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Short Communications

Separation of Isoenzymes of Acid Phosphatase from Needles of *Pinus silvestris* by Partition in Aqueous Polymer Two-Phase Systems.

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The partition of a protein in aqueous polymer two-phase systems is mainly dependent on its net charge and its relative solubility in the two phases. The effect of charge on the partition of a protein is enhanced by introducing into the phase system a polymer which carries a covalently-bound ionizable group.[1] One such polymer is the positively-charged trimethylamino-poly(ethylene glycol) (TMA–PEG). Phase systems containing this polymer have been used to separate the components of various isoenzyme systems.[2] If two components of an isoenzyme system show only small differences in partition behaviour, a multistage procedure such as counter-current distribution (CCD) is needed. On the other hand, if the difference in partition behaviour is large, only a few extractions are necessary for a complete separation.

This paper describes the separation of isoenzymes of acid phosphatase from a crude extract of pine needles by CCD. A two-step batch procedure, which separates the two main components of enzyme activity, electrophoretically distinct from each other, is also described.

Materials and methods. Dextran 500 (Mw 500 000) was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden) and poly(ethylene glycol), (PEG) (Mw 6000) was obtained from Union Carbide (New York, U.S.A). TMA–PEG was prepared from PEG as described by Johansson [3] and contained about 10 mequiv. trimethylamino groups per g polymer. Triton X-100 was obtained from KEBO (Stockholm, Sweden). Other chemicals used were of analytical grade quality. The water used was distilled in a quartz apparatus. The source of acid phosphatase was needles of *Pinus silvestris* taken from the same tree.

Enzyme assay. The phosphatase activity was measured essentially according to Sigma Technical Bulletin No. 104. Only relative activities have been calculated.

Pine needle extract. Fresh pine needles, 1.5 g, were chopped into 1 mm pieces and homogenized with an Ultra-Turrax (Junke & Kunkel, Stauven, Germany) for 30 s in 7.5 ml 750 mM TRIS-citrate buffer pH 5.4, 2 % (w/w) TMA–PEG, and 1 %(w/w) Triton X-100. The homogenate was then incubated at +4 °C for 3 h and centrifuged at 40 000 *g* for 30 min. The supernatant was dialysed at +4 °C overnight against a 20 mM Tris-citrate buffer pH 5.4 containing 0.15 %(w/w) Triton X-100. The dialysate, after removal of precipitated material, was used as pine needle extract.

Electrophoresis. Polyacrylamide gel electrophoresis was carried out in 6 × 100 mm glass tubes as described by Hjerten *et al.*[4]

The gel was composed of 0.375 %(w/v) *N,N'*-methylenbisacrylamide, 7.125 %(w/v) acrylamide (Cyanogum 41 from Merck, Darmstadt, Germany) and 100 mM TRIS, 250 mM boric acid, and 30 mM ethylenediamine tetraacetic acid, pH 7.2. The gels were selectively stained for acid phosphatase activity according to the method described by Shaw and Prasad.[5]

Counter-current distribution. The two phase system was composed of 6.6 %(w/w) dextran, 6.4 %(w/w) TMA–PEG, 0.15 %(w/w) Triton X-100 and 10 mM TRIS-citrate buffer pH 4.1. The CCD was carried out in an automatic thin-layer CCD apparatus described by Albertsson.[6] A plate with 60 cavities numbered 0–59 was used. The settling time was 8 min and the shaking time 40 s. After 58 transfers the content of each cavity was analysed for acid phosphatase activity.

Two-step extraction. The extraction procedure is described in Fig. 3. The upper phase of phase system 1 was extracted twice with fresh lower phase (Phase systems 3 and 5) and the lower phase of system 1 was extracted twice with fresh upper phase (Phase systems 2 and 4). The degree of partition was expressed as the partition ratio G:

$$G = \frac{\text{(Amount of enzyme activity in upper phase)}}{\text{(Amount of enzyme activity in lower phase)}}$$

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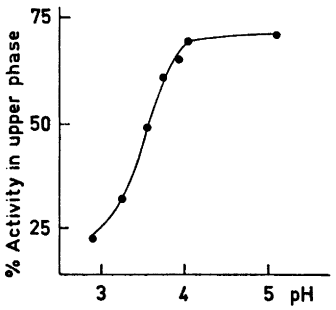

Fig. 1. Extraction of acid phosphatase from needles of *Pinus silvestris* into the upper phase of a phase system containing 6.6 % dextran, 6.4 % TMA–PEG. The pH was raised by titration with TRIS under continuous stirring.

Extraction profile. The extraction of acid phosphatase at different values of pH was performed as described previously.[7]

Gel filtration. The gel filtration was performed in a 1.5 × 70 cm column, filled with Sephadex G-200, (Pharmacia Fine Chemicals, Uppsala, Sweden). The elution buffer was 0.1 M TRIS-citrate, pH 7.5, and the flow rate was 7 ml h^{-1}.

Results and discussion. Acid phosphatase is mainly localized in the lysosomes of the cell. The enzyme was difficult to solubilize, probably due to the membranes surrounding the lysosomes. By addition of Triton X-100 and TMA–PEG to the homogenizing buffer, the extracted enzyme activity increased up to 100 times.

The extraction profile of acid phosphatase is shown in Fig. 1. It is seen that a plateau in enzyme activity is reached at about pH 4. At pH 5 only 70 % of the enzyme activity is found in the upper phase, while 30 % remains in the lower phase. This indicates that the enzyme is composed of at least two components. To investigate this heterogeneity of the enzyme, CCD was used.

Fig. 2 shows the counter-current distribution of acid phosphatase activity. Two peaks were obtained when the enzyme activity was plotted as a function of cavity number. The left peak of enzyme activity was broad and could be fitted to the sum of two theoretical peaks, as shown by the use of the computer program described elsewhere.[8] The material corresponding to each of the two experimental peaks of enzyme activity in Fig. 2 was subjected to gel filtration. No difference in elution volume could be detected. This means that the three fractions possess similar molecular weight. It is therefore probable that the three fractions of enzyme activity obtained by CCD reflect true acid phosphatase isoenzymes.

If the *G*-values of two components greatly differ they may be separated from each other by only two consecutive extractions. The extraction procedure used is shown in Fig. 3. The *G*-value of 12.4 for the isoenzyme component in the upper phase (system 5) shows that this isoenzyme has been highly purified from the component which prefers the lower phase. This latter component has a *G*-value of 0.13 (system 4).

Fig. 4 shows the electrophoretic pattern of acid phosphatase at different stages of the extraction procedure. The electrophoretically fast moving main component, which preferred the upper phase, was found to be homogeneous and in phase-system 5 distinctly separated from the electrophoretically slow-moving main component. This latter component, which preferred the lower phase, was heterogeneous and distinctly separated from the former compo-

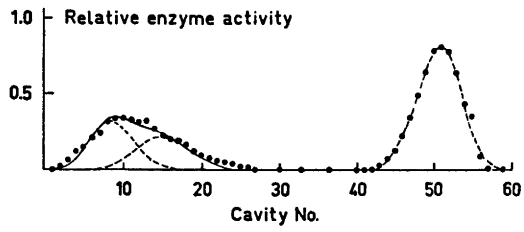

Fig. 2. Distribution of acid phosphatase activity after CCD in identical biphasic systems as used in Fig. 1. The experiment was performed at pH 4.1 and +4 °C. ●, experimental points; – – –, theoretical curves: ———, the sum of theoretical curves. *G*-values calculated according to Hecker[9] were found to be 10.5 and 0.24 for the right and the left main peaks of enzyme activity.

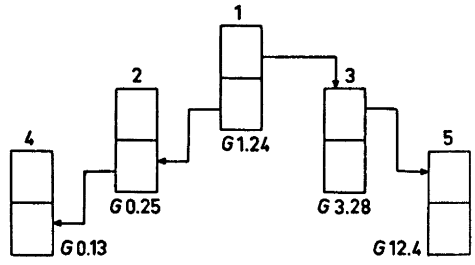

Fig. 3. Extraction of acid phosphatase from pine needles. The upper and lower phase of system 1 is extracted twice with fresh lower and upper phase, respectively. The same phase system as in Fig. 1 was used. The pH of the phase system was 4.0. The *G*-values were calculated for each system and are given in the figure.

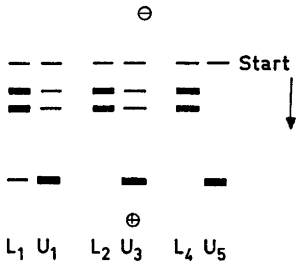

Fig. 4. The electrophoretic pattern of acid phosphatase after polyacrylamide gel electrophoresis. Samples were taken from the phase systems of Fig. 3. L and U correspond to lower and upper phase of the phase systems, respectively. The indices refer to the phase systems of Fig. 3.

nent in phase system 4. The results obtained by electrophoresis shown in Fig. 4, agree with the CCD pattern shown in Fig. 2.

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Substituent Dependent Mass Spectrometric Fragmentation of Monosubstituted Phenyl Propargyl Ethers

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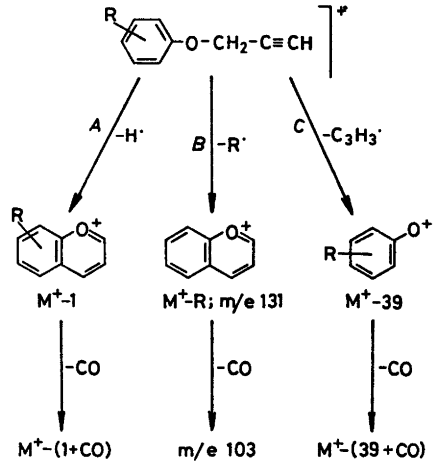
N-Propargylaniline moieties generated by electron impact fragmentation from several different types of molecular ions undergo an intramolecular cyclization to the corresponding quinoline ion.[1] It is reasonable to assume that phenyl propargyl ethers may rearrange in a similar manner giving rise to stable chromanonium ions (*cf.* Scheme 1, routes *A* and *B*). However, from a structural point of view, it is evident that this type of compounds should also be able to decompose by an ether cleavage at an activated benzylic or propargylic[2] site (route *C* in Scheme 1). We have studied a series of monosubstituted phenyl propargyl ethers (compounds *1–15*) and found that their fragmentation patterns follow the proposed routes as outlined in Scheme 1. The relative abundance of the peaks associated with these fragmentations are collected in Table 1. The spectra of compounds *11* and *13* exhibit prominent peaks corresponding to ions formed by fission of the aryl-oxygen bond, a behaviour that may be explained on the basis of an *ortho* effect,[3] which process is exemplified in Scheme 2. Compound *11* also fragments by an unusual route involving a



Scheme 1.