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STRUCTURAL STUDIES OF TWO APYRASES FROM *SOLANUM TUBEROSUM*

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Abstract—The proportion of acid and basic amino acid residues obtained for two homogeneous isoenzymes of apyrase isolated from different clonal varieties of *Solanum tuberosum* (Pimpernel and Desirée) was essentially the same This does not agree with the difference in pI values observed Treatment with asparaginase and glutaminase caused partial inactivation of both enzyme activities in both isoenzymes, and pI values were changed, but not equalized The differences in pI values of the native isoenzymes may still be attributed to different proportions of glutamine and asparagine in the primary structure Leucine is the amino-terminal residue in both isoenzymes Both have two disulphide bridges and one buried sulphydryl group which is not essential for enzyme activity Differences in pI values should thus be attributed to factors other than amino acid composition

INTRODUCTION

Apyrase (ATP-diphosphohydrolase, EC 3 6 1 5) catalyses the hydrolysis of pyrophosphate bonds from nucleoside di and triphosphates with sequential release of two moles of inorganic orthophosphate in the presence of bivalent metal When it is sufficiently purified it does not have phosphomonoesterase activity [1] Apyrases have been isolated from potato tuber in this laboratory [1] The enzyme has also been reported to exist in soluble form in cabbage leaves [2], pea plumules [3] and clover seeds [4] Recently membrane-bound apyrase activities have been described in tissues of several higher plants (cited in refs [5] and [6]) and also in pig pancreas [7] and rat liver [8]

Apyrases isolated from different clonal varieties of *Solanum tuberosum* differ in their rate of hydrolysis of ATP and ADP [9] The enzyme purified from Desirée variety has an ATPase/ADPase ratio of 1 0 and the isoenzyme found in the Pimpernel variety hydrolyses ATP ten times faster than ADP The isoelectric points of these two isoenzymes differ by two pH units but no significant differences have been found in the amino acid residues participating in the catalytic activity of the two isoapyrases [10] The fact that both apyrases appear to have the same enzymatically significant amino acid residues leaves the question of the observed kinetic differences unresolved It therefore seemed adequate to investigate other structural differences such as total amino acid composition, or identity of terminal residues which could account for the different properties of the two isoapyrases The results are reported in the present communication

RESULTS AND DISCUSSION

Purification of isoapyrases by Cibacron Blue-Sepharose column

According to gel-isoelectrofocusing, SDS electrophoresis and PAGE at pH 4 3, the Pimpernel and Desirée isoenzymes obtained from a Cibacron Blue-Sepharose column were *ca* 93% pure with an ATPase sp act of about 10 μkat/mg

Amino acid composition

Table 1 shows that no significant differences exist in the amino acid composition of both isoapyrases The sum of basic or acid amino acids was the same for both isoapyrases within the standard deviation of the method

Tryptophyl residues

In order to establish the tryptophyl residues, reaction with *N*-bromo-succinimide was performed in the presence and in the absence of 10 M urea Figure 1 shows the percentage of 3-methyloxi-indole formation (decrease in *A* at 280 nm) as a function of the consumption of *N*-bromo-succinimide

Figure 1 shows that only in the presence of urea there was a 100% oxidation of tryptophan while in the absence of urea only a 50% decrease in absorbancy was observed It could be thus calculated [11] that both isoenzymes contain two tryptophyl residues of which one is buried since it could be oxidized only in the presence of urea

Sulphur amino acids

A single –SH group could be titrated with DTNB only in the presence of SDS in both isoenzymes Treatment of the enzyme with dithiothreitol before DTNB titration allowed us to determine five –SH groups which agrees with the five cysteic acids found by amino acid analysis of the previously oxidized samples (Table 1) These results indicate that there is one free –SH group and two disulphide bridges per mol of apyrase This –SH group is not catalytically significant [10, 12]

Table 1 Amino acid composition of apyrase isoenzymes

Amino acids	Residues per molecule: Pimpernel* enzyme Normalized to 34 alanines	Desirée* enzyme Normalized to 42 alanines
Lys	26±1 13	26±0 14
His	6±0 21	9±0 90
Arg	10±0 35	10±1 42
Asx	58±1 77	53±0 92
Thr	25†	24±0 07
Ser	34†	30±0 07
Glx	42±1 30	47±0 78
Pro	19±0 38	16±3 20
Gly	42±2 26	41±2 01
Ala	34	42
Val	29†	26±0 15
Met	7±0 15	6±0 32
Ile	21±0 21	24±0 53
Leu	42±1 90	42±0 50
Tyr	27±2 05	27±1 64
Phe	18±0 42	15±1 57
Half-cys	5±0 06	5
Trp‡	2±0 00	2±0 00
Total residues	447	445
MW§	49 430	49 152

*Average value of six analyses Serine and threonine were corrected by extrapolation to 0 time hydrolysis The values of Glx and Asx are total of Glu and Gln and Asp and Asn respectively

†The s d for Thr, Ser and Val of Pimpernel enzyme could not be calculated because the duplicates were oxidized

‡Trp was determined according to ref [11]

§These calculated MW agree with the experimental value of 49 000 obtained previously [10]

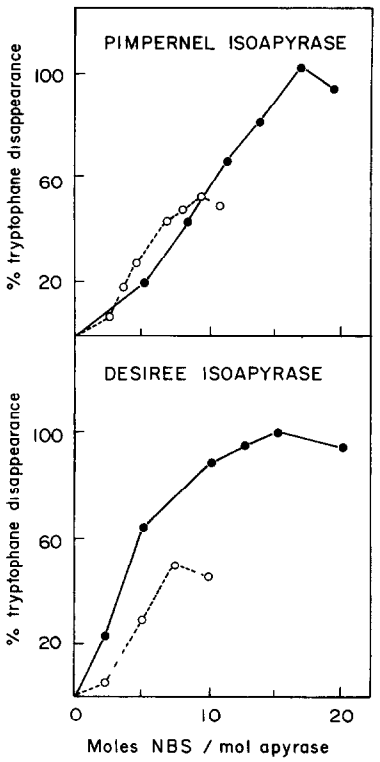

Fig 1 Quantification of tryptophyl residues of isoapyrases Determination of the number of tryptophyl residues was followed by the decrease in absorption at 280 nm, in the presence (●——●) and in the absence (O- - -O) of 10 M urea Amount of protein 1 5 mg

Asparagine and glutamine

Since the molar percentage of basic and acid residues is very similar in both isoenzymes, other evidence has to be sought to explain the difference in pI values previously described which are 8 7 for Pimpernel and 6 7 for the Desirée enzyme [10] Glutamine and asparagine cannot be differentiated from glutamic and aspartic acid through the total amino acid analysis Thus a different distribution between acid and amide residues in both apyrase could explain this difference

In order to elucidate this point, apyrase preparations from both potato varieties were treated with glutaminase or asparaginase and their activity and isoelectrofocusing were studied Asparaginase treatment diminished both enzymic activities (ATPase and ADPase) by 50% in the case of Pimpernel isoenzyme and by 80% in the case of Desirée On the other hand glutaminase inactivated both Pimpernel and Desirée enzymic activities by 25 to 30% This prevented us from carrying out complete deamination of the enzyme, and therefore the isoelectrofocusing which followed this treatment was performed with partially deaminated preparations Thus enzyme species with a different activity and with different charge coexist in our treated samples Table 2 shows the distribution of enzyme activity (except for Pimpernel ADPase) of different charged species formed by enzymatic deamination analysed by isoelectrofocusing

Asparaginase or glutaminase treatment caused the appearance of new bands of more acidic proteins in all cases The pI values of these new bands were up to two pH units lower than those of the original enzymes Part of the apyrase remained unaffected, except for asparaginase treated Desirée enzyme The activity of the more acidic proteins was generally lower than that of the enzyme with the original pI values

It may be thus concluded that the presence of glutamine and asparagine are necessary for full apyrase activity on both isoenzymes The differences of pI observed for the two native isoenzymes cannot be attributed only to a different degree of amidation In that case, the results expected from enzymic deamination would have been a complete equalization of the pI values of both treated enzymes It is, however, clear that glutamine and asparagine in the primary structure account for the more basic character of both protein molecules than could be expected from the total acidic and basic residues

Amino terminal group

TLC of the hydrolysis product of either dansylated isoapyrases showed the presence of DNS-*O*-tyr, ε-*N*-DNS-lys and a spot which co-chromatographed with ε-*N*-DNS-leu, which may thus be identified as the amino terminal residue of both isoenzymes

Table 2 Effect of asparaginase and glutaminase treatment on charge and activity of apyrase isoenzymes, analysed by isoelectrofocusing

	Desirée isoenzyme			
	ATPase		ADPase	
Treatment	pI	nkat	pI	nkat
Control	6 7	62 17	6 7	104 17
Asparaginase	5 8	6 33	6 3	15 13
	4 5	4 17	4 5	0 98
Control	6 7	30 33	6 7	22 50
Glutaminase	6 7	3 63	6 7	4 23
	6 4	2 45	6 4	0 70
	4 1	0 43	5 0	0 28
	3 4	1 33	3 9	0 23

	Pimpernel isoenzyme*	
	ATPase	
Treatment	pI	nkat
Control	8 7	3 53
Asparaginase	8 7	0 50
	7 2	0 33
	6 9	0 85
Control	8 7	94 33
Glutaminase	8 7	17 43
	7 9	4 10
	7 0	3 03
	6 6	2 80

*The yield of ADPase activity is too low to be measured with reliability in the fractions obtained from isoelectrofocusing This is due to the high ATPase/ADPase ratio of Pimpernel isoenzyme Control experiments were performed under identical conditions but without asparaginase and glutaminase

The similarity in amino acid composition, amino-terminus and molecular mass [10] further reinforces the view that apyrases isolated from Pimpernel and Desirée potato varieties are isoenzymes These apyrases differ in the isoelectric point and kinetic parameters The present experiments do not exclude the view that the more basic pI of the Pimpernel isoenzyme may be due to differences of exposure of charged residues to the solvent as well as to a distinct degree of amidation of aspartic or glutamic residues

EXPERIMENTAL

Apyrase activity was followed by Pi liberation from ATP or ADP [1] Two methods of Pi determination were used that of Fiske and SubbaRow [13] for enzymic assays during purification of isoapyrases, and Ernster *et al* [14] after elution of the isoenzyme from isoelectrofocusing gel Proteins were determined by their UV absorption [15] during the isolation of the isoenzymes The method of ref [16] was used only when both enzymes were homogeneous

Purification of the isoenzymes The two apyrases were extracted from cv Pimpernel and cv Desirée of *S tuberosum*. The purification procedures used were the same as previously described in ref [10] except for the last step where the affinity chromatography column had a proportion of 1 1 between Sepharose Cibacron Blue and Sepharose 4B-Cl The enzyme obtained from the purification procedure was eluted with 0 6 M NaCl from a chromatographic column of Sepharose and Cibacron Blue-Sepharose in a 1 1 proportion This was a modification of the last step of enzyme purification [10] in which neither the yield nor the specific activity was changed but a more concd and stable enzyme in a saltless soln was obtained The homogeneity of the two isoenzymes was checked by three procedures disc electrophoresis [17], gel isoelectrofocusing [10] and SDS electrophoresis [18]

Amino acid composition A Beckman Model 118 amino acid analyser was used Samples of *ca* 400 μg enzymes were hydrolysed with 6 M HCl for 24, 48 and 72 hr at 110° [19] Total half-cystine was determined as cysteic acid in a separate sample after performic acid oxidation [20] Tryptophane was determined spectrophotometrically [11] in the presence and absence of 10 M urea The number of –SH groups and –S–S– bridges were established by spectrophotometric titration in the presence of SDS according to refs [21] and [22] respectively

Deamination of isoapyrases by glutaminase and asparaginase treatments The amount of isoenzyme employed was *ca* 600 μg Glutaminase treatment was performed according to ref [23] adding 1 U of this enzyme, at a final vol of 400 μl On the other hand the asparaginase reaction mixture contained 5 U of the corresponding enzyme at a smaller final vol (250 μl) using TES buffer instead of Tris–HCl as described in ref [24] Both assays lasted 22 hr

Isoelectrofocusing Isoapyrase previously treated with asparaginase or glutaminase were subjected to electrofocusing as described in ref [10] The samples were neutralised before applying on the gel Simultaneously native isoapyrases were run as controls

Amino terminal residue The protein was dansylated [25] and dansyl amino acids were identified by two-dimensional chromatography on bilayer micropolyamide plates (Pierce) The method was modified by using the solvent system mentioned in ref [26] R_f values of the 20 dansyl amino acids were determined on this type of polyamide layer

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