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ACID PHOSPHATASE FROM NEEDLES OF *PINUS SILVESTRIS* L

PURIFICATION OF TWO INTERCONVERTIBLE ENZYME FORMS AND CHARACTERIZATION OF A LOW-MOLECULAR WEIGHT FACTOR ASSOCIATED WITH THE ENZYME

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Two main forms of acid phosphatase (orthophosphoric-monoester phosphohydrolase (acid optimum), EC 3 1 3 2) have been isolated from needles of *Pinus silvestris* L The isoelectric points determined by isoelectrofocusing are 3 6 and 9 4 The molecular weights of both forms were estimated as 68 000 The two forms have similar kinetic properties Chromatography of the acidic enzyme form on DEAE-cellulose results in conversion to the basic form Interconversion in the reverse direction was demonstrated to occur on incubation of the basic enzyme form with a pine-needle homogenate or with a low-molecular weight fraction thereof These findings suggest that the interconvertibility between the main enzyme forms is a result of an association of the basic form with a low-molecular weight factor carrying multiple negative charges This factor has been purified and tentatively identified as an oligoribonucleotide or a fragment of RNA The implications of these findings for the application of acid phosphatase isoenzymes as genetic markers in forest trees are discussed

Introduction

During the last decade several workers have studied the variation of electrophoretic isozyme patterns in crude tissue extracts from forest trees An excellent compilation of papers concerning this subject is found in the dissertation of Rudin [1] The purpose of these investigations is to achieve a better understanding of fundamental genetic relationships in forest trees, based on the idea that isoenzymes offer information close to the genome [1] The technique is still developing and the full impact on forest genetics and breeding is yet to be seen According to Allard et al [2], the main benefits of the isoenzyme method are that a large number of gene characters are available for study, that individual alleles at each locus are revealed, and that the method is applicable to a wide group of plant and animal species In addition, the possibility of obtaining reliable results from very young plants, or even seeds, is extremely valuable in forest genetics, where the problem of long generation cycles is predominant The macrogametophyte tissue of gymnosperms is haploid, and this makes it possible to study frequencies of single alleles without interference of both parental genotypes Finally, there is no doubt of the economical value of a well functioning genetic tool that might be developed as a result of such investigations

The isoenzyme method rests upon the assumption that the observed isoenzyme patterns are due to genetically determined variations in electrophoretic mobility However, secondary modifications of the enzyme might give rise to additional variations Therefore it is important to characterize the molecular properties of the isoenzymes that are to serve as genetic markers

In a previous paper [3] it was demonstrated that at least two forms of acid phosphatase (orthophosphoric-monoester phosphohydrolase (acid optimum),

EC 3 1 3 2) are present in homogenates of needles from *Pinus silvestris* In the present paper it is shown that two main forms of the enzyme are mutually interconvertible, an acidic form probably being a complex between a basic form and an oligoribonucleotide factor

Materials and Methods

Enzyme assay The acid phosphatase activity was determined essentially according to Sigma Technical Bulletin No 104 The sample (5–100 μl) was added to 500 μl 100 mM sodium citrate buffer, pH 4 8, room temperature The reaction was initiated by the addition of 500 μl 15 mM *p*-nitrophenyl phosphate in 100 mM sodium citrate buffer, pH 4 8, and stopped by the addition of 2 ml 0 2 M NaOH The amount of product formed was estimated spectrophotometrically at 400 nm using $\epsilon = 18\ 5$ $cm^2\ \mu mol^{-1}$ for *p*-nitrophenolate ion

Protein concentrations Nitrogen concentrations were determined by chemiluminescence after pyrolysis according to the method of Drushel [4] using a Pyro Chemiluminescense digital nitrogen analyser model 703 B (Antek) The samples were injected at a rate of 2 μl/s Protein concentrations were calculated assuming a nitrogen content of 16 0%

Analytical isoelectrofocusing Analytical isoelectrofocusing was performed in precasted polyacrylamide gels, PAG-plates, pH range 3 5–9 5 (LKB) A maximum of 1 200 V, 30 W was applied for 90 min The pH was measured with a surface glass electrode (M2145, Ingold) Protein was stained with Coomassie brillant blue R-250 according to Vesterberg [5] Staining for acid phosphatase activity was performed essentially according to Shaw and Prasard [6] using α-naphthyl phosphate as substrate and Fast Garnet GBC salt as diazo coupler in 0 1 M sodium citrate buffer, pH 4 8/1 mM $MgCl_2$

Partition in aqueous polymer two-phase systems A two-phase system with a final weight of 2 g, including sample, contained 6 6% (w/v) Dextran T 500 (Pharmacia Fine Chemicals), 6 4% (w/v) trimethylaminopoly(ethylene glycol), M_n 6000, prepared according to Johansson [7], and 10 mM sodium citrate buffer, pH 4 3 The two-phase system was thoroughly shaken and finally centrifuged to speed up phase separation Samples were withdrawn from the upper and the lower phases The enzyme activity and the volumes of the phases were determined, and the partition coefficients, K, were calculated according to Albertsson [8]

In this two-phase system the upper phase is enriched in the positively charged polymer, whereas the lower phase is Dextran-rich Negatively charged protein molecules accumulate in the upper phase ($K > 1$), while positively charged protein molecules accumulate in the lower phase ($K < 1$)

Enzyme purification Step 1 400 g needles were homogenized for 2 min with an Ultra-Turrax T-45 (Junke and Kunkel) in 2 0 l 750 mM sodium citrate buffer, pH 5 4/2% (w/v) poly(ethylene glycol) (M_n 6 000)/1% (w/v) Triton X-100 During homogenization the mixture was cooled by the addition of solid CO_2 The homogenate was stirred at 4°C for 1 h, sirved through two layers of cheesecloth, and centrifuged at 20 000 × *g* for 30 min

Step 2 Ice-cold acetone was added to the crude extract obtained in Step 1 to a final concentration of 30% The mixture was then centrifuged at 15 000 × *g* for 10 min

Step 3 Solid $(NH_4)_2SO_4$ was added to the supernatant from step 2 to a final concentration of 30% (w/v) Due to the presence of poly(ethylene glycol) and Triton X-100 this amount of $(NH_4)_2SO_4$ sufficed to saturate the solution Equilibration was accelerated by sonication for 3 min The mixture was centrifuged at 3 000 × *g* for 30 min resulting in a two-phase system The enzyme activity was present in the essentially pigment-free interphase precipitate, which was collected in 80 ml 0 1 M sodium citrate buffer, pH 4 8, and sonicated for 90 s Centrifugation at 20 000 × *g* followed for 20 min

Step 4A The supernatant from Step 3 was passed through a column of Sephacryl S-200 Superfine (Pharmacia Fine Chemicals) which had been equilibrated with 0 1 M sodium citrate buffer, pH 4 8, (column dimensions, 50 × 900 mm, flow rate, 5 ml min^{-1}, 4°C)

Step 5A Fractions containing enzyme activity were pooled and desalted on Sephadex G-25 Fine (Pharmacia Fine Chemicals, column dimensions 50 × 500 mm, flow rate 5 ml min^{-1}, 20 ml/fraction, 4°C) The column was equilibrated with 10 mM sodium citrate buffer, pH 4 8 Fractions containing

activity were pooled and subjected to preparative isoelectrofocusing at 10°C using a 110 ml isoelectrofocusing column (LKB, power 5 W, focusing time 12 h) in a sucrose-stabilized (5–90%, w/v) pH-gradient (3 0–10 5) Ampholines were removed by repeated washing with 0 1 M sodium citrate buffer, pH 4 8, in an ultrafiltration cell using a Pelicon PTGC 0905 filter (Millipore) with a cut-off limit of 10000 daltons

Step 4B After the discovery of the interconvertibility of the two enzyme forms found in Step 5A (see Results) an alternative procedure for the purification was developed The supernatant from Step 3 was equilibrated with 10 mM Tris-HCl, pH 9 0, by ultrafiltration Thus, the sample was diluted 10-times with this buffer and concentrated to its initial volume This procedure was repeated once

Step 5B The filtrate from Step 4B was chromatographed on a DEAE-cellulose column equilibrated with the same buffer (DE-22, Whatman, column dimensions, 25 X 300 mm, flow rate, 2 ml min^{-1}, fraction volume, 20 ml, 4°C) Fractions containing activity were pooled and concentrated by ultrafiltration

Step 6B The concentrated material from Step 5B was filtered through a Sephadex G-150 Superfine column using 100 mM sodium citrate buffer, pH 4 8, (column dimensions, 25 X 700 mm, flow rate 0 5 ml min^{-1}, fraction volume, 5 ml, applied sample volume, 10 ml, 4°C)

Molecular weight determinations Molecular weights were determined by gel filtration on Ultrogel AcA-44 (LKB) using a 100 mM sodium citrate buffer, pH 4 8, (column dimensions, 15 X 700 mm, flow rate, 0 2 ml min^{-1}, fraction volume 1 ml, applied sample volume, 1 ml)

The molecular weights were also estimated by polyacrylamide disc-gel electrophoresis in the presence of sodium dodecyl sulphate (SDS) according to Laemmli [9] Protein bands were stained by Coomassie brillant blue R-250 according to Blakesley and Boezy [10]

Assay of factor interacting with basic enzyme form Purified basic form (15 nkat) of acid phosphatase was incubated for 2 min with sample aliquots to a final volume of 100 μl The buffer was 10 mM sodium citrate, pH 4 3 Under these conditions the factor can bind to the basic enzyme form, yielding the acidic form (see Results) To estimate the extent of formation of the acidic enzyme form, the incubated material was introduced into the aqueous polymer two-phase system described above

Measurement of radioactivity The Cerenkov emission from ^{32}P was measured in the tritium channel in a PRIAS liquid scintillation counter (Packard Instruments) A standard fraction volume of 0 5 ml was used [11]

Purification of low-molecular weight factor Pine cotyledons were raised from seeds on moist filter paper in a humid chamber for 12 days They were then transferred to water culture in a synthetic nutrient solution for 38 days essentially according to Siegenthaler and Depéry [12] (100 μE of light for 14 h/24 h, 23°C) Finally they were allowed to grow for 10 days in a medium containing 250 μCi [^{32}P]-orthophosphate (New England Nuclear) and 10 mmol NH_4SO_4/l, approx pH 7 A crude extract was prepared from 5 g of the green parts of the plants according to Jonsson and Blomquist [3] The extract was subjected to gel filtration on Sephadex G-25 Fine (Pharmacia Fine Chemicals) Column dimensions were 25 X 600 mm and fractions of 16 ml were collected The buffer was 0 1 M sodium citrate, pH 4 8 Fractions containing the factor were pooled and filtered through a column of Sephadex G-15 under the same conditions Fractions from this run containing the factor were pooled and concentrated on a Pelicon PSAC 0905 ultrafilter (Millipore) with a cut-off limit of 1 000 daltons, according to the manufacturer's specifications In the further purification the specific binding of the factor to the basic enzyme form was utilized (see Results) Thus, 3 μkat of purified basic enzyme form was incubated with 500 μl crude factor for 2 min at room temperature The complex formed was separated from excess factor and low-molecular weight impurities on Sephadex G-25 Fine, (column dimensions, 15 X 45 mm, buffer, 10 mM Tris-HCl, pH 9 0) Fractions of 0 5 ml were collected The isolated complex was dissociated by chromatography on DEAE-cellulose, DE-22 (Whatman) By this procedure the basic enzyme form is eluted with the starting buffer (10 mM Tris-HCl, pH 9 0) The factor was recovered after stepwise elution with NaCl in starting buffer All column operations were performed in the cold room (4°C)

Chemicals Analytical grade chemicals were used

Deionized water was further purified in a Milli-Q system (Millipore) *t*-RNA and *r*-RNA prepared from *Escherichia coli* were kindly supplied by Dr Glenn Bjork, Department of Microbiology, University of Umeå, Umeå, Sweden RNA III prepared from baker's yeast, RNA IV from calf liver, DNA from salmon testis, DNA type VIII from *E coli* and RNAase I-A from bovine pancreas (EC 3 1 4 22) were purchased from Sigma Chemical Company DNAase II from porcine spleen (EC 3 1 4 6) was obtained from Worthington, and poly(dA) poly(dT) from Boehringer, Mannheim

Results

Purification of two main enzyme forms

The purification procedure involved Steps 1 to 3, 4A and 5A as described in Materials and Methods A summary of the yields of the enzyme activity is given in Table I

Analytical isoelectrofocusing of the crude extract indicated the presence of several active forms of the enzyme with isoelectric points ranging from about 3 0 to 9 5 (Fig 1) Protein staining revealed protein bands covering the whole pH range (data not shown)

The main effects of the combined acetone and $(NH_4)_2SO_4$ precipitations were volume reduction and removal of resins and pigments, since these steps did not significantly reduce the the number of protein bands Gel filtration on Sephacryl S-200, however, resulted in a substantial purification as shown in Fig 2 Thus, the enzyme activity was found in one peak which was well separated from the two major peaks absorbing at 280 nm The enzymically-active fractions still contained several proteins as determined by analytical isoelectrofocusing (Fig 1) Preparative isoelectrofocusing of this active material resulted in two main fractions with activity maxima at pH 3 6 and 9 4, respectively (Fig 3) This step resulted in a considerable loss of enzyme activity, presumably because the enzyme is sensitive to extreme pH values A short focusing time of 12 h was chosen to give a reasonable resolution in combination with a reasonable recovery of activity

TABLE I

PURIFICATION OF TWO FORMS OF ACID PHOSPHATASE FROM NEEDLES OF *PINUS SILVESTRIS*

Purification step		Total volume (ml)	Total activity (μkat)	Yield (%)
1	Homogenate	1550	217	100
2	Acetone precipitation	2080	176	81
3	$(NH_4)_2SO_4$ precipitation	95	117	54
4A	Sephacryl S-200	75	76	35
5A	Electrofocusing			
	basic form	10	4 3	2
	acidic form	10	6 5	3

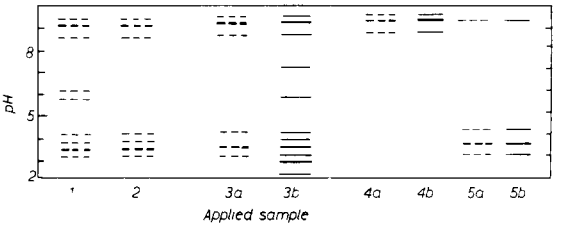


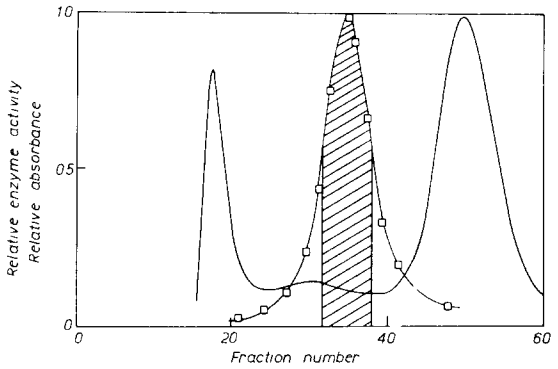
Fig 1 Analytical isoelectrofocusing of various fractions obtained during the purification of acid phosphatase from pine needles Fully drawn lines represent protein-stained bands, while dashed lines represent activity-stained bands The applied samples were 1, homogenate (Step 1), 2, $(NH_4)_2SO_4$ precipitate (Step 3), 3, pooled, active fractions from Sephacryl S-200 chromatography (Step 4A), 4, basic enzyme form obtained by preparative isoelectrofocusing (Step 5A), 5, acidic enzyme form obtained in Step 5A The basic form obtained by the modified preparation method gave a pattern identical to that shown for sample 4

The results of the analytical isoelectrofocusing of the two purified main enzyme forms are shown in Fig 1 The basic fraction gave one major band and two minor bands with isoelectric points ranging from pH 8 5 to 9 5 The acidic fraction gave a similar pattern but in the pH range of 3 0–4 2

In addition, the acidic fraction gave rise to a weak band in the same position as the main band of the basic enzyme form This result was somewhat surprising and gave the first indication that the acidic form might be converted to the basic form

Interconversion of the two main enzyme forms

When the purified acidic form was chromatographed on a DEAE-cellulose column most of the enzyme activity passed through the column without



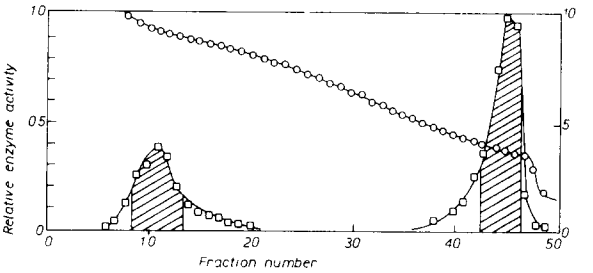


Fig 2 Preparative gel filtration on Sephacryl S-200 ———, A_{280}, □———□, enzyme activity These parameters are expressed relative to their maximal values The shaded area is pooled fractions

Fig 3 Preparative isoelectrofocusing □———□, enzyme activity expressed relative to the maximal value, ○———○, pH, the shaded area is pooled fractions

being adsorbed Analysis of the eluted material by analytical isoelectrofocusing showed that the enzyme had been converted to the basic form As a routine method for the identification of the main enzyme forms, the aqueous polymer two-phase system described in Materials and Methods was employed Table II shows the partition of the enzyme forms in this two-phase system before and after DEAE-cellulose chromatography The acidic form is negatively charged at pH 4 3 and will, thus, partition to the upper phase, whereas the basic enzyme form partitions to the lower phase

The data given in Table II also show that a mixture of enzyme forms, as present in the crude extract, is converted to the basic form on the DEAE-cellulose column A control experiment shows that the purified basic form goes unchanged through the column About 40% of the enzyme activity is lost during DEAE-cellulose chromatography regardless of the isoenzyme composition of the applied material

These results indicate that the acidic form is a complex between the basic form and some negatively-charged factor To test this hypothesis, the basic form was incubated with aliquots of crude extract, or with aliquots of a low-molecular weight fraction obtained by gel filtration of the crude extract on Sephadex G-25 In both cases, incubation resulted in a change of the partition coefficient for the enzyme activity in the two-phase system to a value similar to that obtained with the acidic form The change in partition coefficient occurred over a narrow concentration range (cf Fig 9)

TABLE II

DEAE-CELLULOSE CHROMATOGRAPHY OF VARIOUS ACID PHOSPHATASE SAMPLES

K is the partition coefficient of enzyme activity in the two-phase system described in Materials and Methods

Sample	Total activity (nkat)		Yield (%)	*K*	
	Applied	Eluted		Applied material	Eluted material
Homogenate	143	83	58	2 3	0 1
Acidic form	171	104	61	49	0 2
Basic form	79	47	60	0 1	0 1

Modified purification procedure

This procedure is based on the observation that the basic enzyme form is eluted from the DEAE-cellulose column regardless of the isoenzyme composition of the applied sample Thus, after a buffer change (Step 4B) the material from Step 3 was chromatographed on DEAE-cellulose (Step 5B) followed by gel filtration on Sephadex G-150 (Step 6B) The basic enzyme form obtained by this procedure was indistinguishable from that obtained by the method employed previously (Fig 1) The results, summarized in Table III, show that the modified procedure gives an improved yield of purified enzyme The overall purification of the enzyme was approx 2 800-fold

Molecular weight

Gel filtration of the native enzyme forms indicated that both of them have molecular weights of about 68 000 SDS-polyacrylamide disc-gel electrophoresis in the presence of mercaptoethanol always yields a major component with a molecular weight of 68 000, but two components with lower molecular weights (between 22 000 and 24 000) have occasionally been found for both forms However, it cannot be decided if these low-molecular weight components are genuine subunits, or if they are the result of a limited proteolytic digestion

Kinetic properties

The pH dependencies of the *p*-nitrophenyl phosphate hydrolase activities of the two purified enzyme forms are shown in Fig 4 The basic form has an optimum at pH 4 7 The pH-rate profile of the acidic

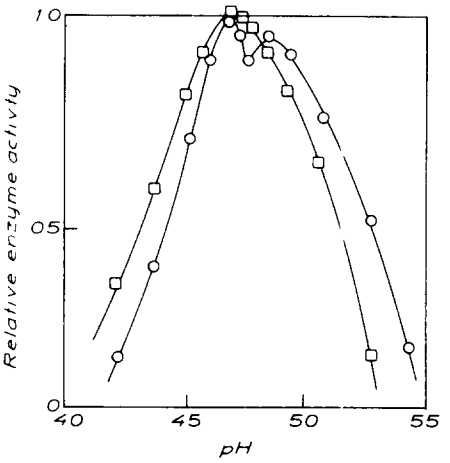


Fig 4 pH-rate profiles of basic and acidic forms of acid phosphatase The assay solution contained 15 mM *p*-nitrophenyl phosphate/100 mM sodium citrate buffer/1 mM $MgCl_2$ Room temperature □——□, basic form, ○——○, acidic form

form appears to be slightly shifted to higher pH values compared to that of the basic form Maxima at pH 4 7 and 4 85 have consistently been observed

The two enzyme forms have practically identical K_m values for *p*-nitrophenyl phosphate, 1 6 mM at pH 4 8

Preliminary results indicate that the two forms have similar substrate specificities as well as similar specificities with respect to various inhibitors

Purification and characterization of low-molecular weight factor

The fractionation on Sephadex G-25 of crude extract from pine needles grown in the presence of

TABLE III

MODIFIED PURIFICATION PROCEDURE

Purification step		Total volume (ml)	Total activity (μkat)	Protein conc μg/ml	Specific activity (kat/kg)	Yield (%)
1	Homogenate	1560	244	6000	0 026	100
2	Acetone precipitation	2100	214	–	–	88
3	$(NH_4)_2SO_4$ precipitation	45	154	–	–	63
4B	Ultrafiltration dialysis/concentration	100	78 0	–	–	32
5B	DEAE-cellulose	140	34	–	–	14
6B	Sephadex G-150	10	18 5	39	73	12

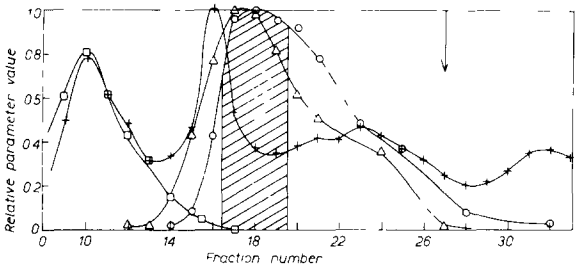


Fig 5 Fractionation of crude extract from pine needles on Sephadex G-25 +———+, absorbance at 280 nm, ○———○, partition coefficient obtained in the assay of the factor, △———△, ^{32}P radioactivity (cpm), □———□, enzyme activity The shaded area represents pooled fractions All these parameters are expressed relative to their maximal values The arrow indicates the peak position of NaCl as determined in a separate run on the same column Further experimental details are given in Materials and Methods

[^{32}P]phosphate is illustrated in Fig 5 The acid phosphatase activity was recovered in the void volume The factor was retarded on the column and showed a maximum in fractions 17–19 Most of the ^{32}P was found in the same fractions

These fractions were pooled and chromatographed on Sephadex G-15 The factor was recovered in the void volume The so prepared, crude factor was incubated with the purified basic enzyme form as described previously Fig 6 shows the elution pattern obtained upon filtration of the mixture on Sephadex G-25 A significant amount of radioactivity was associated with the enzyme fraction The labelled enzyme

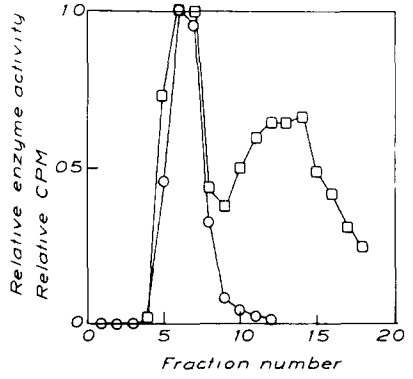


Fig 6 Gel filtration on Sephadex G-25 of basic enzyme form incubated with crude factor ○———○, enzyme activity, □———□, ^{32}P radioactivity (cpm) Parameters expressed relative to their maximal values

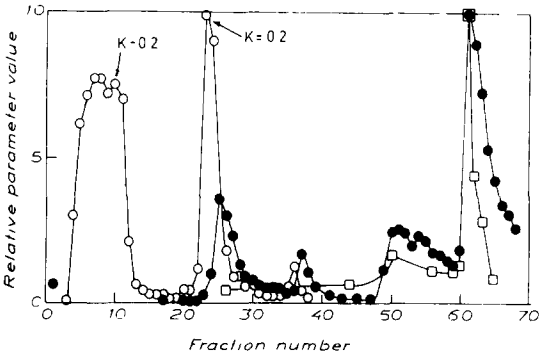


Fig 7 DEAE-cellulose chromatography of acid phosphatase containing ^{32}P-labelled factor ○———○, enzyme activity, ●———●, ^{32}P radioactivity (cpm), □———□, partition coefficient obtained in the assay of the factor The parameters are expressed relative to their maximal values Arrows indicate fractions that were partitioned in the two-phase system The resulting partition coefficients (K 0 2) indicate that these fractions contained basic enzyme form The column was eluted with 10 mM Tris-HCl, pH 9 0 From fraction 20, the eluting buffer contained also 100 mM NaCl, from fraction 35, 200 mM NaCl, from fraction 45, 300 mM NaCl, from fraction 58, 400 mM NaCl

had a partition coefficient of 29 in the two-phase system This is a typical value for the acidic enzyme form

Ion-exchange chromatography of the labelled acidic enzyme form is illustrated in Fig 7 The enzyme activity is eluted as the basic form as indicated by the partition coefficient of 0 2 The major portions of the factor and the radioactivity were

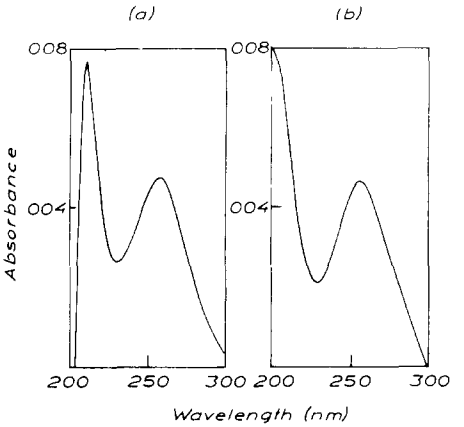


Fig 8 Ultraviolet absorption spectra of (a) purified factor and (b) baker's yeast RNA III

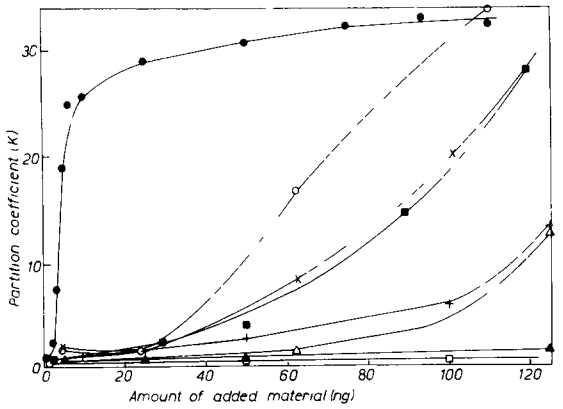


Fig 9 Interaction of purified factor and various nucleic acids with basic form of acid phosphatase (15 nkat) The assay method described in Materials and Method was employed The results are shown as the partition coefficients of enzyme activity, K, obtained after partitioning the incubation mixture in the aqueous polymer two-phase system ●———●, purified factor, □———□, bakers yeast RNA III, △———△, calf liver RNA IV, +———+, *E coli r*-RNA, ■———■, *E coli t*-RNA, ▲———▲, salmon testis DNA, X———X, *E coli* DNA, ○———○, poly (dA) poly (dT)

TABLE IV

EFFECTS OF NUCLEASES ON THE INTERACTION BETWEEN ACID PHOSPHATASE AND THE FACTOR OR VARIOUS NUCLEIC ACIDS

The materials indicated in the table were tested for their ability to convert the basic form of acid phosphatase to an acidic form using the assay described in Materials and Methods Nuclease digestion was performed before incubation with basic enzyme form K is the partition coefficient in the two-phase system employed in the assay The basic enzyme form yields $K \approx 0\,1$, whereas the acidic form yields $K \approx 30$

Material	Digestion time (min)	K
No addition	–	0 1
50 ng factor	–	30
50 ng factor + 1 μg RNAase	2	0 9
50 ng factor + 1 μg RNAase	4	0 7
50 ng factor + 1 μg DNAase	2	19
100 ng poly (dA) poly (dT)	–	29
100 ng poly (dA) poly (dT) + 1 μg DNAase	2	0 1
150 ng *E coli* DNA + 1 μg DNAase	2	0 3
200 ng *r*-RNA	–	21
200 ng *r*-RNA + 1 μg RNAase	2	0 5
200 ng *r*-RNA + 1 μg DNAase	2	18
1 μg RNAase	–	0 1
1 μg DNAase	–	0 1

eluted with 400 mM NaCl Fractions 61 and 62 were pooled and this material was used in further studies of the factor

An ultraviolet absorption spectrum of the purified factor is shown in Fig 8a For comparison the spectrum of RNA III from yeast is shown in Fig 8b The yield of the factor was estimated spectrophotometrically at 257 nm, as 5 μg assuming that the factor has the same specific absorbance as RNA III

The ability of various polynucleotides to complex with the basic enzyme form was tested with the same assay as used for the factor The results presented in Fig 9 suggest that most of the investigated substances can bind to the enzyme However, on a weight basis all of these substances are much less effective than the purified factor

The data presented in Table IV show that the factor is sensitive to RNAase while DNAase has little effect on the ability of the factor to bind to the basic enzyme form DNA loses its binding ability after degradation with DNAase

Discussion

The results presented in this paper show that there are two main forms of acid phosphatase in homogenates of pine needles These forms have widely different isoelectric points, but their enzymatic properties are quite similar The two forms are interconvertible, and the results suggest that the acidic enzyme form (p*I* 3 6) is a complex between the basic form (p*I* 9 4) and some unknown factor Apparently, this complex is stable enough to remain essentially undissociated during isoelectrofocusing (cf Fig 1) However, the complex is completely dissociated by DEAE-cellulose which seems to bind the factor with a high affinity

The striking 6 pH-unit difference in the isoelectric points of the basic and acidic enzyme forms is strong evidence that the factor carries multiple negative charges even at relatively low pH values

The results allow some conclusions to be made concerning the chemical nature of the factor Firstly, the fact that ^{32}P-label is associated with the factor throughout the purification procedure strongly suggests that the factor contains orthophosphate groups Secondly, the ultraviolet absorption spectrum of the purified factor indicates that the factor is a nucleo-

tide derivative Thirdly, the observations that the effect of the factor is abolished by RNAase but not by DNAase are evidence that the factor is an oligoribonucleotide or a fragment of RNA

The data allow only a crude estimate of the molecular size of the factor Thus, the factor is retained on a filter with the cut-off limit of 1 000 daltons but passes through a filter with the cut-off limit of 10 000 daltons The factor is retarded on Sephadex G-25 but appears in the void volume of Sephadex G-15 The fractionation range of G-25 is 100–5 000 daltons for Dextrans, while G-15 excludes Dextrans larger than 1 500 daltons according to the manufacturer's specifications Therefore, one might tentatively conclude that the factor has a molecular weight of 1 500–3 000 and is composed of 5–10 ribonucleotide residues

The question remains whether the purified factor is a unique oligoribonucleotide or a mixture of chemical species Thus, it was shown that various nucleic acids can interact with the basic enzyme form to yield complexes behaving like the acidic enzyme form in the two-phase system employed (Fig 9) However, the isolated factor is much more effective on a weight basis than the tested nucleic acids There is one additional piece of evidence indicating that the factor might be a well-defined molecule or, at least, an oligonucleotide of well-defined size The basic enzyme form gives rise to three distinct bands on isoelectric focusing, and the acidic form yields a similar pattern (Fig 1) If the purified factor contained a mixture of binding species, one might expect that isoelectric focusing would yield a broad, diffuse zone rather than the distinct bands actually observed

Although it is obvious that the factor is present in homogenates from pine needles, the question remains whether it is associated with the enzyme in vivo or an artifact of the homogenization procedure Unfortunately, it is practically impossible to extract enzymes by mild methods from pine needles because of the mechanical properties of this material However, it is possible to prepare a chloroplast-enriched fraction [13] from pine cotyledons, and preliminary experiments show that this fraction contains almost exclusively the acidic form of acid phosphatase It is tempting to speculate that the acidic form of acid phosphatase is present in the cell, and that the oligonucleotide has a biological role, perhaps in regulating the localization of the enzyme or its interaction with membranes

Clearly, the presence of the interconvertible forms of acid phosphatase in tissue extracts from *P silvestris* is an undesirable complication in studies of genetic variants of this enzyme One could perhaps avoid these complications by treating the extract with DEAE-cellulose to remove the negatively-charged factor prior to the electrophoretic isoenzyme analysis However, relevant genetic information might be obtained even without such treatment, since the distinct bands observed on isoelectrofocusing of the purified enzyme forms might represent true genetic variants of the enzyme

Rudin and Ekberg [14] included acid phosphatase in an extensive genetic study of isoenzymes in the haploid macrogametophyte tissue of *P silvestris* seeds They interpreted their zymograms in terms of two loci for acid phosphatase designated PHOS-MA and PHOS-MB The PHOS-MA locus showed a variation which could be attributed to the presence of three alleles in the investigated population However, each one of these alleles gave rise to two electrophoretic bands of acid phosphatase activity Since all the enzyme variants from the PHOS-MA locus migrated towards the anode (pH 7 2), they are probably complexed with the negatively-charged factor The double-band pattern is difficult to explain, but one can speculate that extracts of macrogametophyte tissue contain two differently charged forms of the oligonucleotide factor or that the factor partly dissociates during the electrophoretic run

The PHOS-MB locus was observed either as a relatively weak band migrating slowly towards the anode or as a null allele, i e , the absence of a band It is possible, of course, that the macrogametophyte contain an acid phosphatase that is not synthesized in needles, but it seems more likely that the postulated PHOS-MB locus represents the basic main form of the enzyme In the present study, variants of this enzyme form were found to have isoelectric points above pH 8 5 (Fig 1) Consequently, they would be expected to migrate towards the cathode and out of the gel under the conditions used by Rudin and Ekberg Conceivably, however, a genetic variant of the basic enzyme form having a lower isoelectric point could have been present in the material studied by these

authors Another possibility is that the basic enzyme form binds negatively-charged components of the buffer used in the gel electrophoresis experiments, for example, borate ions or EDTA, thus explaining the occasional appearance of a band attributed to the PHOS-MB locus by Rudin and Ekberg In this connection it is interesting to note that the postulated alleles did not occur with equal frequency in the single tree reported to have a heterozygous PHOS-MB locus [14]

In conclusion, the results of the present study amply illustrate the importance of a biochemical characterization of enzyme systems that are to be used in genetic isoenzyme studies This may be laborious but it seems to be the best way to eliminate conclusively variations that are genetically irrelevant

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