IDENTIFICATION AND SUBCELLULAR LOCALIZATION OF TWO ISOENZYMES OF APYRASE FROM *SOLANUM TUBEROSUM*

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

Abstract—Two forms of ATP-diphosphohydrolase were identified in *Solanum tuberosum* tuber var. Ultimus. Their hydrolytic activity ratios (ATPase/ADPase) were over 10 for form A and 1 for form B. In the potato tuber homogenate the hydrolytic activity ratio is 3.0, as a result of contributions of the two forms of apyrase. These two apyrases (A and B) were partially separated and the possibility that they are produced as an artifact by partial proteolysis or subunit aggregation was excluded. The subcellular localization of the Ultimus isoapyrases was studied by differential centrifugation. These enzymes are localized in distinct compartments. The high ratio enzyme (A) lies mainly in the soluble fraction, while the low ratio apyrase (B) is principally bound to membranes. The two isoapyrases differ greatly in their kinetic properties and p*I*, but only slightly in M_r. Both enzymes immunocross-react with antiapyrase Desirée, which is important for isoenzyme detection by the immunowestern blot. This is the first example of two isoenzymes of apyrase in the same variety of *S. tuberosum*.

INTRODUCTION

Apyrase catalyses the splitting of pyrophosphate bonds of tri- and diphosphates with liberation of orthophosphate. Phosphomonoesters are not split by potato apyrase and the only structural requirement for the substrate is the length of the pyrophosphate chain, although organic di- and triphosphates are more readily split than the inorganic phosphates [1]. Bivalent metal ions such as Ca^{2+}, Mn^{2+}, Mg^{2+}, Co^{2+} or Zn^{2+} are always required [2].

This enzyme has been observed in animal tissues such as rat secretory tissues [3], bovine aorta [4] and rat synaptosome [5, 6]. Apyrase has also been found in plant tissues. In soluble form it has been found, for example in cabbage leaves [7], clover seeds [8], potato tuber [9, 10] and potato buds [11]. It has also been observed in an insoluble form in potato tuber [12, 13], in pea stem [14, 15] and in chick pea roots [16].

Our group recently initiated studies on animal apyrase [3] but we have been studying plant ATP-diphosphohydrolase for several years. We have demonstrated that this activity is present in all varieties of *S. tuberosum* that we have tested. Their kinetic properties and p*I* depend on the clonal variety of the isolated apyrase [10]. The ATPase/ADPase ratio ranged from 1.0 to more than 10.0.

We have purified to homogeneity two apyrases from *Solanum tuberosum*. Pimpernel apyrase catalyses the hydrolysis of the γ-phosphoryl group of ATP 10 times faster than the β-phosphoryl group of ADP (ATPase/ADPase = 10), while the Desirée enzyme catalyses both reactions at the same rate (ATPase/ADPase = 1). These two apyrases were characterized [17] and it was shown that the amino acid composition [18] and the possible amino acid residues at the active site did not explain the kinetic or the p*I* differences. In order to explain these differences we chose to work on var. Ultimus whose ATPase/ADPase ratio is 3.0 in the extract. We found that there are two forms of apyrase present in this potato tuber; one with a high ATPase/ADPase ratio and the other with a low ratio. The mixture of these two enzymes resulted in an intermediate ratio in the extract. These isoapyrases differ in their kinetic properties, in their superficial charge and their subcellular distribution. We had not been able earlier to demonstrate the coexistence of isoenzymes of apyrase in the same potato tuber variety.

RESULTS AND DISCUSSION

Identification of isoenzymes of apyrase

The purification scheme employed for Pimpernel and Desirée enzymes [17] was used for the Ultimus apyrase. The ATPase/ADPase ratio was *ca* three, after the initial purification steps (salt precipitation and gel filtration). The last step of the separation was a pseudoaffinity chromatography through a Cibacron Blue–Sepharose column, which partially resolved apyrase activity into two peaks, one with a high ATPase/ADPase ratio (Ultimus A, ATPase/ADPase = 10) which eluted with 1.4 M NaCl and the other with a low ratio (Ultimus B, ATPase/ADPase = 1.0) which eluted with 1.6 M NaCl.

Isoelectrofocusing

In order to eliminate the possibility that the two peaks with a different ATPase/ADPase ratio were an artifact of the affinity chromatography, this separation procedure

was replaced by isoelectrofocusing and by ion exchange chromatography.

The enzymic fraction eluted from a gel filtration column (Sephadex G-100) was submitted to electrofocusing in a pH gradient ranging from 3.0 to 10.0 (Fig. 1). Two peaks with apyrase activity were eluted from the polyacrylamide gel; one with a p*I* of 5.5 (low ATPase/ADPase ratio) and the other with p*I* of 6.6 (high ratio). This result indicates that the two peaks with different ATPase/ADPase ratio are different enzymes and do not arise as an artifact.

Ion exchange chromatography

The apyrase fraction eluted from the Sephadex G-100 column was chromatographed through a CM-Sephadex column. Two peaks with apyrase activity were eluted with abrupt changes in ionic strength. The first peak had a high ATPase/ADPase ratio and was released with 0.35 M NaCl, the second peak had a low ratio and was liberated from the column with 0.5 M NaCl (Fig. 2-upper). Each peak was pooled, concentrated and passed twice more through the same exchanger. This procedure allowed us to obtain each apyrase free from the other, as seen in Fig. 2-lower for the low ratio enzyme. The same result was observed with apyrase Ultimus A (results not shown). These experiments confirmed that the two isoapyrases were not produced as an artifact of the purification procedure.

Partial purification in the presence of protease inhibitors

Another possibility that must be ruled out is interconversion of one apyrase into the other by proteolysis. The two tests employed were the use of protease inhibitors and addition of external proteases.

The inhibitors used were phenylmethylsulphonyl fluoride (PMSF) and pepstatin. A better recovery of enzyme activity was obtained when the purification was done in the presence of the above inhibitors as compared to a control with thioglycolic acid (TGA). In both cases two peaks of apyrase activity with different ATPase/ADPase ratios eluted from CM-Sephadex columns. These results show that addition of protease inhibitors during purification is very useful in order to obtain a high enzymic yield;

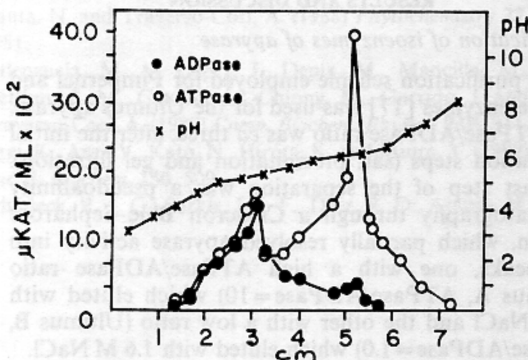
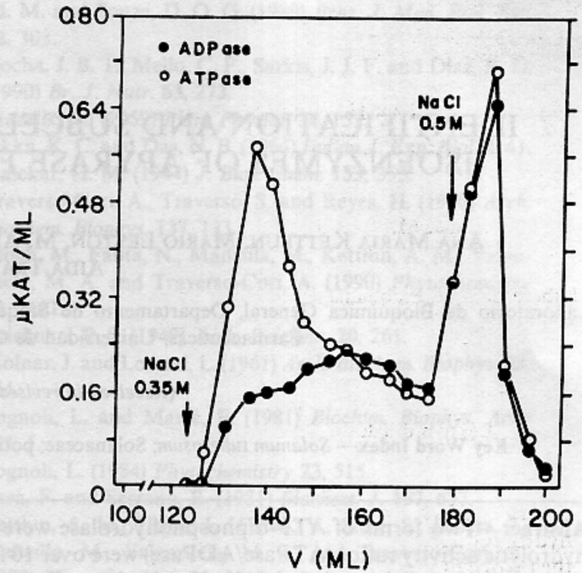


Fig. 1. Gel electrofocusing of Ultimus apyrase. The enzymic fraction obtained from a Sephadex G-100 column was added before the gel polymerization. Ampholines ranged from pH 3 to 10 (x–x) pH profile, (○–○) ATPase activity and (●–●) ADPase activity measured in μkat ml^{-1}.



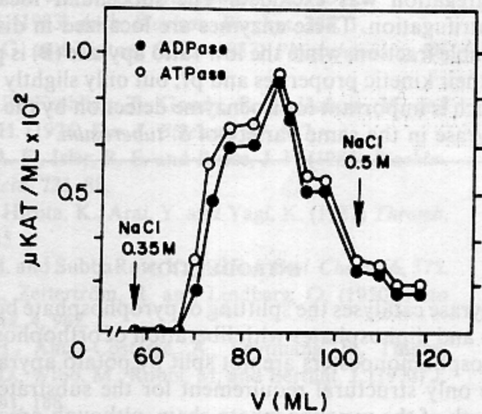


Fig. 2. Reiterative ion-exchange chromatography of apyrase Ultimus B. Columns were filled with CM-Sephadex and equilibrated with 50 mM succinate buffer pH 6. Elution was accomplished with abrupt changes in ionic strength, (○–○) ATPase activity, (●–●) ADPase activity. (Upper) First passage through the CM-Sephadex column; 26.9 μkat of ATPase activity and 25.7 μkat of ADPase activity, obtained from pseudoaffinity chromatography, were added to the column. (Lower) Third passage through the ion exchanger; 0.48 μkat of ATPase and 0.47 μkat of ADPase activity, eluted from two successive passages through CM-Sephadex, were added to the column.

but these compounds do not prevent the detection of two apyrases with different kinetic properties in the tuber of *S. tuberosum* var. Ultimus.

Ultimus A and Ultimus B were also preincubated independently with α-chymotrypsin. Both ATPase and ADPase activities of the two apyrases decreased (Table 1), but there was no significant change in the ATPase/ADPase ratio. A similar result was obtained when preincubation was done in the presence of trypsin. These results show that the apyrases do not interconvert due to a proteolytic activity of the type of trypsin or chymotrypsin.

Table 1. Effect of α-chymotrypsin on apyrase activity and on the ATPase/ADPase ratio of Ultimus enzyme A and B

	ATPase		ADPase		
Preincubation time (min)	(μkat ml^{-1})	% of residual activity*	(μkat ml^{-1})	% of residual activity	ATPase/ADPase ratio
High ratio					
0.5	36.6	100.0	5.8	100.0	6.4
2.0	36.6	100.0	5.8	100.0	6.4
5.0	33.8	92.0	5.8	100.0	5.9
9.0	32.7	86.0	4.7	81.1	6.8
15.0	25.0	68.2	3.9	68.1	6.4
Low ratio					
0.5	51.0	100.0	23.5	100.0	2.2
2.0	55.5	91.9	22.0	93.6	2.5
5.0	36.0	70.6	21.5	91.5	1.7
9.0	26.0	50.9	17.0	72.3	1.5
15.0	25.0	49.0	14.5	61.7	1.7

*The percentages are calculated with respect to a control in which α-chymotrypsin was replaced by 0.1 M borate pH 8. Assays were performed as described in Experimental. Each determination was performed in triplicate.

Determination of M_r

The possibility that the two forms of apyrase found in the potato var. Ultimus differ in states of aggregation of the same subunit was rejected by determination of M_r under both native and denaturing conditions. The M_r of the native enzyme was determined by gel filtration through a Sephacryl S-200 column. The M_r was 46500 for apyrase B and 45300 for apyrase A. Slab gel electrophoresis in the presence of SDS was also used to determine the M_r under denaturing conditions. The values were 51100 for apyrase B and 48300 for apyrase A. These experiments demonstrate that both apyrases are constituted by only one polypeptide subunit and that the apyrase of low ATPase/ADPase ratio is slightly larger than the apyrase of high ratio.

Immunowestern blot

Both apyrases A and B immunocross-reacted with polyclonal and monospecific antibodies prepared against apyrase Desirée. This property was used to identify the apyrases. The protein patterns obtained from a slab SDS-PAGE of apyrase at different purification stages, when stained with Coomassie Blue, revealed two minor protein bands with M_r *ca* 48000 that were present in the extract and were concentrated during the purification procedure.

In the Western blot the two proteins that immuno-cross-react with antiapyrase Desirée and whose M_rs correspond to that of the standard apyrase (homogeneous Desirée enzyme), are the ones which are concentrated during the purification. These results indicate that the two bands with slightly different M_rs that react with antibodies prepared against Desirée enzyme, are probably the two isoapyrases present in the var. Ultimus tuber and that they are present in the initial extract.

Subcellular localization

Evidence for the subcellular localization of the Ultimus isoapyrases was obtained by differential centrifugation. We studied the corresponding distributions of both ATPase and ADPase activities in each fraction, together with the classical enzyme marker activities. The microsomal fraction, P_3, was not contaminated either with mitochondrial or with cytosolic fractions because the respective enzyme markers (glutamate dehydrogenase and lactate dehydrogenase) were not detected. The 100000 *g* pellet (P_3) contained 5′-nucleotidase activity, suggesting the presence of plasma membrane in this microsomal fraction.

The results obtained in a study related to the apyrase activity are summarized in Table 2. In the extract (H), the ATPase/ADPase ratio was *ca* 3.0, while in the insoluble fractions (P_1, P_2, P_3) the ratio was low (near 1) and in the soluble fraction (S) this ratio was high (above 5). These results indicate that the two isoapyrases have different subcellular sites, the low ratio apyrase being preferentially bound to membranes while the Ultimus A enzyme is principally in the soluble fraction. This is further confirmed by isoelectrofocusing of the solubilized fractions (Fig. 3). In the solubilized extract (Fig. 3-upper), two peaks with apyrase activity can be seen, one with low ATPase/ADPase ratio with a p*I* of 5.0 and the other with a high ratio and a p*I* of 6.0. In the soluble fraction (Fig. 3-middle,) one principal peak of high ratio and a second with intermediate ATPase/ADPase ratio were found. It is important to note that the high ratio enzyme is unstable to isoelectrofocusing while the low ratio apyrase is stable. In the solubilized microsomal fraction, (Fig. 3-lower) two peaks with apyrase activity were also observed. The principal peak had an ATPase/ADPase ratio of one and a p*I* of 5.4. There was also a very small peak with a higher ratio and p*I*.

Table 2. Subcellular distribution of isoapyrases of *S. tuberosum* var. Ultimus

Fraction	Protein (mg g^{-1} tissue)	ATPase activity (μkat × 10^2 g^{-1} tissue)	Distribution (%)	s.a. (μkat mg^{-1} protein)	ADPase activity (μkat × 10^2 g^{-1} tissue)	Distribution (%)	s.a. (μkat mg^{-1} protein)	ATPase/ADPase ratio
H	4.460	52.58	100.00	12.03	18.35	100.00	0.042	2.86
P_1	0.069	0.27	0.51	0.04	0.21	1.11	0.029	1.30
P_2	0.258	1.35	2.57	0.05	0.55	4.92	0.035	1.50
P_3	0.422	9.02	17.14	0.21	6.35	4.58	0.150	1.40
S	3.216	34.97	66.49	0.11	6.40	34.87	0.020	5.46
Total recovery			86.71			75.50		

H = Homogenate; P_1 = 1000 *g* pellet; P_2 = 12 000 *g* pellet; P_3 = 100 000 *g* pellet. S = 100 000 *g* supernatant. These values are the average of five independent experiments.

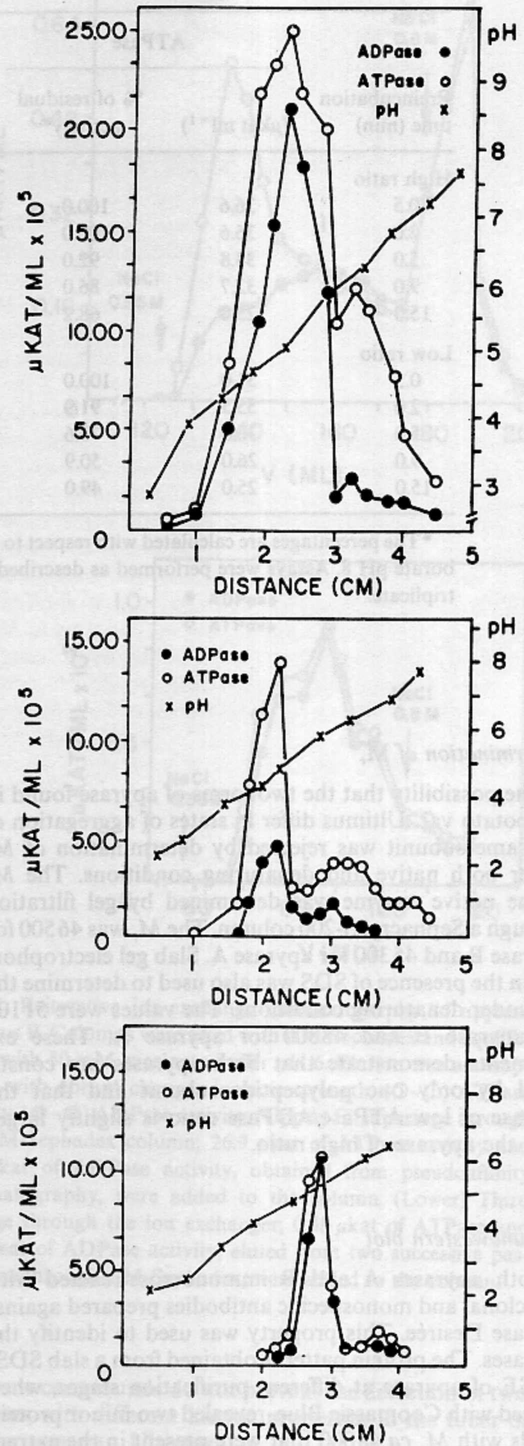


Fig. 3. Isoelectrofocusing of different subcellular fractions and apyrase activity determination. Ampholines ranged from pH 3 to 10 (x–x) pH profile, (○–○) ATPase activity and (●–●) ADPase activity were measured each 0.2 cm of gel. (Upper) Homogenate solubilized with 2 M NaCl. (Middle) 100 000 *g* supernatant. (Lower) 100 000 *g* pellet solubilized with 2 M NaCl.

Solubilization of the microsomal apyrase was easy and only a short incubation with 2 M NaCl required. There was no change in the ATPase/ADPase ratio with solubilization, indicating that the ratio is characteristic and is independent of the state of binding of the enzyme to membranes.

These results are important because they support the proposed hypothesis for distinct subcellular distribution of both isoapyrases, Ultimus A and B. Outwardly apyrase B is preferentially bound to membranes so it is found largely in the P_3 fraction when we measure the activity either directly or after isoelectrofocusing. Nonetheless Ultimus B might also be found in the soluble fraction because the ATPase/ADPase ratio of this fraction is 5.5, which is much lower than that of the purified enzyme (A), which is more than 10. This last enzyme (Ultimus A) is almost exclusively located in the cytosol with no more than a small percentage bound to membranes.

The different subcellular distributions of both Ultimus apyrases give additional evidence for the conclusion that these two fractions with apyrase activity are different isoenzymes, kinetically different, that coexist in the tuber of this variety of *S. tuberosum* where they may play different roles, because they are localized in separate compartments of the cell.

Effect of inhibitors

The effect of some specific inhibitors of ATPase and adenylate kinase on the cytosolic and microsomal fractions are summarized in Table 3. The low sensitivity towards ouabain excludes the presence of (Na^+, K^+)-ATPase in both subcellular fractions. Mitochondrial ATPase may also be excluded because ATPase activity of both fractions (P_3 and S) is not inhibited by oligomycin and, on the contrary, both P_2 and H fractions are inhibited 24.4 and 15.7% by this substance, respectively. This result is expected because these fractions (P_2 and homogenate) contain mitochondria.

Table 3. Effect of inhibitors on Ultimus apyrases A and B obtained from subcellular fractionation

Inhibitors	Concentration	Substrate	Inhibition (%) P_3	S
DES	10 mM	ATP	1.1	12.9
		ADP	3.4	11.6
Azide	10 mM	ATP	36.2	36.8
		ADP	47.3	67.8
Ouabain	5 mM	ATP	0.0	0.0
		ADP	0.0	0.0
Oligomycin	0.9 mg ml^{-1}	ATP	0.0	0.0
		ADP	*—	—
Ap5A	0.5 mM	ATP	0.0	0.0
		ADP	0.0	0.0

Assay conditions as described in the Experimental. Each experiment was done in triplicate.

*Not determined.

Diethylstilbestrol (DES) inhibits transport of ATPase in fungi and plant cells [19], and at low concentration (below 1 mM) it inhibits ATPase and ADPase activities of various plant microsomal preparations [14, 20]. In potato tuber var. Ultimus, ATPase and ADPase activities present in both the soluble and microsomal fractions are slightly inhibited by DES.

The adenylate kinase inhibitor adenylyl (3',5')-adenosine pentaphosphate (Ap5A) [21] did not change the ATPase and ADPase activities. Azide inhibits apyrase activities of S and P_3 fractions of *S. tuberosum* var. Ultimus. This inhibition by azide was also found in membrane-bound apyrase of several animal tissues [22, 23] and of potato tuber [3].

Experiments with these inhibitors suggest that in the P_3 and S fractions of potato tuber var. Ultimus there exists an ATPase activity that does not correspond to (Na^+, K^+)-ATPase or to mitochondrial ATPase. There is also an ADPase activity which is not due to the coupled action of adenylate kinase and ATPase but corresponds to an ATP-diphosphohydrolase. Isoelectrofocusing of these fractions (P_3 and S) demonstrates that each isoenzyme has ATPase and ADPase activities, but with different ATPase/ADPase ratio.

EXPERIMENTAL

Materials. Reagent grade chemicals were used. Apyrase was obtained from a pure strain of *S. tuberosum* cv. Ultimus supplied by the Instituto de Invesigaciones Agropecuarias "La Platina", Santiago, Chile.

Enzyme assays. Apyrase activity was estimated using ATP (ATPase activity) or ADP (ADPase activity) as substrate, at a final concn of 2 mM, in the presence of 5 mM $CaCl_2$ and 100 mM Na-succinate, pH 6. Inorganic phosphate (Pi) was determined by the methods of ref. [24] or ref. [25], according to the sensitivity required. A unit of activity (μkat) is equivalent to 1 μmol of Pi liberated per sec at 30°. The 5'-nucleotidase activity was measured according to ref. [26] and glutamate dehydrogenase activity was assayed as described in ref. [27]. Lactate dehydrogenase activity was measured as described in ref. [28].

Apyrase purification. Both apyrases were partially separated as previously reported [17]. The last step of Cibacron-Blue F-3GA linked to Sepharose was replaced either by gel electrofocusing or by a CM-Sephadex column equilibrated with 50 mM Na-succinate, pH 6. The elution was done with either abrupt changes in ionic strength (0.35 and 0.45 M NaCl) for enzyme A and B respectively, or with linear gradients (0–2 M NaCl). Partial purification in the presence of protease inhibitors was performed as described above. The last step of separation was on a CM-Sephadex column; the enzyme was eluted with a linear gradient of ionic strength (0–2 M NaCl). In this purification scheme thioglycolic acid (TGA) was replaced by 100 μM PMSF and 1 μM pepstatin.

Protein determination. Protein concentrations were measured at A_{280} or by the method of ref. [29].

Gel electrofocusing: The gel mixture was as described in ref. [30]. Ampholine sols (Sigma) ranged from pH 3 to 10. The sample was added before polymerization so it was homogeneously distributed along the gel. Voltage was constant at 300 V. The current decreased to a constant value in *ca* 110 min. Protein bands were stained with Coomassie Brilliant Blue [31]. In a parallel experiment, gel activity was measured after elution of the proteins from gel slices (0.2 cm) with 0.1 M NaCl overnight at 4°.

Partial proteolysis of apyrase Ultimus A and B with added proteases. Enzymes A and B from a Cibacron Blue–Sepharose column (*ca* 1 mg) were preincubated during variable times with the proteolytic enzymes (0.4 mg). When α-chymotrypsin was used, preincubation was performed in 10 mM borate, pH 8. When the experiments were done with trypsin, the buffer was 10 mM (*N*,*N*-bis-2-hydroxyethyl)-2-aminoethane sulphonic acid (BES) pH 7.5.

M_r *determination*: The M_r was estimated by gel filtration [32] using a Sephacryl S-200 column (1.16 × 55 cm) equilibrated with 300 mM NaCl in 50 mM Na-succinate pH 4 and 20 mM TGA. M_r was also estimated under denaturating conditions by slab SDS polyacrylamide gel electrophoresis in slabs of (0.03 × 6 × 10 cm); gel mixtures as described in ref. [33]. The M_r was calculated according to ref. [34].

Immunowestern blot. SDS/polyacrylamide gel eletrophoresis was performed on 12.5% gels as described in ref. [33]. Proteins were transferred to nitrocellulose paper as described in ref. [35]. Binding of the antibody to the nitrocellulose-immobilized proteins was done by the method of ref. [36]. Binding was assessed by detection of a coloured product produced by peroxidase-conjugated goat anti-(rabbit IgG) in the presence of 4-chloro-1-naphthol plus H_2O_2.

Subcellular fractionation. The procedure followed was that described in ref. [3] but the extraction buffer was 25 mM 2-(*N*-morpholino)ethanesulphonic acid (MES), pH 6, 250 mM sucrose, 4 mM cysteine, 1 mM PMSF and 10 mM KCl. The solubilization of the microsomal fraction was achieved by adding solid NaCl to the resuspended 100000 *g* pellet up to 2 M. This sample was left at room temp. for 10 min with occasional agitation on a Vortex mixer.

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REFERENCES

1. Del Campo, G., Puente, J., Valenzuela, M. A., Traverso-Cori, A. and Cori, O. (1977) *Biochem. J.* **167**, 525.
2. Valenzuela, M. A., Kettlun, A. M., Mancilla, M., Calvo, V., Fanta, N. and Traverso-Cori, A. (1988) *Phytochemistry* **27**, 1981.
3. Valenzuela, M. A., López, J., Depix, M., Mancilla, M., Kettlun, A. M., Catalán, L., Chiong, M., Garrido, J. and Traverso-Cori, A. (1989) *Comp. Biochem. Physiol.* **93B**, 911.
4. Yagi, K., Arai, Y., Kato, N., Hirota, K. and Miura, Y. (1989) *Eur. J. Biochem.* **150**, 509.
5. Schadeck, R. J. G., Sarkis, J. J. F., Diaz, R. D., Araujo, H. M. M. and Souza, D. O. G. (1989) *Braz. J. Med. Biol. Res.* **22**, 303.
6. Rocha, J. B. T., Mello, C. F., Sarkis, J. J. F. and Diaz, R. D. (1990) *Br. J. Nutr.* **63**, 273.
7. Mazelis, M. (1959) *Plant Physiol.* **34**, 153.
8. Sikka, K. C. and Das, N. B. (1964) *Indian J. Exp. Biol.* **2**, 41.
9. Kalckar, H. M. (1944) *J. Biol. Chem.* **153**, 355.
10. Traverso-Cori, A., Traverso, S. and Reyes, H. (1970) *Arch. Biochem. Biophys.* **137**, 133.
11. Anich, M., Fanta, N., Mancilla, M., Kettlun, A. M., Valenzuela, M. A. and Traverso-Cori, A. (1990) *Phytochemistry* **29**, 1411.
12. Krishnan, P. S. (1949) *Arch. Biochem.* **20**, 261.
13. Molnar, J. and Lorand, L. (1961) *Arch. Biochem. Biophys.* **93**, 354.
14. Tognoli, L. and Marré, E. (1981) *Biochim. Biophys. Acta* **642**, 1.
15. Tognoli, L. (1984) *Phytochemistry* **23**, 515.
16. Vara, F. and Serrano, R. (1981) *Biochem. J.* **197**, 637.
17. Kettlun, A. M., Uribe, L., Calvo, V., Silva, S., Ribera, J., Mancilla, M., Valenzuela, M. A. and Traverso-Cori, A. (1982) *Phytochemistry* **21**, 551.
18. Mancilla, M., Kettlun, A. M., Valenzuela, M. A. and Traverso-Cori, A. (1984) *Phytochemistry* **23**, 1397.
19. Serrano, R. (1983) *Arch. Biochem. Biophys.* **227**, 1.
20. Carter, S. G. and Tipton, C. L. (1987) *Phytochemistry* **26**, 2191.
21. Feldhaus, P., Frohlich, T., Goody, R. S., Isahow, M. and Schirmer, H. (1975) *Eur. J. Biochem.* **57**, 197.
22. Knowles, A. F., Isler, R. E. and Reece, J. F. (1983) *Biochim. Biophys. Acta.* **731**, 88.
23. Miura, Y., Hirota, K., Arai, Y. and Yagi, K. (1987) *Thromb. Res.* **46**, 685.
24. Fiske, C. M. and SubbaRow, Y. (1925) *J. Biol. Chem.* **66**, 375.
25. Ernster, L., Zettertröm, R. and Lindberg, O. (1950) *Acta Chem. Scand.* **4**, 942.
26. Beaufay, H., Amar-Costesec, A., Feytmans, E., Thines-Sempoux, D., Wibo, M., Robbi, M. and Berthat, J. (1974) *J. Cell. Biol.* **61**, 188.
27. Leighton, F., Poda, B., Beaufay, H., Beaudhuin, P., Coffey, J. W., Flower, S. and de Duve, C. (1968) *J. Cell. Biol.* **37**, 482.
28. Bergmeyer, H. V., Gawehn, K. and Grassed, M. (1974) *Methods in Enzymatic Analysis*, Vol. 1, pp. 425. Academic Press, London.
29. Bradford, M. M. (1976) *Analyt. Biochem.* **72**, 248.
30. Pearce, F. L., Banks, B. E. C., Banthorpe, D. V., Berry, A. R., Davis, H. S. and Vernon, C. (1972) *Eur. J. Biochem.* **29**, 417.
31. Winter, A. E. K. and Anderson, V. (1977) *LKB Application Note.* **250**. December.
32. Andrews, P. (1964) *Biochem. J.* **91**, 222.
33. Laemmli, U. K. (1970) *Nature* **227**, 680.
34. Weber, K. and Osborn, M. (1969) *J. Biol. Chem.* **244**, 4406.
35. Towbin, H., Staehelin, T. and Gordon, J. (1979) *Proc. Natl Acad. Sci. U.S.A.* **76**, 4350.
36. Burnette, W. N. (1981) *Analyt. Biochem.* **112**, 195.