aqueous solution; \(\triangle\), at -20°C in aqueous solution. (b) The enzyme in salt extract of nuclei was assayed in the presence of ethylene glycol at concentrations of 10, 20, 30, 40 and 50\% (v/v) in the assay mixture (see under 'Experimental'); 50\(\mu\)l volumes of enzymes solution were used in the assay.

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Table 1. Summary of the results obtained by using aqueous or ethylene glycol solutions

<table>
<thead>
<tr>
<th>Method used</th>
<th>Temperature</th>
<th>Specific enzyme activity (munits/mg of protein) at last step</th>
<th>Purification factor</th>
<th>Total yield of enzyme activity from thymus (munits/g net weight of tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aqueous</td>
<td>4°C</td>
<td>250</td>
<td>2500</td>
<td>2</td>
</tr>
<tr>
<td>†50 % (v/v) ethylene glycol</td>
<td>4°C</td>
<td>1083</td>
<td>7220</td>
<td>6.5</td>
</tr>
<tr>
<td>‡50 % (v/v) ethylene glycol</td>
<td>-10°C</td>
<td>1880</td>
<td>9235</td>
<td>7.0</td>
</tr>
<tr>
<td>30 % (v/v) ethylene glycol</td>
<td>-10°C</td>
<td>822</td>
<td>7527</td>
<td>5.0</td>
</tr>
</tbody>
</table>

*, †, ‡ have been taken from our previously published work (Tsopanakis et al., 1976, 1977, 1978).
However, a curve was obtained, which can be described by a kinetic equation of second or higher order, with respect to ethylene glycol. Thus ethylene glycol seems to behave as an inhibitor of the polymerase and this parabolic behaviour can be explained if more than one molecule of glycol is binding on the same or other active sites of the enzyme.

Purification results are shown in Table 1, where methods in aqueous solutions and 50% (v/v) ethylene glycol at 4°C and -10°C and also 30% (v/v) ethylene glycol at -10°C are compared. The specific activity of the enzyme purified in 50% ethylene glycol is 4.4 times greater at 4°C and 7.5 times greater at -10°C than in aqueous solutions.

The yield of enzyme activity in glycol preparations increased approx. 3.5 times from the aqueous ones. It is noteworthy that the salt-extraction step gave a 30-fold purification and a total yield of about 500%, compared with a 23-fold purification and 147% yield in aqueous solutions (Tsopanakis et al., 1977, 1978). This may be attributed to the removal of an inhibitor during extraction or to loss of poly(ADP-ribose) glycohydrolase, which is inhibited by ethylene glycol (C. Tsopanakis & S. Shall, unpublished work). When a lower concentration of ethylene glycol (30%, v/v) was used throughout the procedure, lower overall yield and specific activities were obtained than with 50% ethylene glycol (Table 1).

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Electron-Transfer Mechanisms in Multihaem Cytochromes

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Cytochrome c₃ is a four-haem protein isolated from sulphate-reducing bacteria (Le Gall & Postgate, 1973). N.m.r. (Dobson et al., 1974; McDonald et al., 1974; Moura et al., 1977) and e.p.r. (DerVartanian, 1973; DerVartanian & Le Gall, 1974) have been shown to be powerful tools for obtaining structural information of multihaem cytochromes. The electron-exchange mechanism in multihaem cytochromes is a complex process due to the large number of redox centres present per molecule. There are two possible electron-exchange processes: (i) the intramolecular mechanism between the haems within the same molecule and (ii) the intermolecular mechanism between the haems of different protein molecules.

A convenient way of studying the electron-transfer mechanism in cytochrome c₃ is to follow the n.m.r. spectra at different contributions of the reduced (diagrammatic) and oxidized (paramagnetic) forms (McDonald et al., 1974). Analysis of the patterns obtained