Pergamon

PII: S0031-9422(96)00259-2

Phytochemistry, Vol. 43, No. 2, pp. 359–365, 1996

0031-9422/96 $15.00 + 0.00

DIFFERENTIAL INHIBITION OF EUKARYOTE PROTEIN KINASES BY CONDENSED TANNINS

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(*Received in revised form* 26 *February* 1996)

Key Word Index—Condensed tannins; procyanidin; prodelphinidin; protein kinase; enzyme inhibition.

Abstract—Condensed tannins, isolated from a variety of plant sources, were characterized according to the constituent flavans, being based on procyanidin and/or prodelphinidin and having a *cis* or *trans* stereochemistry at positions 2 and 3. All the tannin preparations are potent inhibitors of rat liver cyclic AMP-dependent protein kinase catalytic subunit (cAK) with IC_{50} values (concentrations for 50% inhibition) ranging from 0.009 to 0.2 μM. The tannin preparations are very good inhibitors of rat brain Ca^{2+}- and phospholipid-dependent protein kinase C (PKC) (IC_{50} values in the range 0.3–7 μM), wheat embryo Ca^{2+}-dependent protein kinase (CDPK) (IC_{50} values in the range 0.8–7 μM) and of calmodulin (CaM)-dependent myosin light chain kinase (MLCK) (IC_{50} values in the range 7–24 μM). One of the most effective preparations, that from the leaves of *Ribes nigrum*, has IC_{50} values with respect to cAK, PKC, CDPK and MLCK of 0.009, 0.6, 2.0 and 16 μM, respectively. In general, the order with respect to sensitivity to inhibition by these condensed tannins is cAK > PKC > CDPK > MLCK. The *Ribes nigrum* preparation is a competitive inhibitor of cAK with respect to both ATP and synthetic peptide substrate. These condensed tannin preparations are the most potent plant-derived inhibitors of cAK yet found. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

Plants elaborate a large variety of defensive secondary metabolites [1] and a number of such bioactive compounds have been shown to interact with protein kinases involved in eukaryote cell signal transduction. Thus flavonoids variously inhibit the catalytic subunit of cyclic AMP-dependent protein kinase (cAK) [2], Ca^{2+}-and phospholipid-dependent protein kinase (PKC) [3], CaM-dependent myosin light chain kinase (MLCK) [4, 5] and plant Ca^{2+}-dependent protein kinase (CDPK) [5]. Several xanthones are potent inhibitors of cAK and CDPK but are weaker inhibitors of MLCK [6]. A number of plant-derived anthraquinones variously inhibit PKC, cAK, CDPK and MLCK [7]. PKC, cAK and CDPK are inhibited by a variety of plant-derived, catechin-related compounds, the concentrations for 50% inhibition (IC_{50} values) of the more potent inhibitors of these protein kinases being about 1 μM [8]. The most potent plant-derived protein kinase inhibitors found so far are various gallic acid esters (hydrolysable tannins) from the medicinal plant *Phyllanthus amarus* [9]. Increasing number of esterified gallic acid residues in such compounds correlates with increasing affinity for cAK. The more effective hydrolysable tannins of this kind are potent inhibitors of cAK (IC_{50} values about 0.2 μM) but are less effective as inhibitors of PKC, CDPK and MLCK [9].

Other plant-derived inhibitors of eukaryote protein kinase include the stilbene derivative piceatannol, an inhibitor of receptor tyrosine kinase (RTK) [10], the isoflavone genistein (also an RTK inhibitor) [11], the benzophenanthridine alkaloid chelerythrine (an inhibitor of PKC) [12] and the diferuloyl compound curcumin, an inhibitor of phosphorylase b kinase [13] and of PKA and PKC [14]. Some non-aromatic, non-phenolic plant defence compounds are protein kinase inhibitors. Thus a set of amphiphilic, acidic triterpenoids (including 18α- and 18β-glycyrrhetinic acids, ursolic acid and oleanolic acid) are very selective inhibitors of cAK [15]. These compounds are inactive or very poor as inhibitors of CDPK, PKC and MLCK [15]. Similarly, the carotenoid crocetin is a very selective inhibitor of cAK (IC_{50} 3 μM) while being a very poor inhibitor of PKC, CDPK and MLCK [15].

We have previously shown that a range of condensed tannins deriving from the cladodes of *Phyllocladus trichomanoides*, the bark of *Pseudotsuga menziesii* and the heartwood of *Acacia melanoxylon* are potent inhibitors of PKC and CDPK while being in general

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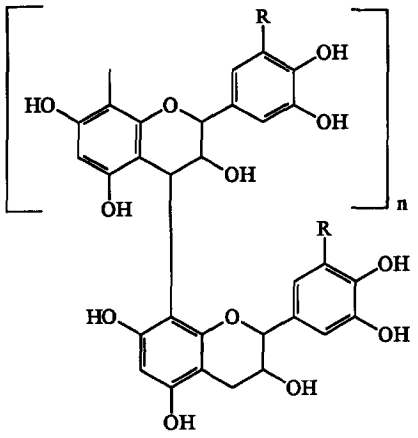

Fig. 1. The structure of condensed tannins. Condensed tannins are polymers consisting of flavan monomer units linked together by mostly C-4 to C-8 and to a lesser extent C-4 to C-6. Where R = H it is a procyanidin and where R = OH it is a prodelphinidin. The sterochemistry at the 2- and 3-position can be *cis* or *trans* as detailed in Table 1. The mean molecular weights of the condensed tannin preparations studied are also listed in Table 1.

relatively poorer inhibitors of cAK [8]. Increasing degree of catechin polymerization correlates with increasing inhibitory effectiveness [8]. The procyanidin dimer, trimer and tetramer from the bark of *P. menziesii* have IC_{50} values for PKC and CDPK of about 1 μM [8]. The present paper shows that a variety of condensed tannin preparations from a variety of other sources are the most potent plant-derived inhibitors of cAK yet found.

RESULTS AND DISCUSSION

Isolation of condensed tannins

Condensed tannins are polymers consisting of flavan monomers, such as procyanidin and prodelphinidin units, linked together mostly by C-4 to C-8 and to a lesser extent by C-4 to C-6 (Fig. 1). The stereochemistry of the C-2 and C-3 substituents in the heterocyclic ring can be *cis* or *trans*. A number of condensed tannins were purified from a variety of plant sources as described in Experimental. The condensed tannin preparations are heterogeneous in composition. However the relative proportions of compounds with *cis* or *trans* stereochemistry, the ratio of procyanidin to prodelphinidin in each preparation and estimates of mean molecular weight have been determined as previously described [16] for the various preparations (Fig. 1; Table 1).

Inhibition of cAK by condensed tannins

All of the tannin preparations are potent inhibitors of the catalytic subunit (cAK) of rat liver cyclic AMP-dependent protein kinase (PKA) with preparations **2–18** having IC_{50} values for cAK (concentrations for 50% inhibition of the enzyme) of about 10–100 nM (Table 2). Plots of cAK activity versus tannin preparation concentration are presented in Fig. 2. The least effective preparation is that of apple tannins (**1**; IC_{50} value 168 nM) which contains only procyanidins (average M_r 1800) (Table 1). It is notable that by and large the condensed tannin preparations that are less effective as inhibitors of cAK (**2**, **7**, **9**, **10**, **4**, **5** and **1** in order of decreasing effectiveness) with IC_{50} values in the range 22–168 nM have 2- and 3-*cis*/*trans* ratios of 80/20 to

Table 1. The composition and relative stereochemistry proportions of condensed tannin preparations*

Tannin preparation	*cis*/*trans*	Procyanidin/prodelphinidin	M_N†
1 *Malus communis* tannins	—	100/0	1800
2 *Sorghum vulgare* seed	92/8	80/20	2500
3 *Grevillea robusta* leaf	39/61	72/28	5000
4 *Photinia glabrescens* leaf	100/0	97/3	3800
5 *Lotus corniculatus*	80/20	87/13	1800
6 *Ribes grossularia* fruit	63/37	77/23	2700
7 *Chaenomeles sinensis* fruit	100/0	94/6	unknown
8 *Ribes sanguineum* leaf	10/90	12/88	3300
9 *Vitis vinifera* leaf	85/15	95/5	2900
10 *Phoenix canariensis* frond	100/0	100/0	unknown
11 *Lotus pedunculatus*	20/80	80/20	3000
12 *Salix caprea* catkins	78/22	38/62	unknown
13 *Feijoa sellowiana* fruit	70/30	85/15	unknown
14 *Ribes nigrum* leaf	6/94	13/87	4300
15 *Acacia mearnsii* polymer‡	—	—	unknown
16 *Aesculus hippocastanum* unripe fruit	100/0	97/3	1750
17 *Ribes rubrum* fruit	24/76	78/22	2700
18 *Vaccinium corymbosum* fruit	100/0	96/4	3500

*The structure type is detailed in Fig. 1.

†M_N was assumed as 3000 for the unknown M_N tannin preparations.

‡This is a mixed type different from the others.

Table 2. Inhibition of protein kinase by condensed tannin preparations

Preparation	IC_{50} (μM)*			
	cAK	CDPK	PKC	MLCK
1	0.168	7	6.7	11
2	0.022	2	1.0	15
3	0.010	0.8	0.5	7
4	0.052	2	0.4	8
5	0.070	2	0.7	24
6	0.016	2	0.7	15
7	0.024	2	0.6	21
8	0.012	2	0.5	16
9	0.028	2	0.6	16
10	0.040	2	0.3	18
11	0.009	2	0.5	21
12	0.031	2	0.5	14
13	0.021	2	0.5	15
14	0.023	2	0.3	15
15	0.019	1	0.5	17
16	0.026	3	1.1	21
17	0.009	2	0.6	16
18	0.011	2	0.5	13

*Rat liver cAK, wheat germ CDPK, avian MLCK and rat brain PKC were assayed using synthetic peptide substrates as described in the Experimental in the presence or absence of increasing concentrations of the test compounds. Test compounds were added dissolved in 10% DMSO to give 2% final DMSO concentration (cAK and CDPK assays) or 1.7% final DMSO concentration (PKC and MLCK assays). Concentrations for 50% inhibition (IC_{50} values) were determined by interpolation from plots of protein kinase activity versus inhibitor concentration.

100/0. In addition compounds **2**, **7**, **9**, **10**, **4**, **5** and **1** have high proportions of procyanidin/prodelphinidin ranging from 80/20 to 100/0 (Table 1). Most of the more effective condensed tannin cAK inhibitors found here (**17**, **11**, **3** and **8**; IC_{50} values 9, 9, 10 and 12 nM, respectively) have a relatively low ratio of 2- and 3-position *cis/trans* (ranging from 10/90 to 39/61) and a substantial proportion of prodelphinidin component (procyanidin/prodelphinidin ratios ranging from 12/88 to 80/20 (Table 1). However notable exceptions to this pattern are the preparation from *Vaccinium corymbosum* fruit (**18**) and *Ribes nigrum* leaf (**14**). Preparation **18** is a potent cAK inhibitor (IC_{50} value 11 nM) while having all *cis* components and being largely procyanidin in composition (procyanidin/prodelphinidin ratio 96/4) (Table 1). Conversely, preparation **14**, which has a *cis/trans* ratio of 6/94 and a procyanidin/prodelphinidin ratio of 13/87 is less effective than **18** as an inhibitor of cAK (IC_{50} value 23 nM; Tables 1 and 2). In addition, condensed tannin preparations **6**, **13** and **12**, that have intermediate *cis/trans* ratios of 63/37 to 78/22 and procyanidin/prodelphinidin ratios of 38/62 to 85/15, have IC_{50} values of 16, 21 and 31 nM respectively (Tables 1 and 2). While noting that there are a number of exceptions to this, the general trend is that the condensed tannin preparations that are more effective as cAK inhibitors have a higher proportion of 2- and 3-*trans* configuration compounds and have a substantial prodelphinidin component. All of the preparations examined are potent inhibitors of cAK.

Inhibition of CDPK, PKC and MLCK by plant condensed tannin preparations

Preparations **2–18** have similar effectiveness as inhibitors of rat brain PKC with IC_{50} values in the range of 0.3 to 1.0 μM and are slightly less effective as inhibitors of wheat embryo CDPK (IC_{50} values in the range 0.8–3 μM) (Table 2). However condensed tannin preparations **2–18** are much less effective as inhibitors of calmodulin (CaM)-dependent MLCK with IC_{50} values for the protein kinase in the range of 7–24 μM (Table 2). Preparation **1** is a much poorer inhibitor of cAK, PKC and CDPK than the other preparations examined (IC_{50} values 168 nM, 6.7 μM and 71 μM, respectively) while being one of the better inhibitors of MLCK (IC_{50} value 11 μM) (Table 2).

Mechanism of inhibition of cAK by condensed tannins

The mechanism of inhibition of cAK by the most potent condensed tannin preparations, namely preparation **17**, was examined by determining Lineweaver–Burk (double-reciprocal) plots of v_0^{-1} versus (substrate concentration)$^{-1}$ in the presence of absence of various concentrations of the inhibitor (Figs 3A–3C). Preparation **17** inhibits cAK in a fashion that is competitive with respect to ATP (K_i 0.048±0.005 μM, mean± deviation from two determinations) (Figs 3A and 3B). However **17** inhibits cAK in a fashion that is non-competitive with respect to the synthetic peptide substrate kemptide (LRRASLG) (K_i 0.035 μM) (Fig. 3C).

DISCUSSION

The present results show that a range of condensed tannin preparations from a variety of plant sources are potent inhibitors of eukaryote protein kinases. The relative protein kinase sensitivity to these inhibitors is in the order cAK > PKC, CDPK > MLCK (Table 2). These condensed tannin preparations are the most potent plant derived cAK inhibitors yet found, the most potent preparations having IC_{50} values of about 10 nM (Table 2). The IC_{50} values for PKC and CDPK of these tannin preparations are similar to those found for the procyanidin dimer, trimer and tetramer from the bark of *P. menziesii* (about 1 μM) [8]. However the IC_{50} values for cAK of the more potent present preparations (9–30 mM) are about 100–1000 times lower than the IC_{50} values for cAK of the *P. menziesii* procyanidins [8].

Condensed tannins are expected to interact with proteins in general [17, 18]. However the present preparations exhibit a marked specificity for cAK and least affinity for MLCK (Table 2). Thus the most

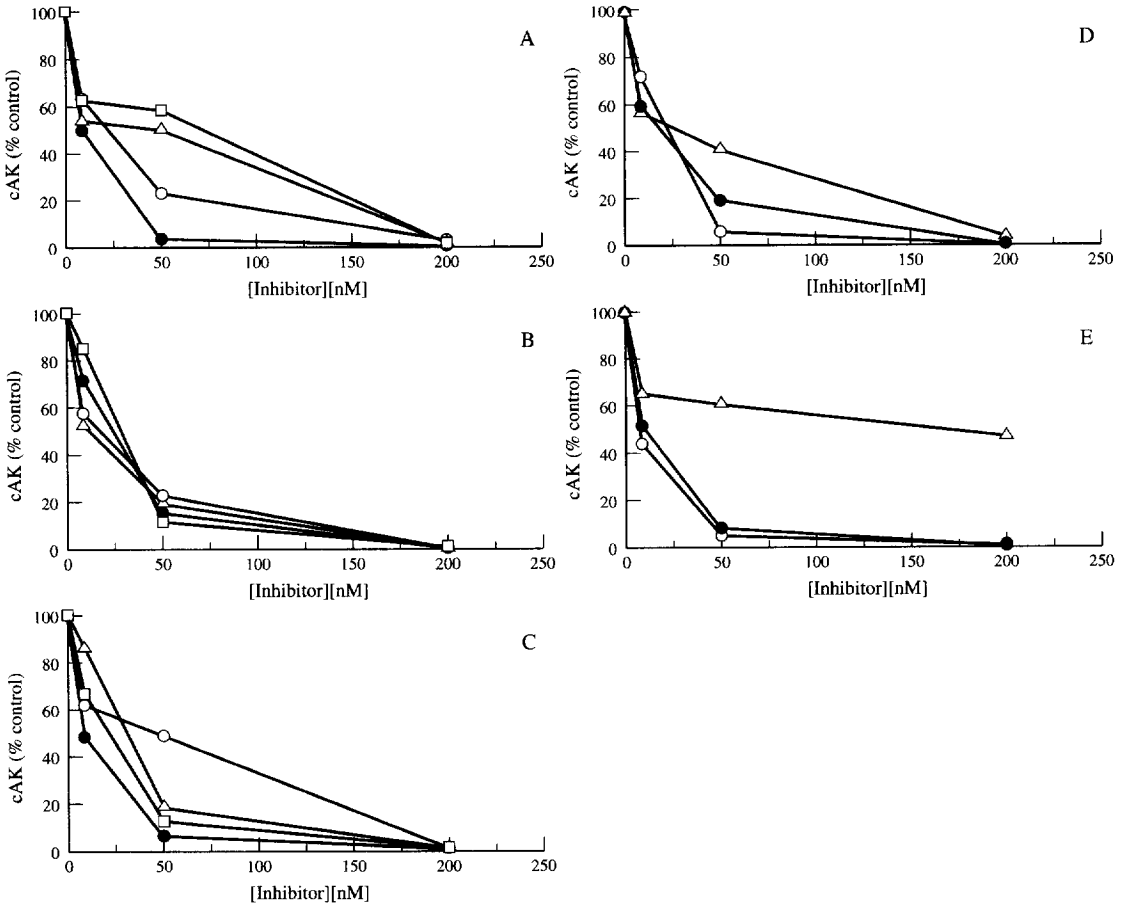

Fig. 2. Inhibition of cAK by plant tannin preparations. Rat liver cAK was assayed with 20 μM kemptide as substrate in the standard assay conditions containing 2% DMSO and increasing concentration of each tannin preparation. The cAK activity is expressed as % control (no added inhibitor). (A), ○ – ○, 2; ● – ●, 3; △ – △, 4; □ – □, 5; (B), ○ – ○, 6; ● – ●, 7; △ – △, 8; □ – □, 9; (C), ○ – ○, 10; ● – ●, 11; △ – △, 12; □ – □, 13; (D), ○ – ○, 14; ● – ●, 15; △ – △, 16; (E), ○ – ○, 17; ● – ●, 18; △ – △, 1.

potent preparation (**17**) has IC_{50} values for cAK, PKC, CDPK and MLCK of 9 nM, 0.6 μM, 2.0 μM and 16 μM, respectively (Table 2). Naturally-occurring protein kinase inhibitors showing selectivity for cAK include hydrolysable tannins from *P. amarus*, the most potent of which has IC_{50} values for cAK, CDPK, PKC and MLCK of 0.2, 2, 26 and 56 μM, respectively [9]. A number of amphiphilic triterpenoids (specifically, those having a 3-hydroxy and a distal carboxy at the opposite end of an otherwise non-polar triterpenoid nucleus) are selective inhibitors of cAK [15], as is the carotenoid crocetin [15]. However the IC_{50} values for cAK of these non-phenolic compounds are 100–1000 times higher than those for the better condensed tannin preparations described here [15].

The most selective synthetic cAK inhibitor so far found is the isoquinoline derivative H-89 (*N*-[2-(*p*-bromocinnamylamino)ethyl] - 5 - isoquinolinesulphonamide) which has K_i values for cAK, PKC and MLCK of 50 nM, 31 μM and 28 μM, respectively [19]. The only apparent physical structural feature common to H-89 and the condensed tannin constituents is a planar, heterocyclic, bicyclic ring associated with more mobile polar residues (Fig. 1). H-89 [19] and the condensed tannin preparation **17** (Figs 3A and 3B) inhibit cAK in a fashion that is competitive with respect to ATP. The binding of ATP to cAK involves interaction of the adenine moiety with a hydrophobic pocket determined in part by two antiparallel β strands (β1 and β2) and hydrogen bonding of the phosphate groups to the so-called 'phosphate anchor' loop linking these strands [20, 21]. A feasible model is that the planar, hydrophobic ring of H-89 and a flavan ring of the condensed tannins bind to the purine-binding hydrophobic site with the more mobile, polar groups being involved in hydrogen bonding interactions with other parts of the active site. The small synthetic peptide substrate kemptide (LRRASLG) binds to cAK at a site removed from this purine-binding site [20, 21]. Consistent with this, tannin preparation **17** inhibits cAK in a fashion that is noncompetitive with respect to kemptide (Fig. 3C) but competitive with respect to ATP (Figs 3A and B).

The 100-fold difference in affinity of the condensed tannins for cAK and plant CDPK is consistent with a

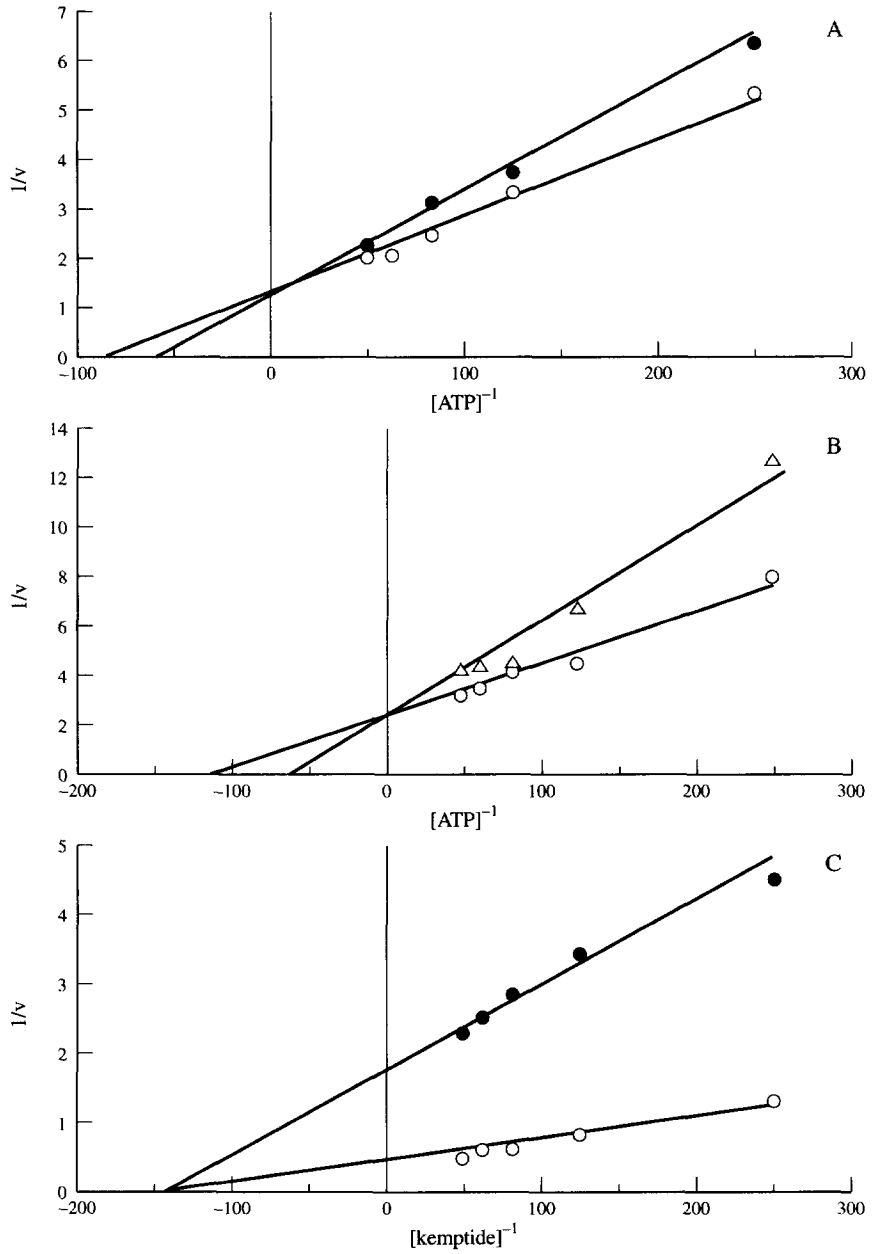

Fig. 3. Inhibition of cAK by the tannin preparation **17**. Protein kinase activity was measured in the standard assay conditions in the presence or absence of various concentrations of preparation **17** and either ATP concentration (A and B) or kemptide (LRRASLG) concentration (C) was varied. Double-reciprocal plots of the kinetic data are presented (v^{-1} is in arbitrary units). (A), ○ – ○, no addition; ● – ●, 20 nM preparation **17**; (B), ○ – ○, no addition; △ – △, 40 nM preparation **17**; (C), ○ – ○, no addition, ● – ●, 110 nM preparation **17**.

defensive function involving the cAK of fungi and animal herbivores as a target. There is no evidence for the presence of cAK in higher plants [22]. The present data suggest that this enzyme, which is subject to inhibition by a large number of plant defensive compounds [2–15], may be a very specific target for condensed tannins. Compartmentation of such compounds away from the cytosol, sequestration in hull [6], bark or wood [8] and a relatively low affinity for the plant CDPK (Table 2) would protect the tannin-producing plant cells from functional impairment caused by inhibition of CDPK. Inhibition of animal or fungal cAK would not necessarily be lethal but would impair cyclic AMP-mediated regulation of signalling, metabolism and specific gene expression critical for optimal function [23, 24]. As previously suggested [9], endocytosis [25] after binding of polyphenolics to externally-oriented membrane protein domains represents a possible mechanism of entry of these inhibitory compounds into target cells. While these condensed tannins have an

extraordinarily high affinity for cAK, it remains possible that other structurally-related proteins are also high affinity sites of action of these condensed tannins in the target organisms.

EXPERIMENTAL

Tannin preparation isolation and analysis. Fresh fruit or leaf material was extracted twice in a Waring Blendor with acetone–water (7:3) as solvent, filtered to remove plant debris and then salt was added to separate the upper acetone layer. The acetone was evapd *in vacuo* at <40° and aqueous residue, diluted with water if necessary, was extracted with CHCl, (2×) and EtOAc (6×). The solution was dialysed overnight against distilled water. An equal volume of MeOH was added to this solution which was then applied to a column of Sephadex LH-20 (4 × 20 cm), than had been pre-swollen in MeOH–water (1:1). The absorbed tannin was washed with 1–2 l of the same solvent and the purified polymer eluted as a discrete, visible band with acetone– water (7:3). The acetone was removed *in vacuo* at <40° and the water was removed by freeze-drying to yield the tannin as a light-tan, fluffy solid. The tannins were analysed by ^{13}C NMR chiroptical, and degradative methods as described in detail elsewhere [26].

Materials. [γ-^{32}P]ATP (specific activity 4,000 Ci mmol^{-1}) was obtained from Bresatec, Adelaide, Australia. Kemptide (LRRASLG), epidermal growth factor receptor-derived synthetic peptide (EGFRP; VRKRTLRRL-NH_2) and myosin light chain-based synthetic peptide (MLCP; KKRAARATSNVFA-NH_2) were obtained from Auspep, Melbourne, Australia. ATP and dithiothreitol were obtained from the Sigma Chemical Co.

Protein kinase isolation and assay. Rat brain PKC (specific activity 0.6 μmol min^{-1} mg protein^{-1} with 3.5 μM EGFRP as substrate), chicken gizzard myosin light chain kinase (MLCK) (specific activity 0.05 μmol min^{-1} mg protein^{-1} with 20 μM MLCP as substrate), wheat embryo CDPK (specific activity 0.01 μmol min^{-1} mg protein^{-1} with 1.0 mg ml^{-1} histone type III-S as substrate) and rat liver cyclic AMP-dependent protein kinase catalytic subunit (cAK) (specific activity 0.3 μmol min^{-1} mg protein^{-1} with 20 μM kemptide as substrate) were extensively purified and assayed in standard assay conditions as described previously [8]. PKC, MLCK, CDPK and cAK were assayed employing synthetic peptides as substrates, namely 3.5 μM EGFRP, 17 μM MLCP, 20 μM MLCP and 20 μM kemptide, respectively.

Inhibitor IC_{50} values (concns for 50% inhibition of particular protein kinases in the standard assay conditions) were determined from interpolation of plots of protein kinase activity versus inhibitor concn. Control protein kinase activity (no added inhibitor) was routinely determined in sextuplet and assays with inhibitor included were determined in duplicate. All assay results were corrected by subtraction of blank values from assays conducted in the absence of added protein kinase. The standard deviations associated with control protein kinase assays were about 10% of mean values. Inhibitor compounds were routinely dissolved in 10% (w/v) DMSO and added to protein kinase assays to give the following DMSO concns: 1.7% (w/v) (MLCK and PKC assays) and 2% (w/v) (cAK and CDPK assays). Control protein kinase assays (without added inhibitor) were conducted with inclusion of the appropriate concn of DMSO.

Acknowledgements—This work was supported by a grant to G. M. P. from the Australian Research Council. B. H. W. was supported by a La Trobe University Postgraduate Scholarship.

REFERENCES

1. Harborne, J. B. and Baxter, H. (1993) *Phytochemical Dictionary, A Handbook of Bioactive Compounds From Plants*. Taylor and Francis, London.
2. Jinsart, W., Ternai, B. and Polya, G. M. (1992) *Biol. Chem. Hoppe-Seyler* **373**, 205.
3. Ferriola, P. C., Cody, V. and Middleton, E. (1989) *Biochem. Pharmacol.* **38**, 1617.
4. Rogers, J. C. and Williams, D. L. (1989) *Biochem. Biophys. Res. Commun.* **164**, 419.
5. Jinsart, W., Ternai, B. and Polya, G. M. (1991) *Biol. Chem. Hoppe-Seyler* **372**, 819.
6. Jinsart, W., Ternai, B., Buddhasukh, D. and Polya, G. M. (1991) *Phytochemistry* **31**, 3711.
7. Jinsart, W., Ternai, B. and Polya, G. M. (1992) *Biol. Chem. Hoppe-Seyler* **373**, 903.
8. Polya, G. M. and Foo, L. Y. (1994) *Phytochemistry* **35**, 1399.
9. Polya, G. M., Wang, B. H. and Foo, L. Y. (1995) *Phytochemistry* **38**, 307.
10. Johnson, M. S., Wolbers, W. B., Noble, J., Fennell, M. and Mitchell, R. (1995) *Mol. and Cell. Endocrinol.* **109**, 69.
11. Akiyama, T., Ishida, J., Nakagawa, S., Ogawara, H., Watanabe, S., Itoh, N., Shibuya, M. and Fukami, Y. (1987) *J. Biol. Chem.* **262**, 5592.
12. Herbert, J. M., Augereau, J. M., Gleye, J. and Maffrand, J. P. (1990) *Biochem. Biophys. Res. Commun.* **172**, 993.
13. Reddy, S. and Aggarwal, B. B. (1994) *FEBS Letters* **341**, 19.
14. Hasmeda, M. and Polya, G. M., (1996) *Phytochemistry* **42**, 599.
15. Wang, B. H. and Polya, G. M. (1995) *Phytochemistry* **41**, 55.
16. Foo, L. Y. and Porter, L. J. (1981) *J. Sci. Food Agric.* **32**, 711.
17. Haslam, E. (1981) in *The Biochemistry of Plants* (Conn, E. E., ed.), Vol. 7, p. 527. Academic Press, New York.
18. Porter, L. J. (1989) in *Methods in Plant Biochemis-*

try (Dey, P. M. and Harborne, J. B., eds), Vol. 1, *Plant Phenolics* (Harborne, J. B., ed.), p. 389. Academic Press, New York.
19. Chijiwa, T., Mishima, A., Hagiwara, M., Sano, M., Hayashi, K., Inoue, T., Naito, K., Toshioka, T. and Hidaka, H. (1990) *J. Biol. Chem.* **265**, 5267.
20. Knighton, D. R., Zheng, J., Eyck, L. F. T., Ashford, V. A., Xuong, N., Taylor, S. S. and Sowadski, J. M. (1991) *Science* **253**, 407.
21. Taylor, S. S., Knighton, D. R., Zheng, J., Sowadski, J. M., Gibbs, C. S. and Zoller, M. L. (1993) *TIBS* **18**, 84.
22. Polya, G. M., Chung, R. and Menting, J. (1991) *Plant Science* **79**, 37.
23. Cohen, P. (1989) *Ann. Rev. Biochem.* **58**, 453.
24. Cohen, P. (1992) *TIBS* **17**, 408.
25. Mellman, I., Fuchs, R. and Helenius, A. (1986) *Ann. Rev. Biochem.* **55**, 663.
26. Czochanska, Z., Foo, L. Y., Newman, R. H. and Porter, L. J. (1980) *J. Chem. Soc., Perkin* **1**, 2278.