Phytochemistry, 1969, Vol. 8, pp. 823 to 826. Pergamon Press. Printed in England

THE INFLUENCE OF POLYPHENOLS ON POTATO PHOSPHORYLASE

GARY E. BLANK* and ERNEST SONDHEIMER

Department of Chemistry, College of Forestry at Syracuse University, Syracuse, New York 13210

(*Received* 25 *October* 1968)

Abstract—The effects of a number of naturally occurring polyphenols on crude potato phosphorylase were reinvestigated in order to determine the mechanism of their action. While crystalline muscle phosphorylase is only weakly inhibited by 10^{-3} M chlorogenic acid, crude potato phosphorylase is 5 to 6 times more strongly inhibited. This difference in responses is due to the presence of *o*-diphenol oxidase in the potato preparation and most of the effective inhibitors are believed to be *o*-quinones. These probably attack sulfhydryl groups of the potato phosphorylase. The behavior of *o*-diphenols which contain primary amino groups in the side-chain is anomalous since they are either only weakly inhibitory or actually activate.

INTRODUCTION

IT HAS been known for a number of years that phenols can profoundly affect the activity of peroxidases; monohydric substances are activators and dihydric phenols inhibitors.[1] This has aroused considerable interest in connection with indoleacetic acid-induced growth since the possibility exists that the effects observed *in vitro* may have physiological significance. In addition to their effects on peroxidases, phenols have been reported to modify the activity of a number of other plant enzymes, including oxidative decarboxylases[2] and phosphorylase.[3] Extensive studies on the interaction of phenols, particularly chlorogenic acid, with crude potato phosphorylase have been reported by Schwimmer.[3] Because of the widespread coexistence of chlorogenic acid-like compounds and phosphorylase in plants any interaction between phenols and this important enzyme could have physiologically detectable consequences. These considerations prompted us to reexamine the influence of phenols on the activity of potato phosphorylase in an attempt to ascertain the mechanism and significance of these interactions.

RESULTS

Inhibition of Potato Phosphorylase by Phenols

Ammonium sulfate fractionation of potato extracts yields a phosphorylase preparation which is inhibited by chlorogenic acid.[3] Results with other compounds, some of which have been evaluated previously, are summarized in Table 1. While potato phosphorylase is a PLP-†containing enzyme, the addition of PLP to the incubation mixture has no effect, either on the activity of the enzyme or on the per cent inhibition caused by chlorogenic acid. How-

* During this investigation G. E. Blank held a National Defense Education Act fellowship.

† PLP = pyridoxal-5-phosphate.

[1] W. A. ANDREAE, *Nature* **170**, 83 (1952).

[2] M. MAZELIS, *J. Biol. Chem.* **237**, No. 1, 104 (1962).

[3] S. SCHWIMMER, *J. Biol. Chem.* **232**, 715 (1958).

TABLE 1. EFFECTS OF PHENOLS ON THE ACTIVITY OF POTATO PHOSPHORYLASE

Compound	Inhibition (%)	Compound	Effect
Catechol	52	3,4-Dimethoxycinnamic acid	6% Inhibition
Chlorogenic acid	46	Adrenaline	6% Inhibition
Caffeic acid	41	L-Dopa	4% Inhibition
Dihydrochlorogenic acid	40	Protocatechuic acid	1% Inhibition
Hydroquinone	16	L-Tyrosine	3% Activation
p-Coumaric acid	11	Dopamine	15% Activation
Resorcinol	10	L-Dopa methyl ester	24% Activation

All compounds were tested at 10^{-3} M and the primer concentration was 0·05 mg/ml. The phosphorylase was isolated by the procedure of Green and Stumpf[11] and contained *o*-diphenol oxidase.

ever, the addition of 0·02 N sodium hydrosulfite to the assay mixture reduced the chlorogenic acid inhibition five- to six-fold. Similarly, dialysis of the phosphorylase preparation against 0·02 M KCN decreased the chlorogenic acid inhibition two-fold. In the presence of the phosphorylase preparation the 324 nm peak of chlorogenic acid decreased and a new broad peak near 277 nm formed. This type of spectral change is characteristic of the enzymatic oxidation of chlorogenic acid.[4] The above observations, therefore, indicate that the phosphorylase preparation was contaminated with *o*-diphenol oxidase and that oxidation of chlorogenic acid enhances its inhibitory properties.

The purification procedure for phosphorylase from potatoes could be readily modified, using cyanide, to yield an active preparation with very low *o*-diphenol oxidase activity. When this preparation was used at a concentration at which the rate of inorganic phosphate release was comparable to that obtained with the *o*-diphenol oxidase-contaminated mixture, the inhibition with 10^{-3} M chlorogenic acid was reduced five-fold. Addition of an *o*-diphenol oxidase preparation from potatoes with chlorogenic acid brought the inhibition back to the original level. Crystalline muscle phosphorylase was only slightly inhibited by chlorogenic acid; 10^{-3} M gave approximately 10 per cent inhibition.

Oxidation of chlorogenic acid in the presence of *o*-diphenol oxidase is a complex process yielding several transient intermediates and various end products. The first enzymatic step is the formation of an *o*-quinone requiring one atom oxygen per mole chlorogenic acid. Subsequently, hydroxychlorogenic acids and hydroxyquinones are formed spontaneously. The latter intermediate polymerizes nonenzymatically to yield colored products. Pierpoint[5] has shown that barbituric acid traps the *o*-quinone intermediate without interfering in the rate of the enzymatic phase of the oxidation.

When 5×10^{-3} M barbituric acid was added to the phosphorylase *o*-diphenol oxidase mixture the inhibition with 10^{-3} M chlorogenic acid was decreased from 39 per cent to 0 ± 4 per cent. This suggests that the major species responsible for the inhibition of phosphorylase are *o*-quinones.

Sites of Attack on Phosphorylase

According to Pierpoint many compounds containing primary or secondary amino groups, heterocyclic nitrogen and sulfhydryl groups can react with the *o*-quinone of chlorogenic acid without inhibiting *o*-diphenol oxidase.[5] Table 2 summarizes the effects of several of these

[4] E. C. SISLER and H. J. EVANS, *Biochim. Biophys. Acta* **28**, 638 (1958).
[5] W. S. PIERPOINT, *Biochem. J.* **98**, 567 (1965).

compounds on a reconstituted potato phosphorylase *o*-diphenol oxidase mixture exposed to 10^{-3} M chlorogenic acid and shows that only cysteine is an effective antagonist. In view of the fact that potato phosphorylase is considered a "SH-enzyme",[6] this suggests that the inhibition caused by oxidized chlorogenic acid is due to addition of the *o*-quinone of chlorogenic acid to one or more sulfhydryl groups of the phosphorylase.

TABLE 2. ANTAGONISM OF THE CHLOROGENIC ACID INHIBITION OF PHOSPHORYLASE BY COMPOUNDS THAT REACT WITH *o*-QUINONES

Phosphorylase	Oxidase	Test compound	% Inhibition
+	None	None	8
+	+	None	39
+	+	$1{\cdot}2 \times 10^{-2}$ M L-lysine	30
+	+	5×10^{-3} M L-histidine	30
+	+	5×10^{-3} M L-tryptophan	39
+	+	Serum albumin*	31
+	+	5×10^{-3} M L-cysteine	5

* Serum albumin was added at a concentration three times as high as the total proteins in the potato extracts.

Potato phosphorylase freed of *o*-diphenol oxidase by CN^- treatment was used. The *o*-diphenol oxidase used was prepared from potatoes and had no phosphorylase activity.[12] All samples contain 10^{-3} M chlorogenic acid and 0·05 mg/ml starch primer. In the absence of inhibition the P_i release during 30 min incubation was approximately 0·4 mg/ml.

DISCUSSION

The phenols listed in Table 1 were all tested at 10^{-3} M levels, yet they vary considerably in their effects on the phosphorylase preparation, ranging from 52 per cent inhibition to 24 per cent activation. The differences in inhibitory effects can be explained on the assumption that high inhibitory activity requires that the phenol be a good substrate for *o*-diphenol oxidase. Thus *p*-coumaric acid, resorcinol, 3,4-dimethoxycinnamic acid and tyrosine, lack the *o*-dihydric phenol structure and are therefore not oxidizable by *o*-diphenol oxidase. Hydroquinone was oxidized only after a 1 hr lag phase by a purified potato *o*-diphenol oxidase preparation.[7] Neumann *et al.*[7] have found that a crude *o*-diphenol oxidase preparation from potatoes catalyzed the oxidation of chlorogenic acid and catechol rapidly while 3,4-dihydroxybenzoic acid was a very poor substrate. Significantly, the latter compound does not inhibit the *o*-diphenol oxidase-containing phosphorylase preparation, (Table 1). That the *N*-containing *o*-dihydric phenols behave differently from the *N*-free phenols is shown by the fact that at 10^{-3} M levels, adrenaline and dopa have very low if any inhibitory activity while 2-(3,4-dihydroxyphenyl)-ethylamine (dopamine) and dopa methyl ester are somewhat activating. These data are similar to Schwimmers' who found 17 per cent activation with 5×10^{-2} M dopa.[3]

A possible site of interaction of the *o*-quinones and phosphorylase at pH 6 is at —SH groups. The *o*-quinone of chlorogenic acid will also react with compounds having primary and secondary amino groups but these reactions become significant only at higher pH values. This is consistent with the work reported by Alberghina[8] who found that at pH 7·8 lysine

[6] Y. P. LEE, *Biochim. Biophys. Acta* **43**, 25 (1960).
[7] J. NEUMANN, G. LEGRAND, G. LEHONORE and J. LAVOLLAY, *Compt. Rend.* **251**, 3091 (1960).
[8] F. A. N. ALBERGHINA, *Life Sci.* **3**, 49 (1966).

and histidine antagonize the inhibition of glucose-6-phosphate dehydrogenase by oxidized chlorogenic acid. However, in our work pH 6·0 was used and under these conditions only cysteine was effective in antagonizing the chlorogenic acid inhibition of phosphorylase. Since serum albumin has a very low SH content its ineffectiveness as an antagonist is not surprising. While it has been shown that cysteine can decrease the rate of oxidation of chlorogenic acid by *o*-diphenol oxidase this is not believed to be the reason for its effectiveness in decreasing the inhibitory action of chlorogenic acid. Pierpoint's results indicate that when the ratio of cysteine to chlorogenic acid is greater than 1·5:1, adduct formation with the *o*-quinone does occur and enzymatic oxygen uptake is not greatly interfered with.[5]

The isolation of active plant enzymes is often hampered by the presence of large amounts of phenols and their oxidases. Unlike phosphorylase, not all enzymes are resistant to reducing agents or oxidase inhibitors. Furthermore, this approach does not eliminate interactions between the unoxidized phenols and the enzyme. In a recent report, Loomis and Battaile[9] discuss the utility of soluble and insoluble polyvinylpyrrolidone for the isolation of plant enzymes.

While it is true that chlorogenic acids, *o*-diphenol oxidase and phosphorylase are present in potato tubers there is no evidence to suggest that this system has a regulatory role in the intact cell. However, it has been known for years that when potato tubers are infected by *Phytophthora infestans* starch may accumulate around the site of infection.[10] These infections are characterized by the loss of cellular integrity, an abnormally high level of polyphenols and their oxidation products, the very conditions that could lead to phosphorylase inhibition. It might be of interest therefore to ascertain whether or not phosphorylase activity is decreased in the periphery of necrotic spots of infected potatoes.

EXPERIMENTAL

Muscle phosphorylase a (α-1,4-glucan, orthophosphateglucosyltransferase, 2.4.1.1) was obtained from Worthington Biochemical Corporation. Chlorogenic acid was isolated from unroasted coffee beans and was twice recrystallized. Catalytic reduction of chlorogenic acid yielded dihydrochlorogenic acid, m.p. 168°. Crude potato phosphorylase was isolated according to the $(NH_4)_2SO_4$ fractionation procedure of Green and Stumpf.[11] Potato phosphorylase with low *o*-diphenol oxidase (*o*-diphenol:oxygen oxidoreductase, 1.10.31) activity was prepared by adding 2×10^{-3} M KCN during each of the $(NH_4)_2SO_4$ precipitations in the Green and Stumpf procedure. The final preparation was dialyzed against 0·2 M sodium acetate buffer, pH 6·8. Only 5% oxidation of chlorogenic acid was detectable after 30 min incubation with this preparation, whereas the original Green and Stumpf preparation gave complete oxidation in a few seconds. *o*-Diphenol oxidase was isolated from potatoes with $(NH_4)_2SO_4$ fractionation, column chromatography, and inactivation of phosphorylase by heating for 3 min at 68°.[12]

Potato phosphorylase was assayed in 0·2 M sodium acetate buffer, pH 6·0, with 0·10 and 0·02 per cent solutions of Merck's soluble starch at 37°. The final concentration of glucose-1-P was $1{\cdot}1 \times 10^{-2}$ M. Duplicate samples were generally incubated for 30 min and an aliquot then pipetted into cold 5% trichloroacetic acid. Determination of the phosphate released was by the assay described by Schwimmer and Weston.[13] Muscle phosphorylase was assayed according to the method of Illingworth and Cori.[14] Inhibition was determined after a 30 min reaction time, and the inhibitors were added to the solutions containing the enzymes prior to substrate.

[9] W. D. LOOMIS and J. BATTAILE, *Phytochem.* **5**, 423 (1966).
[10] K. O. MÜLLER, "Hypersensitivity", in *Plant Pathology* (edited by J. G. HORSFALL and A. E. DIMOND), pp. 484–485, Academic Press, New York (1959).
[11] D. E. GREEN and P. K. STUMPF, *J. Biol. Chem.* **142**, 355 (1942).
[12] F. A. M. ALBERGHINA, *Phytochem.* **3**, 65 (1963).
[13] S. SCHWIMMER and W. J. WESTON, *J. Biol. Chem.* **220**, 143 (1956).
[14] B. A. ILLINGWORTH and G. T. CORI, *Biochem. Prep.* (edited by E. E. SNELL) **3**, 1 (1953).