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# PROPERTIES OF TWO APYRASES FROM *SOLANUM TUBEROSUM*

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**Key Word Index**—*Solanum tuberosum*; Solanaceae; potato; enzymic hydrolysis of pyrophosphate bonds; apyrase; pyrophosphohydrolase; plant isoenzyme; active site of isoenzymes.

**Abstract**—Two homogeneous isoenzymes of apyrase from Pimpernel and Desirée varieties of *Solanum tuberosum* were obtained by affinity chromatography on agarose–Cibacron Blue or agarose–ATP-phosphonate columns. Both enzymes split P–O–P bonds of organic and inorganic di- and triphosphates. The ratio of ATPase/ADPase is different for the two apyrases: 10 for Pimpernel and 1 for Desirée. All these activities require bivalent metals. Both isoapyrases have the same MW (49 000) but differ in their p*I* (8.74 for Pimpernel and 6.69 for Desirée). The optimum pH of hydrolysis of organic di- and triphosphates is 6 (except for Pimpernel ADPase) and 5 for inorganic substrates. Chemical modification of tryptophan, tyrosine, arginine and carboxylic residues decreased all enzymic activities of both enzymes. Protection by substrates and inactivation rates of the individual activities are different for each isoenzyme.

## INTRODUCTION

Apyrases or diphosphohydrolases (EC 3.6.1.5) have been described in several plant tissues [1]. In potato this enzyme exists in more than one molecular form depending upon the clonal variety from which the potato tuber was obtained. These apyrases coming from different clonal sources exhibit different electrophoretic migration patterns [2]. P–O–P bonds are split both in organic and inorganic substrates with release of orthophosphate. The presence of bivalent metals is always required. There is no splitting of $\alpha$–$\beta$ P–O–P bonds in ATP and thus no intact pyrophosphate is released [3]. Phosphomonoesters are not split by potato apyrases and the only structural requirement for the substrate is the length of the pyrophosphoric chain, although organic pyrophosphates are more efficiently attacked than their unsubstituted analogues [4].

In the present work two apyrases obtained from *Solanum tuberosum*: var. Pimpernel and var. Desirée have been purified to homogeneity. Pimpernel apyrase catalyses the hydrolysis of the $\gamma$-phosphoryl group of ATP 10 times faster than the $\beta$-phosphoryl group of ADP (ATPase/ADPase = 10),* while Desirée enzyme catalyses both reactions at the same rate (ATPase/ADPase = 1).

Since the two isoenzymes catalyse the same reaction and have the same qualitative specificity for P–O–P bonds, it was thought worth exploring the participation of amino acid residues in the enzymic reaction, which could eventually clarify the kinetic differences. It has been thought that tyrosine residues could be connected with the activities of apyrases, since the browning of enzyme preparations as well as oxidation with mushroom tyrosinase is paralleled by a definite decrease of activities [5].

The present work analyses differences in electrophoretic pattern and kinetic behaviour of isoapyrases as well as the effect of chemical modifications of aromatic, acid and basic amino acid residues.

## RESULTS AND DISCUSSION

### *Purification of two apyrases by affinity chromatography columns*

Apyrases from two single clonal varieties of *Solanum tuberosum*, Pimpernel and Desirée, were purified by a factor of 2000 and 5000, respectively (Table 1). The purification factor for inorganic pyrophosphatase activity is lower than that for ATPase and ADPase activities. This is probably due to the elimination of contaminating pyrophosphatases present in large amounts in the potato extract. The purified enzymes showed a single band either by electrophoresis at pH 4.3 or by gel electrofocusing. This single protein has the four hydrolytic activities.

The average sp. act. of both enzymes after pooling and concentration of the eluted fractions from the affinity chromatography columns was between 8.3 and 11.6 $\mu$kat/mg of protein. The sp. act. measured at the time of the elution from either of the affinity

*Abbreviations: ATPase, adenosine triphosphatase; ADPase, adenosine diphosphatase; PPPase, tripolyphosphatase; PPase, pyrophosphatase; TNM, tetranitromethane; *p*-CMB, *p*-chloromercuribenzoate; DTNB, bis-dithionitrobenzoic acid.

Table 1. Purification of the apyrases

| | Total protein* (mg) | Total activity ($\mu$kat) | | | | Sp. act. ($\mu$kat × $10^2$/mg) | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | ATPase | ADPase | PPPase | PPase | ATPase | ADPase | PPPase | PPase |
| PIMPERNEL ENZYME | | | | | | | | | |
| Extract | 36 800 | 208.3 | 29.2 | 32.5 | 35.0 | 0.57 | 0.08 | 0.08 | 0.10 |
| First $(NH_4)_2SO_4$ ppt | 5170 | 86.1 | 14.5 | 22.2 | 17.8 | 1.67 | 0.28 | 0.43 | 0.33 |
| Second $(NH_4)_2SO_4$ ppt | 480 | 63.1 | 9.7 | 8.1 | 4.5 | 13.20 | 2.00 | 0.97 | 0.97 |
| Third $(NH_4)_2SO_4$ ppt | 120 | 61.3 | 7.4 | 5.7 | 1.5 | 52.00 | 6.30 | 4.80 | 1.20 |
| Sephadex G–100 | 11.4 | 47.5 | 4.5 | 3.8 | 0.5 | 417.00 | 39.00 | 33.70 | 4.70 |
| Affinity chromatography: | | | | | | | | | |
| (a) Agarose AMP-PCP | 0.9 | 10.2 | 0.9 | 0.9 | 0.03 | 1090.0 | 101.8 | 98.3 | 3.7 |
| (b) Agarose Cibacron | 1.9 | 21.2 | 1.7 | 2.0 | 0.10 | 1100.0 | 87.2 | 105.0 | 4.8 |
| DESIRÉE ENZYME | | | | | | | | | |
| Extract | 30000 | 67.9 | 65.7 | 11.3 | 11.3 | 0.21 | 0.22 | 0.03 | 0.03 |
| First $(NH_4)_2SO_4$ ppt | 1730 | 54.3 | 49.9 | 6.7 | 3.9 | 3.10 | 2.80 | 0.38 | 0.23 |
| Second $(NH_4)_2SO_4$ ppt | 304 | 47.5 | 47.9 | 5.0 | 2.6 | 15.50 | 15.80 | 1.60 | 0.85 |
| Third $(NH_4)_2SO_4$ ppt | 120 | 40.7 | 38.1 | 4.6 | 1.3 | 34.00 | 31.70 | 3.80 | 1.10 |
| Sephadex G–100 | 9.5 | 20.2 | 19.0 | 1.9 | 0.6 | 215.00 | 201.70 | 20.20 | 6.20 |
| Affinity chromatography: | | | | | | | | | |
| (a) Agarose AMP-PCP | 0.5 | 4.8 | 3.9 | 0.4 | 0.1 | 1060.0 | 860.0 | 85.0 | 26.7 |
| (b) Agarose Cibacron | 0.8 | 8.8 | 10.3 | 1.5 | 0.3 | 1110.0 | 1210.0 | 58.3 | 21.7 |

*All data refer to 1 kg of peeled potatoes.

Table 2. Effect of bivalent metals on the enzymic activities of potato apyrases

| | Pimpernel enzyme Relative activity (%) | | | | Desirée enzyme Relative activity (%) | | | |
|---|---|---|---|---|---|---|---|---|
| Addition* | ATPase† | ADPase† | PPPase‡ | PPase‡ | ATPase† | ADPase† | PPPase‡ | PPase‡ |
| $Ca^{2+}$ | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| $Mn^{2+}$ | 87 | 170 | 113 | 64 | 123 | 96 | 65 | 41 |
| $Cd^{2+}$ | 72 | 134 | 107 | 50 | 125 | 90 | 85 | 41 |
| $Mg^{2+}$ | 57 | 65 | 41 | 42 | 85 | 72 | 48 | 41 |
| $Co^{2+}$ | 50 | 60 | 22 | 23 | 85 | 86 | 28 | 24 |
| $Zn^{2+}$ | 37 | 51 | 17 | 10 | 72 | 103 | 30 | 20 |
| $Sr^{2+}$ | 29 | 24 | 5 | 20 | 45 | 34 | 21 | 50 |
| $Ni^{2+}$ | 20 | 24 | 7 | 6 | 28 | 37 | 25 | 19 |
| $Cu^{2+}$ | 10 | 20 | 5 | 5 | 23 | 35 | 14 | 17 |
| None | 7 | 3 | 7 | 9 | 27 | 16 | 16 | 29 |

*Metal used at a final concn of 5 mM.
†Buffer used: 100 mM MES pH 6.
‡Buffer used: 100 mM sodium acetate pH 5.

Table 3. Kinetic parameters of the hydrolysis of ATP, ADP and PPP by potato apyrases

| Pimpernel enzyme | ATPase | | | ADPase | | | PPPase | | |
|---|---|---|---|---|---|---|---|---|---|
| Temp. | $K_m$ (mM) | $V_m$ ($\mu$kat) | $V_m/K_m$ | $K_m$ (mM) | $V_m$ ($\mu$kat) | $V_m/K_m$ | $K_m$ (mM) | $V_m$ ($\mu$kat) | $V_m/K_m$ |
| 20° | 0.05 | 15.5 | 310 | 0.14 | 1.2 | 8.3 | 0.11 | 1.0 | 9.1 |
| 30° | 0.06 | 20.2 | 336 | 0.25 | 1.7 | 6.7 | 0.12 | 1.4 | 11.8 |
| 40° | 0.22 | 29.3 | 133 | 0.27 | 2.5 | 9.3 | 0.19 | 2.3 | 12.3 |
| $E_a$(cal/mol) | | 12580* | | | 12090* | | | 4300† | |
| Correlation (%) | | 99.4 | | | 99.5 | | | 98.6 | |
| Desirée enzyme | ATPase | | | ADPase | | | PPPase | | |
| Temp. | $K_m$ (mM) | $V_m$ ($\mu$kat) | $V_m/K_m$ | $K_m$ (mM) | $V_m$ ($\mu$kat) | $V_m/K_m$ | $K_m$ (mM) | $V_m$ ($\mu$kat) | $V_m/K_m$ |
| 20° | 0.024 | 11.2 | 465 | 0.07 | 9.2 | 130 | 0.27 | 1.0 | 3.7 |
| 30° | 0.025 | 15.5 | 620 | 0.10 | 14.7 | 147 | 0.35 | 1.5 | 4.3 |
| 40° | 0.080 | 22.3 | 279 | 0.13 | 24.0 | 185 | 0.60 | 2.3 | 3.8 |
| $E_a$(cal/mol) | | 12400† | | | 12100† | | | 4820† | |
| Correlation (%) | | 98.3 | | | 99.0 | | | 99.4 | |

All experiments were run at least in triplicate. There was a maximum dispersion of 20% between parameters calculated from different experiments.

Assay conditions for $K_m$ and $V_m$ determination as described in Experimental, with substrate concn ranged from 0.125 to 0.66 mM for ADP and PPP, and 0.05 to 0.15 mM for ATP.

*Substrate concn: 4 mM; metal concn: 10 mM.

†Substrate concn: 2 mM; metal concn: 5 mM.

columns was 2 or 3 times higher than the figures mentioned above, probably because of instability of the enzyme at low protein concentration during the elution, since these preparations at protein concentration of *ca* 5 mg/ml were very stable with a half-life of 52 months at 4°.

*Comparative properties of two apyrases*

*MW.* Both enzymes from the different potato varieties have the same MW of 49 000 as measured by gel filtration [6]. This value agrees with earlier evidence of an apyrase of high ATPase/ADPase ratio as determined by several methods [2, 3].

*Isoelectric point.* Pimpernel enzyme with a p*I* of 8.74 is a basic protein in contrast with the more acidic Desirée apyrase with a p*I* of 6.69.

*Bivalent metal dependence of enzymic activities.* Table 2 shows that the specificity of metal requirement of the four enzymic activities of the Pimpernel and Desirée enzymes is not very strict. The hydrolytic activities of Pimpernel were less than 10% in the absence of added metal whereas the Desirée apyrase had higher residual activities. The residual enzymic activity in the Desirée enzyme decreased to less than 10% only after addition of 50 mM EDTA. The simplest interpretation of this fact is that the $K_m$ for metal in the reactions catalysed by the Desirée enzyme could be close to the concentration of contaminating $Ca^{2+}$ remaining after treatment with chelating agents (15–25 $\mu$M).

*Optimum pH of enzymic activities.* In the presence of $Ca^{2+}$ both ATPase and ADPase activities of Desirée apyrase had the same optimum pH of 6 with half-maximum velocities at pH 4.6 and 8.4. The Pimpernel enzyme, on the other hand, had different pH optima for ATPase and ADPase. The values for the former activity were the same as for the Desirée enzyme, whereas ADPase activity had a pH optimum of 8 with inflections at 5.2 and 11.4. The inorganic hydrolase activities of both enzymes, PPPase and PPase, had an optimum pH of 5 with inflections around pH 4 and 6. This is a very unusual property because most of the acid pyrophosphatases described in the literature are not bivalent metal dependent, although a bacterial acid pyrophosphatase stimulated by $Co^{2+}$ has been reported [7].

Enzymic activities of both apyrases in the presence of $Mn^{2+}$ instead of $Ca^{2+}$ had optimum pH and inflections shifted between half and one unit towards the acid side. These differences could be at least in part due to the larger association constant of $Mn^{2+}$ with the substrates [8].

*Kinetic and thermodynamic parameters of enzymes.* Table 3 summarizes $K_m$ and $V_m$ of both enzymes at different temperatures. For both enzymes $K_m$ for ATPase activity increased four-fold over a 20° rise in temperature, and the $V_m$ values were doubled. On the other hand, $K_m$ and $V_m$ for ADPase and PPPase activities of both enzymes were doubled in this temperature range. If the $V_m/K_m$ ratio is used as a measure of catalytic efficiency, the ATPase activity of both enzymes had a maximum value at 30° but ADPase increased up to 40°.

The energies of activation were the same for

Table 4. Cr-ATP and AMP-PCP as inhibitors of enzymic activities of isoapyrases

| Inhibitors | ATPase $K_i$(mM) | ADPase $K_i$(mM) |
|---|---|---|
| Pimpernel enzyme | | |
| Cr-ATP | 0.33 | 0.12 |
| AMP-PCP* | 1.35 | 0.91 |
| Desirée enzyme | | |
| Cr-ATP | 0.02 | 0.04 |
| AMP-PCP | 0.07 | 0.02 |

Assay conditions as described in Table 2. Temp. 30°. Cr-ATP concn ranged from 0.06 to 0.30 mM. The concn of AMP-PCP used on Desirée enzyme ranged from 0.025 to 0.25 mM for ATPase and 0.5 to 1 mM for ADPase. All experiments were run at least in duplicate.

*$K_i$ values taken from ref. [4].

ATPase and ADPase activities measured in both enzymes; however, $E_a$ calculated for PPPase was *ca* 40% of the value found for ATP and ADP (Table 3). The identity of $E_a$ obtained for both apyrases is consistent with a common mechanism for both enzymes.

The $E_a$ for PPase activity could not be determined since precipitation of pyrophosphate with $Ca^{2+}$ at higher temperatures could introduce a factor of non-homogeneity in the reaction.

*Chromium nucleotides and AMP-PCP as inhibitors of apyrases.* Cr-ATP and AMP-PCP were not substrates but competitive inhibitors of ATPase and ADPase activities of Pimpernel and Desirée enzymes. These compounds were fairly good inhibitors of the enzymic activies. For Desirée apyrase the $K_i$ values of these analogues were similar to or even smaller than $K_m$ (Tables 3 and 4), but this was not the case for the ADPase activity of Pimpernel. The stronger interaction of Cr-ATP or AMP-PCP with the Desirée enzyme compared with that of Pimpernel could be related to structural differences in the interaction between the active sites and the different moieties of the substrate ($\beta$ or $\gamma$ phosphoryl of ATP or $\beta$ of ADP).

Cr-ADP was not hydrolysed by apyrase but it was destroyed non-enzymically in the presence of $Ca^{2+}$. The decomposition of this complex was followed spectrophotometrically, measuring the appearance of free Cr at 800 nm [9]. This did not allow us to measure $K_i$ for Cr-ADP. This instability of the complex in the presence of $Ca^{2+}$ has not been described, and it may lead to artifacts if not taken into account.

*Participation of amino acid residues in enzymic reaction.* Aromatic amino acid residues (tyrosine and tryptophane) participate in ATPase and ADPase activities of Pimpernel enzyme [5]. Treatment with electrophilic reagents such as TNM, *N*-acetyl imidazole and iodine reduced the ATPase activity of this enzyme much faster than its ADPase activity with concomitant disappearance of tyrosine groups. TNM affects the PPPase activity of Pimpernel enzyme to the same extent as its ATPase activity. The same was observed for the three enzymic activities of Desirée pyrophosphohydrolase (Table 5).

The participation of thiol groups has been excluded by the insensitivity of apyrase to *p*CMB and DTNB and thus nitration by TNM may be regarded in this case as specific for tyrosine groups.

ATP protected ATPase and ADPase activity of the two isoenzymes from inactivation by nitration. The other substrates were less effective or were completely ineffective.

We have suggested that the high ATPase/ADPase ratio of the Pimpernel enzyme could be accounted for by the involvement of distinct tyrosine residues [5]. It could be expected that in a pyrophosphohydrolase with an ATPase/ADPase ratio of one, nitration by TNM should affect both enzymic activities to the same extent, and this was confirmed for the Desirée apyrase (Table 5). Its PPPase activity was also reduced to a comparable extent.

On the other hand, TNM affects the ADPase activity of Pimpernel enzyme to about half the extent of the other two activities, thus supporting the interpretation of distinct tyrosine residues in ATPase or ADPase activities of this isoenzyme.

Tryptophane residues are apparently essential for all the enzymatic activities of the two pyrophosphohydrolases. The course of reduction of ATPase and PPPase activities by 2-hydroxy-5-nitrobenzyl bromide was almost identical in the Pimpernel enzyme. The ADPase activity of this apyrase was less inactivated by this alkylating reagent (Table 5). The three enzymic activities of Desirée enzyme showed the same parallel decay as can be seen from the results summarized in Table 5. This pattern is consistent with the one observed for TNM inactivation.

Experimental models [10] have shown that tryptophane residues interact with the adenine moiety of nucleotides, and therefore this residue could participate in substrate binding. Protection from alkylation by 2-hydroxy-5-nitrobenzyl bromide suggests that Ca nucleotides and Cr substrate analogues may interact with a tryptophane residue at the active site.

The role of the carboxylic groups in the reaction catalysed by apyrase was suggested by the pH profiles and by the inactivation by carbodiimide [5]. Table 5 shows that in Pimpernel enzyme the three enzymic activities were affected to the same extent by carbodiimide in the presence of glycine methyl ester, whereas with Desirée apyrase PPPase activity was suppressed to the extent of one-third of ATPase and ADPase activities. Carraway and Koshland [11] reported that the reaction can be reversed by treatment with neutral hydroxylamine. This treatment did not change the inactivation pattern of apyrase. Substrates in the presence or absence of $Ca^{2+}$ did not protect Pimpernel apyrase against inactivation by amidation. On the other hand, the same substrates ATP, ADP and PPP with or without $Ca^{2+}$ protected the three enzymatic activities of the Desirée enzyme from the carbodiimide reaction.

Results with model compounds suggest that guani-

Table 5. Effect of modifying reagents on apyrase activities* (percentage of inactivation)

| Reagent | ATPase | | | ADPase | | | PPPase | | |
|---|---|---|---|---|---|---|---|---|---|
| | No addition | Protecting agent added (10 mM) | | No addition | Protecting agent added (10 mM) | | No addition | Protecting agent added (10 mM) | |
| Pimpernel enzyme | | | | | | | | | |
| TNM | 54 | ATP | 25 | 25 | ATP | 4 | 50 | ATP | 50 |
| (50 mM; 20 min) | | ADP | 43 | | ADP | 4 | | ADP | 47 |
| | | PPP | 50 | | PPP | 24 | | PPP | 57 |
| 2-Hydroxy-5-nitro- | 60 | Ca ATP | 43 | 37 | Ca ATP | 21 | 59 | Ca ATP | 40 |
| benzyl bromide | | Ca ADP | 49 | | Ca ADP | 27 | | Ca ADP | 46 |
| (20 mM; 4 min) | | Ca PPP | 46 | | Ca PPP | 22 | | Ca PPP | 34 |
| | | Cr–ATP | 18 | | Cr–ATP | 13 | | Cr–ATP | 28 |
| | | Cr–ADP | 25 | | Cr–ADP | 19 | | Cr–ADP | 16 |
| Carbodiimide | 76 | ATP | 64 | 63 | ATP | 56 | 58 | ATP | 53 |
| (60 mM; 10 min) | | ADP | 73 | | ADP | 56 | | ADP | 45 |
| | | PPP | 66 | | PPP | 63 | | PPP | 59 |
| 2, 3-Butanedione | 55 | ATP | 6 | 52 | ATP | 8 | 50 | ATP | 0 |
| (56 mM; 60 min) | | ADP | 23 | | ADP | 25 | | ADP | 6 |
| | | PPP | 57 | | PPP | 53 | | PPP | 48 |
| | | Ca PPP | 0 | | Ca PPP | 0 | | Ca PPP | 0 |
| Desirée enzyme | | | | | | | | | |
| TNM | 77 | ATP | 37 | 73 | ATP | 35 | 64 | ATP | 42 |
| (50 mM; 20 min) | | ADP | 33 | | ADP | 33 | | ADP | 43 |
| | | PPP | 38 | | PPP | 33 | | PPP | 64 |
| 2-Hydroxy-5-nitro | 48 | Ca ATP | 33 | 49 | Ca ATP | 23 | 46 | Ca ATP | 21 |
| benzyl bromide | | Ca ADP | 23 | | Ca ADP | 20 | | Ca ADP | 26 |
| (20 mM; 4 min) | | Ca PPP | 44 | | Ca PPP | 44 | | Ca PPP | 43 |
| | | Cr–ATP | 18 | | Cr–ATP | 13 | | Cr–ATP | 28 |
| | | Cr–ADP | 25 | | Cr–ADP | 18 | | Cr–ADP | 17 |
| Carbodiimide | 68 | ATP | 42 | 78 | ATP | 37 | 23 | ATP | 0 |
| (30 mM; 15 min) | | ADP | 51 | | ADP | 38 | | ADP | 4 |
| | | PPP | 62 | | PPP | 50 | | PPP | 15 |
| 2, 3-Butanedione | 60 | ATP | 4 | 47 | ATP | 9 | 24 | ATP | 0 |
| (28 mM; 30 min) | | ADP | 10 | | ADP | 14 | | ADP | 0 |
| | | PPP | 40 | | PPP | 47 | | PPP | 9 |

Apyrases were treated with the protein reagents either with or without further additions. After incubating for the time specified, the reaction was stopped by dilution and the enzyme was assayed with ATP, ADP or PPP.

*Enzyme activity in the absence of modifying reagents was taken as 100%.

dino groups of arginine residues act by neutralizing negative charges on phosphate substrates [12], thus facilitating nucleophilic attack. The existence of essential arginine residues at the ATP binding site of enzymes has been studied in phosphoryl transfer enzymes by means of 2, 3-butanedione [13]. The rates of inactivation of the three enzymic activities by butanedione in Pimpernel apyrase were about the same (Table 5), whereas in Desirée enzyme the inactivation of these three activities was different, ATPase being the most affected. The pattern is the opposite to that of TNM, where the dissociation of activities was observed for the Pimpernel and not for the Desirée enzyme and suggests again different regional environments for the same residue in each isoenzyme. The substrates, ATP and ADP, protected the three activities of both enzymes from inactivation, but PPP was an inefficient protector in both isoenzymes. Addition of $Ca^{2+}$ to PPP allows almost complete protection for all three enzymic activities, suggesting that a highly negative compound like PPP at pH 8 needs partial charge neutralization by a bivalent metal ion in order to interact with residues at the active site. The slight protection by PPP in the absence of $Ca^{2+}$ in Desirée enzyme agrees with a lower $K_m$ valuc for $Ca^{2+}$ with this enzyme, compared with Pimpernel apyrase. This may again be explained by the metal $K_m$ values for Desirée enzyme being similar to the concentration of contaminating $Ca^{2+}$ ion of the incubation medium.

The inactivation of both apyrases was almost completely reversed upon removal of excess of butanedione and borate, suggesting that only arginine groups were modified [14] and excluding the possibility that lysine residues were involved.

The optimum pH activities of both enzymes were unaffected by modification with TNM, 2-hydroxy-5-nitrobenzyl bromide, carbodiimide or butanedione with regard to ATPase, ADPase and PPPase activities.

The different effect of modifiers of aromatic amino acid residues could be a basis for an explanation of the kinetic differences between the two isoenzymes. Sp. act. for ADPase of the Pimpernel isoenzyme is ten times lower than that of the Desirée variety, but sp. act. of the PPPase is the same for both isoenzymes. This is in agreement with the fact that chemical modification of aromatic amino acid residues affects the ADPase of Pimpernel less than that of Desirée.

The difference in kinetic behaviour of the two isoenzymes from the clonal varieties of *S. tuberosum* may be related to their different charges, as expressed in their $\Delta pI$ of *ca* 2 units and to different reactivities of aromatic and arginyl residues to chemical modification.

The results obtained with chemical modifiers of amino acid residues suggest that even though the active sites of Pimpernel and Desirée apyrases seem to contain the same residues, the spatial distribution of accessibility, as well as the conformational changes induced by substrates or inhibitors are qualitatively different for the two isoenzymes.

## EXPERIMENTAL

Apyrase activity was assayed as previously described [3] by measuring the liberation of Pi from ATP or ADP. PPPase and PPase activities were assayed in a similar way: 0.1 M NaOAc, pH 5; 5 mM $CaCl_2$ and 2 mM PPP or PP.

Protein contents were measured by the Lowry method [15] or by $A_{280}$.

*Apyrase purification.* The two apyrases were obtained from homogeneous strains of *S. tuberosum* cv Pimpernel and cv Desirée, obtained by clonal selection, and supplied by the Instituto de Investigaciones Agropecuarias 'La Platina', Santiago, Chile. Both enzymes were prepared as previously reported [5]. The last step of CMC was replaced by a column either of (a) ATP–phosphonate or (b) Cibacron Blue F3G-A linked to agarose. (a) An aliquot containing 40 $\mu$kat of apyrase was applied to a column (1.1 cm × 28 cm) of agarose ATP–phosphonate previously equilibrated with 50 mM MES, pH 6. The enzyme was eluted at the same pH by a linear gradient (0.0–30 mM) of ATP in 2 mM EDTA, at a total vol. of 25 ml. The maximum peak of activity emerges between 5 and 6 mM ATP. (b) An apyrase prepn containing *ca* 700 $\mu$kat was applied to a column (1.6 cm × 83 cm) of agarose Cibacron Blue previously equilibrated either with 100 mM MES or 50 mM Na succinate at pH 6. The enzyme was eluted by a linear gradient (0–4 M) of NaCl, in pH 6 buffer of a total vol. of 700 ml. The enzyme eluted at *ca* 1.8 M NaCl.

*Disc electrophoresis.* The purified enzyme soln was submitted to PAGE at pH 4.3 [16]. The enzymic activities were determined as reported before [2].

*Gel electrofocusing.* The gel mixtures used were as given in ref. [17]. Ampholine solns (LKB-Produkter AB, Bromma, Sweden) ranged between pH 3 and 10 or 7 and 10. The upper reservoir contained 0.02 M NaOH (cathode) and the lower (anode) 0.2% of $H_2SO_4$. The voltage was constant at 300 V. The current decreased to a constant value in *ca* 90 min. Protein bands were stained with Coomassie Brilliant Blue [18]. Enzymic activities and pH were measured after elution from the gel with 0.2 M NaCl prepared in recently boiled $H_2O$.

*Synthesis of* 5′-($\beta$, $\gamma$-methylene) triphosphate (AMP–PCP). The synthesis and purification were as outlined in ref. [19]. The product was identified by PC [19] and paper electrophoresis at 4°, 300 V, for 3 hr in 25 mM Na citrate, pH 5, 10 mM EDTA.

*Binding of AMP–PCP to agarose.* Sepharose 4B was activated by BrCN [20] and coupled to AMP–PCP oxidized with periodate according to ref. [21]. The amount of bound nucleotide was *ca* 0.7 $\mu$mol/ml of gel [22].

*Binding of Cibacron Blue F3G–A to agarose.* 5.7 mg Cibacron Blue were coupled per ml of Sepharose Cl–4B [23, 24].

*MWs* of both isoenzymes were estimated by gel filtration [6].

*Optimum pH determination.* The four enzymatic activities of both apyrases were determined at various pHs at 30° with Good's buffers, [25] or $\beta$-alanine. No appreciable inactivation of the enzymes in the absence of substrate occurred even at extreme pHs.

*Measurement of kinetic and thermodynamic parameters.* The appearance of Pi was measured by the *iso*-BuOH–$C_6H_6$ method [26]. $K_m$ and $V_{max}$ at different temps were calculated by the method of least squares. For measuring the temp. dependence of the three enzymic activities of the Desirée enzyme and the PPPase of Pimpernel apyrase the substrate and $Ca^{2+}$ concn were those used as in the assay but ATPase and ADPase activities of the Pimpernel enzyme were measured using 4 mM substrate and 10 mM $Ca^{2+}$. These expts were done at five or more different temps. between 0° and 60°. The activation energies ($E_a$) were calculated from Arrhenius plots. The plots were curved above 30° for ATPase and PPPase activities and above 40° for ADPase activity.

*Activation by bivalent metals.* Usual precautions were taken to avoid contamination with extraneous metal ions [27]. The residual $Ca^{2+}$ contamination was determined by atomic absorption spectrophotometry.

*Synthesis of* Cr (*III*)*—adenine nucleotide complexes.* Cr–ATP and Cr–ADP were synthesized according to ref. [9]. A better yield of Cr–ADP than reported [9] was obtained by changing the relative concns of $Cr(NO_3)_3$ and ADP from 1:2 to 1:1.

*Calculation of* $K_i$ *values for* Cr–*ATP and AMP–PCP.* The method of ref. [28] was used to calculate the inhibition constant.

*Modification of amino acid residues.* Chemical modification of apyrases by TNM, 2-hydroxy-5-nitrobenzyl bromide, carbodiimide and *p*CMB were performed by the methods previously described [5]. The reaction with 2,3-butanedione was performed at 30° in 40 mM Na-borate buffer, pH 8 [29]. The loss of ATPase and ADPase activities of apyrase by this reagent was largely reversed upon removal of excess of butanedione and borate by passage through a Sephadex G-25 column (6.2 cm × 10.7 cm) equilibrated in 100 mM Tris–HCl, pH 8 and 1 M KCl [14]. The reaction with DTNB was performed according to ref. [30].

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