PURIFICATION AND CHARACTERIZATION OF TWO ISOAPYRASES FROM *SOLANUM TUBEROSUM* VAR. ULTIMUS

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Key Word Index—*Solanum tuberosum*; Solanaceae; potato; isoenzymes; apyrase; ATP-diphosphohydrolase.

Abstract—Two isoenzymes of ATP-diphosphohydrolase (apyrase) were extracted and purified from *S. tuberosum* var. Ultimus. Their hydrolytic activity ratios (ATPase/ADPase) were 1.0 (apyrase B) and *ca* 15.0 (apyrase A). They were characterized and compared with apyrases of other varieties of *S. tuberosum*. Ultimus apyrases, like the other apyrases, did not hydrolyse esteric bonds but only pyrophosphate bonds of organic and inorganic compounds. The optimum pH of all the studied hydrolytic activities of the Ultimus apyrases A and B was 6, except for the ADPase of enzyme A which was 8. Both enzymes require bivalent metal ions for catalytic activity. The activation order for both Ultimus enzymes was: $Ca^{2+}>Mn^{2+}> Mg^{2+}>Co^{2+}>Zn^{2+}$. Chemical modification of tryptophan, tyrosine, arginine and carboxylic residues decreased all enzymic activities of both apyrases. The modification of histidine residues reduced the ATPase and ADPase activities of the low ratio apyrase and the ATPase of the high ratio enzyme but did not affect its ADPase activity. Neither of the Ultimus apyrases showed the participation of -SH groups in the active site. The pI values obtained were: 5.45 for apyrase B and 6.56 for apyrase A. The absorption and the fluorescence spectra of the Ultimus isoenzymes were coincident. The amino acid composition of both isoenzymes is very similar, the number of histidines being the most remarkable difference. The amino acid composition of both isoenzymes does not explain the difference of one pH unit in the isoelectric point between the Ultimus enzymes A and B.

INTRODUCTION

A large number of plant [1] and animal [2] tissues contain a pyrophosphohydrolase commonly called apyrase (EC 3.6.1.5). This enzyme hydrolyses P–O–P bonds of both organic and inorganic substrates with release of orthophosphate ions plus a phosphomonoester [3]. Apyrase is activated by metal cations, the major effect is produced by Ca^{2+} but other ions such as Mn^{2+}, Co^{2+}, Mg^{2+} and Zn^{2+} also stimulate enzymic activity [4].

Different isoapyrases have been found, depending on the variety of *Solanum tuberosum* used as a source of apyrase. Enzymes extracted from potatoes, var. Pimpernel and var. Desirée, have been purified to homogeneity and characterized [5, 6]. These apyrases are similar in M_r, metal ion dependence, purification scheme [5] and amino acid composition [6], but they are very different in isoelectric point and in the ratio of ATPase/ADPase activity [5]. This ratio is 10.0 for Pimpernel enzyme and 1.0 for Desirée apyrase.

In a preceeding paper [7] we demonstrated the coexistence of two isoapyrases in potato tuber cv Ultimus. These isoenzymes differ in the ATPase/ADPase ratio, in the isoelectric point and slightly in their M_r (48 000 for apyrase B and 46 500 for apyrase A). They also differ in their subcellular localization. The enzyme Ultimus A with a high ATPase/ADPase ratio > 10.0 is mainly in a soluble fraction, while Ultimus B enzyme with (ATPase/ADPase ratio *ca* 1.0) is largely membrane bound. In the present work we have purified and characterized the two isoapyrases, Ultimus A and Ultimus B.

RESULTS AND DISCUSSION

Purification of Ultimus A and B

The scheme employed for the separation of both isoapyrases is shown in Table 1. In the five initial steps of the sequence the ATPase/ADPase ratio was maintained at *ca* 3.0. After pseudoaffinity chromatography through a Cibacron Blue-Sepharose column, two peaks with different ratios of apyrase activity were partially separated (results not shown). The last stage of the purification scheme is represented in Fig. 1, which shows the elution pattern from a CM-Sephadex column of a fraction enriched with apyrase of low ATPase/ADPase ratio. Similar results were obtained when the initial fraction was enriched with apyrase A (not shown).

The separation scheme gave a low yield, but a very high purification factor (more than 2000 times for both isoapyrases). Other alternative techniques such as: chromatofocussing, preparative electrofocussing, dye-ligand affinity chromatography and hydroxylapatite chromatography were used in order to improve the yield. All these methods were inadequate because the purity decreased when the yield increased.

Table 1. Purification sequence of Ultimus apyrases A and B

Fraction	Total activity (μkat) ATPase	ADPase	Specific activity (μkat × 10^2 mg^{-1}) ATPase	ADPase	Recovery (%) ATPase	ADPase	Purification (folds) ATPAse	ADPase	ATPase/ADPase ratio
Extract	340	122	1	<1	100	100	1	1	2.8
Fraction A	334	115	10	3	98	94	7	7	2.9
Fraction B	238	71	56	17	70	58	40	33	3.4
Fraction C	172	57	142	48	51	47	101	94	3.0
Sephadex G-100	115	39	747	250	34	32	533	495	3.0
F3GA A*	24	1	4190	247	7	1	2990	485	16.9
Sepharose B†	29	16	2280	1290	8	13	1630	2550	1.8
CM A	4	<1	1660	67	1	<1	1190	133	25.0
Sephadex B	8	8	3340	3440	2	7	2390	6910	1.0

All data refered to 1 kg of peeled potatoes.
*Apyrase A (high ATPase/ADPase ratio).
†Apyrase B (low ATPase/ADPase ratio).

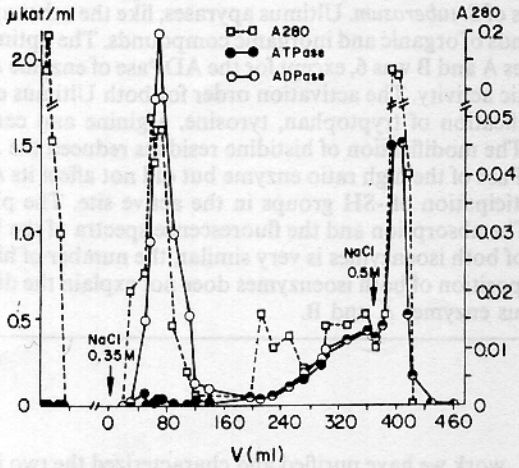

Fig. 1. Ultimus isoapyrases separation by ion exchange chromatography. The column (15.4 × 1.8 cm) was filled with 39 ml of CM-Sephadex, equilibrated with 50 mM succinate buffer pH 6, 2 μkat ATPase and 1 μkat ADPase diluted 1:5 in succinate 5 mM pH 6, were added.

Enzyme purity. Apyrase A and B obtained from the last step of the purification sequence previously described, showed a single band with isoelectrofocussing or SDS electrophoresis. This indicates that the purified isoenzymes were homogeneous.

Enzyme stability. On storage of the isoapyrases (highly purified, with a final protein concentration of at least 1 mg ml^{-1}) at −20° for six months, the ATPase activity of apyrase A was 47% of the original, while the ADPase activity of apyrase A and ATPase and ADPase activities of apyrase B decreased by less than 30%. In the high ratio apyrase A, the ATPase activity is less stable than the ADPase activity, while in the low ratio apyrase, both activities decrease at similar rates.

Apyrase characterization

Isoelectric point. Apyrase A and B are very different in their pI values. Apyrase A is more basic with pI 6.56; apyrase B is more acidic with pI 5.45. When compared with other apyrases such as Pimpernel (a high ratio enzyme) and Desirée (a low ratio enzyme) we observe the same tendency, with the Pimpernel apyrase being more basic than the Desirée apyrase [5].

Substrate specificity. These results are summarized in Table 2. Ultimus apyrases do not hydrolyse phosphate monoesters, like the Pimpernel and the Desirée enzymes [3]. This behaviour is common to all apyrases studied and is reflected in changes in the *ATP*ase/XPase ratio. The most obvious difference between apyrases A and B was the ATPase/ADPase ratio.

Optimum pH. Both ATPase and ADPase activities of the apyrase B were maximum at pH 6.0 with half maximal activity at pH 4.8 and 7.6. A different result was obtained with the ADPase activity of apyrase A which has an optimum pH of 8.0, with half maximal activity at 6.2 and 8.5. The pH profile of the ATPase activity was similar to that obtained with apyrase B. These results agree with those obtained with Pimpernel and Desirée enzymes [5]. In the high ratio enzyme (Pimpernel) the optimum pH for the ATPase activity was 6 while the ADPase activity has an optimum pH at 8. In the low ratio enzyme (Desirée), both activities have a maximum at pH 6.

Effect of bivalent metal ions. Table 3 summarizes the effects of some metal ions on apyrase Ultimus A and B activities. Both isoenzymes need a metal ion in order to catalyse hydrolysis. This is a common characteristic of all the plant apyrases [8–10]. When no metal ion was added apyrase activities ranged between 4 and 15% because of traces of metal ions. Addition of EDTA decreased enzymic activity <7%. Ca^{2+} is the best activating metal ion for both Ultimus apyrases.

On comparison of the effects of bivalent metal ions on both apyrases of low ATPase/ADPase ratio (Desirée and Ultimus), different patterns were observed because in the Ultimus B enzyme, Ca^{2+} was the best activating metal ion for ATPase activity, while Mn^{2+} was better for the Desirée enzyme. The high ratio apyrase (Ultimus A and Pimpernel) also differ; ADPase activity of Pimpernel enzyme was more stimulated by Mn^{2+} than by Ca^{2+}, while in the Ultimus A apyrase Ca^{2+} was the best activating metal ion for both ATPase and ADPase activities.

Table 2. Substrate specificity of *S. tuberosum* var. Ultimus apyrases

Substrate	High ATPase/ADPase ratio ATPase / XPase*	ratio apyrase %	Low ATPase/ADPase ratio ATPase / XPase*	ratio apyrase %
ATP	1	100.0	1	100.0
ADP	17	5.9	1	104.7
PPP	19	5.4	16	6.3
PP	102	1.0	49	2.0
5'-AMP	16000	<0.1	11500	<0.1
p-NO_2-Phenyl phosphate	8000	<0.1	11500	<0.1
Glucose-1-P	6710	<0.1	4000	<0.1

The assay medium contained 100 mM succinate buffer pH 6.0, 5 mM Ca^{2+} and the corresponding 2 mM substrate.

*XPase activity = ADPase, PPPase, PPase, 5'-AMPase, pNPPase and G-1-Pase.

Table 3. Effect of bivalent metal ions on the enzymic activities of Ultimus apyrases

Addition	Apyrase A Relative activity (%) ATPase	ADPase	Apyrase B Relative activity (%) ATPase	ADPase
Ca^{2+}	100	100	100	100
Mn^{2+}	77	88	80	70
Mg^{2+}	54	50	48	65
Co^{2+}	43	50	43	60
Zn^{2+}	32	44	39	66
None	4	8	15	9
EDTA	4	5	7	5

Metal ion used at a final concentration of 5 mM.
Buffer used: 100 mM MES pH 6.0.
EDTA used at a final concentration of 1 mM.

Table 4. Amino acid composition of Ultimus apyrases

Amino acid	Residues per molecule Apyrase A	Apyrase B
Asx	48	49
His	6	12
Pro	20	20
Lys	29	29
Glx	42	44
Arg	12	14
Tyr	28	26
Ile	19	22
Ser	31	33
Val	23	22
Leu	42	41
Gly	43	43
Ala	40	44
Met	8	6
Phe	15	15
Thr	23	23
Trp*	3	3
Cys	nd	nd
Total residues	413	448
M_r ($\times 10^{-3}$)	46.7	48.0

The values of Glx and Asx are the total of Glu plus Gln and Asp plus Asn respectively.
*Trp was determined spectrophotometrically according to ref. [20].

Amino acid composition. Table 4 shows a significant coincidence between the total number of each amino acid residue in both Ultimus apyrases. The most striking difference is in the number of histidine residues. Ultimus A contains six of these while the low ratio Ultimus B has 12.

The sum of basic or acid amino acids was very similar for both isoapyrases. The slight differences do not explain the difference of one pH unit in the pI values of these enzymes. This could result from the different degrees of amidation of the carboxylic residues of both isoapyrases, or differences between the primary, secondary and tertiary structures of both isoapyrases which would produce a greater solvent exposure of the carboxylic groups in apyrase B than in apyrase A.

The amino acid compositions of the four apyrases, Ultimus A and B, Pimpernel and Desirée [6] were very similar. The only significant difference was again in the number of histidine residues when apyrases of a low or high activity ratio are compared. The Pimpernel apyrase has 50% fewer histidines than the Desirée apyrase. This difference is even greater in the Ultimus apyrases where the low ratio enzyme has twice as many histidine residues than the high ratio Ultimus A.

In the low ratio apyrases, with their high histidine content, this amino acid may be at the active site and differentially accelerate hydrolysis of ADP over ATP. However, differences in the numbers of histidine residues may change tertiary structures and ATPase/ADPase ratios. We attempted to distinguish between these hypotheses by chemically modifying the histidine residues.

Chemical modification of amino acid residues of Ultimus isoapyrases. A summary of the inactivation percentage of both Ultimus apyrases (A and B) with the modifying reagents and the effect of the substrates ATP and ADP in the modification reaction is shown in Table 5. Since the ATPase and ADPase activities of both Ultimus isoapyrases were drastically reduced by tetranitromethane (TNM) and *N*-Br-succinimide (NBS), the aromatic amino

Table 5. Effect of modifying reagents on apyrase activities of Ultimus enzymes A and B

	Apyrase Ultimus A				Apyrase Ultimus B			
	ATPase		ADPase		ATPase		ADPase	
Reagent	No addition inact (%)	Protecting agent (10 mM) inact (%)	No addition inact (%)	Protecting agent (10 mM) inact (%)	No addition inact (%)	Protecting agent (10 mM) inact (%)	No addition inact (%)	Protecting agent (10 mM) inact (%)
TNM 50 mM	51.0	ATP 10.9	34.0	ATP 0.0	55.0	ATP 0.0	60.4	ATP 8.0
20 min, 0°		ADP 24.5		ADP 7.5		ADP 7.3		ADP 14.1
Carbodiimide 30 mM	52.8	ATP 50.2	46.8	ATP 26.0	44.6	ATP 42.5	43.2	ATP 40.1
20 min, 0°		ADP 49.6		ADP 31.5		ADP 22.9		ADP 23.1
2,3-Butanedione 28 mM	16.5	ATP 10.0	25.0	ATP 2.5	52.0	ATP 12.1	54.1	ATP 14.0
60 min, 30°		ADP 4.5		ADP 9.5		ADP 24.3		ADP 22.6
NBS 10 μM	65.0	ATP 61.0	54.0	ATP 42.0	73.0	ATP 78.5	64.0	ATP 64.8
5 min, 20°		ADP 58.0		ADP 52.0		ADP 75.0		ADP 69.9
DTNB 50 μM	0.0	ATP 0.0	0.0	ATP 0.0	0.0	ATP 0.0	0.0	ATP 0.0
20 min, 20°		ADP 0.0		ADP 0.0		ADP 0.0		ADP 0.0
Diethylpyrocarbonate 50 mM	61.0	ATP 58.0	0.0	ATP 0.0	59.0	ATP 5.0	61.0	ATP 0.0
12 min, 30°		ADP 60.0		ADP 0.0		ADP 58.0		ADP 61.0

After incubation for the specified time, the reaction was stopped by dilution and the enzyme was assayed with ATP or ADP. Enzyme activity in the absence of modifying reagent was taken as 100%.

The protecting agents added were 10 mM ATP or ADP.

acids tyrosine and tryptophane appear to participate. ATPase activity of apyrase Ultimus A declines more than the ADPase activity when the tyrosine residues were nitrated with TNM; the same happens with Pimpernel apyrase. In the low ATPase/ADPase ratio apyrases (Desirée and Ultimus B), both ATPase and ADPase activities decreased at similar rates. These results can be explained by the participation of tyrosines with different solvent exposure in the ATPase activity with respect to the ADPase activity in the high ratio apyrases. Meanwhile, in the low ratio apyrases, tyrosine residues with similar solvent accessibility would be involved.

The importance of carboxylic amino acid residues was suggested by the pH profiles. The modification of carboxylic amino acid residues by carbodiimide caused the inactivation of both ATPase and ADPase activities of the two Ultimus apyrases.

Modification of arginyl residues by 2,3-butanedione produced a greater inactivation of both enzymatic activities of the low ratio enzyme (apyrase B), compared with the inactivation produced in apyrase A. Similar results were observed with Pimpernel (high ratio) and Desirée (low ratio apyrase) [5].

No inactivation was produced by the modification of cysteinyl residues with bis-dithionitrobenzoic acid (DTNB); hence none of the Ultimus apyrases have -SH groups involved in their enzymatic activities. Neither Pimpernel [11] nor Desirée apyrases [5] have -SH groups essential for their hydrolytic activities.

Histidyl residues were modified with diethylpyrocarbonate. This modification produced the inactivation of both hydrolytic activities of apyrase B. A different effect was observed in apyrase A, where the ATPase activity was considerably decreased while the ADPase activity was unaffected by modification with diethylpyrocarbonate. The modification of histidine residues by diethylpyrocarbonate was performed in the presence of Ca^{2+} because the inactivation was enhanced with the addition of this metal ion. Ca^{2+} binds to Desirée apyrase in the absence of substrate [4], and this binding may expose some histidine residues, increasing the degree of modification, and thus the inactivation percentage. These results, together with the difference in the number of histidines between apyrases A and B, indicates that perhaps histidyl residues are involved in both enzymatic activities of apyrase B, but only in the ATPase activity of apyrase A. This may explain the difference in the ATPase/ADPase ratio of both Ultimus isoapyrases.

Absorption and fluorescence spectra. The absorption spectra of apyrases A and B were coincident with a maximum at 277 nm and a minimum at 250 nm. The fluorescence emission spectra of both Ultimus apyrases were also similar, with an emission maximum at 350 nm.

EXPERIMENTAL

Materials. All chemicals were reagent grade. Apyrases were obtained from a pure strain of *Solanum tuberosum* cv Ultimus.

Apyrase activity. Assayed by measuring the liberation of Pi [12, 13] from ATP or ADP as previously described [14]. A unit of activity (μkat) is equivalent to 1 μmol Pi liberated sec^{-1} at 30°.

Protein. Measured by the method of ref. [15] or by A_{280} using bovine serum albumin (BSA) as standard.

Apyrase purification. The two apyrases were obtained from an homogenous strain of *S. tuberosum* cv Ultimus, obtained by clonal selection. Both apyrases were prepared as previously reported [5]. Two fractions were pooled from a Cibacron Blue-Sepharose column, the first enriched with apyrase A and the other with apyrase B. These fractions were concd by ultrafiltration, diluted (1:5) in Na succinate (5 mM, pH 6), and passed independently through CM-Sephadex columns (15 × 1.4 cm) previously equilibrated with succinate buffer (50 mM, pH 6). Differential elution was achieved with abrupt changes in ionic strength, 0.35 and 0.5 M NaCl for apyrases A and B, respectively.

Apyrase purity. The degree of contamination was determined by isoelectrofocussing [16] and by SDS-PAGE [17].

Substrate specificity. The assays were performed in 100 mM succinate buffer pH 6 and 5 mM Ca^{2+} at 30° for 5 min. The compounds tested as substrates had a final concn of 2 mM and were: ATP, ADP, PPPi, PPi, 5'-AMP, *p*-nitrophenylphosphate and glucose 1-phosphate. The released Pi was measured by the method of ref [12].

Optimum pH. The buffers used were: Na acetate pH 4.5 and pH 5.5; 2-*N*-morpholinoethanesulphonic acid (MES) pH 5.5, pH 6.0, pH 6.5 and pH 7.0; *N*-tris(hydroxymethyl-2-aminoethanesulphonic) acid (TES) pH 7.0, pH 7.5, pH 8.0 and pH 8.5. The buffers were adjusted to ionic strength of 5.5 millimho.

Bivalent metal ions effect. Usual precautions were taken to avoid contamination with extraneous metal ions [18].

Amino acid. Analyses were performed in Chiron Co, Emeryville U.S.A., by Dr Carlos George-Nascimento. Samples of homogeneous apyrases (A and B) were hydrolysed with 6 M HCl for 24 hr at 110°. The mixture of amino acids was derivatized with phenylisothiocyanate and separated by reverse phase HPLC [19]. A column of 0.46 × 15 cm (Waters Pico Tag) was used; the solvent gradient was: solvent A, buffer Na acetate-triethylamine, pH 6.4, and solvent B was 6.0% acetonitrile. The data were analysed by a computer program developed in the Biochemistry Department of Chicago University.

The number of Trp residues was determined spectrophotometrically [20] in the presence of 10 M urea.

Modification of amino acid residues in the apyrases by TNM, carbodiimide, NBS, DTNB, 2,3-butanedione and diethylpyrocarbonate were performed by methods previously described [21]. The modification reaction was stopped by diluting 20- to 100-fold with 0.1 M NaCl. Controls were performed with the respective solvents. The individual modification conditions such as temp, time, pH, and modifying concn are listed in Table 5. The protecting agents added were either 10 mM ATP or 10 mM ADP.

Absorption and fluorescence spectra. Homogeneous apyrases A and B eluted from a CM-Sephadex column were dialysed exhaustively against 0.1 M NaCl and adjusted to a protein concn of *ca* 0.5 mg ml^{-1}. The absorption spectra were measured in a Shimadzu UV-160 spectrophotometer between 200 and 350 nm.

The fluorescence spectra were recorded in a Perkin Elmer S-5 Luminescence Spectrometer. Fluorescence spectra were obtained with excitation at 277 nm and the emission was monitored between 200 and 420 nm.

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