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PURIFICATION AND PROPERTIES OF HYDROXYCINNAMOYL CoA QUINATE HYDROXYCINNAMOYL TRANSFERASE FROM POTATOES

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Abstract—A rapid method for the purification of hydroxycinnamoyl CoA quinate hydroxycinnamoyl transferase (CQT) from potato tubers which had been stored at low temperatures is described. The method involves affinity chromatography on Blue Sepharose with biospecific desorption of CQT with its substrate, CoA. Elution of the Blue Sepharose column with a gradient of CoA leads to the resolution of CQT, a protein with MW of *ca* 41500, into 3 peaks of activity; the largest peak elutes first. This fraction is purified ×1440 and gives a single band of protein after PAGE which suggests a high degree of purity. The properties of the 3 fractions of CQT, with respect to substrates and to a number of inhibitors, are described. The first and last eluting CQT fractions are specific for quinate and show no activity towards shikimate. The second peak, however, shows a small activity towards shikimate but this is thought to be due to an underlying peak of a shikimate specific enzyme. The major peak of CQT activity found in potatoes stored at 0° is absent from those stored at 10° throughout the period after harvest.

INTRODUCTION

The enzyme hydroxycinnamoyl CoA quinate hydroxycinnamoyl transferase (CQT) catalyses the reversible exchange of CoA thioester and quinate groups in the synthesis and breakdown of chlorogenic and *p*-coumaroyl quinic acids [1–3]. The activity of the enzyme has been shown to be stimulated by treatments with light [4–6] and low temperature storage [7, 8] which lead to the accumulation of chlorogenic acid. It thus seems likely that, in conjunction with hydroxycinnamate CoA ligase, CQT plays a role in the endergonic processes involved in the synthetic reactions leading to esterification of quinic acid with hydroxycinnamoyl residues. The fact that CQT can also cleave chlorogenic and *p*-coumarylquinic acids [2] in the presence of CoA to form the CoA thioesters suggests that it may also play a role in the catabolism of the quinate esters.

The present work forms part of a study of the mechanism of the activation of CQT activity which can be induced by low temperature storage of commodities such as tomatoes, potatoes and sweet potatoes [7, 8]. The present paper describes a method for purification of CQT by affinity chromatography and some properties of the purified enzyme.

RESULTS AND DISCUSSION

Purification of the enzymes

CQT was purified from potatoes, which had been stored at 0° for 103 days after harvest, using $(NH_4)_2SO_4$ precipitation, desalting on Sephadex G-25 followed by elution from a column of DEAE-cellulose using a linear gradient of KCl between 0 and 0.4 M. Under these conditions, CQT elutes as a major peak containing *ca* 90% of the total activity at a salt concentration of *ca* 0.08 M and a minor peak with *ca* 10% of the total activity eluting at 0.12 M KCl [3]. Table 1 shows that this procedure leads to a 12.5-fold purification with retention of 68% of the original activity.

The major peak after DEAE-cellulose chromatography was applied after dialysis to a column of Blue Sepharose equilibrated with 5 mM phosphate pH 7 containing 1 mM DTE. This led to the retention of 83% of the applied protein and 100% of the applied activity. The column was eluted with a linear gradient of CoA from 0 to 0.4 mM (see Fig. 1) and this resulted in the elution of 3 peaks of CQT activity and of *ca* 7.6% of the applied protein. The major peak (I), which was eluted at a CoA concentration of *ca* 0.22 mM, contained 79% of the applied activity but only 0.5% of the applied protein (Table 1). The second CQT peak (II) eluting at 0.29 mM contained 20.7% of the applied activity and 0.8% of the applied protein while the third peak (III) eluting at 0.39 mM had 18% of the activity and 6.3% of the applied protein. This represents overall degrees of purification of 1440-, 248- and 28-fold respectively for the 3 CQT peaks. For peak I the overall purification of 1440-fold involved the retention of 42% of the original tissue activity and to a final specific activity of 279 nkat/mg protein (molecular activity of 670 assuming a MW of CQT of 40000). This fraction was stable during storage and retained 67% of its original activity during 10 month storage at −20°.

It can be seen from the results with CQT that the adsorption step on to the Blue Sepharose column appears to be relatively non-specific. The specificity is bestowed by the fact that low concentrations of CoA, the co-facto

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Table 1. Purification of CQT from potato tubers stored at 0° for 103 days

	Volume (ml)	Total activity (nkat)	Total protein (mg)	Specific activity (nkat/mg protein)	Yield (%)	Purification
Crude extract, 100 g fr. wt	262	104	519	0.20	100	1
$(NH_4)_2SO_4$ (30–85%)	30	90.9	413	0.22	87.4	1.1
Post-Sephadex G25	80	117	359	0.33	112.5	1.65
Post-DEAE-cellulose (major peak)	28	71.2	28.5	2.50	68.5	12.5
Post-dialysis of DEAE peak	335	54.4	28.1	1.94	52.3	9.7
Post-Blue Sepharose						
Peak I	47	43.2	0.155	279	41.5	1440
Peak II	35	11.3	0.228	49.6	10.9	248
Peak III	38	9.8	1.77	5.5	9.4	28

of the enzyme, can be used to desorb CQT activity in a very specific manner. Stellwagen *et al.* [9, 10] have proposed that the binding of proteins to the blue chromophore of Blue Sepharose (Cibacron Blue F3G-A) was diagnostic for the presence of a 'dinucleotide fold' in their structure. However, other enzymes, such as *E. coli* polynucleotide phosphorylase, appears to bind via the polynucleotide binding region [11]. The fact that 83% of the applied protein is retained on the Blue Sepharose column at low salt concentrations, and that the adsorption is sensitive to the salt concentration, suggests that simple non-specific ionic binding may be important in the retention of some proteins.

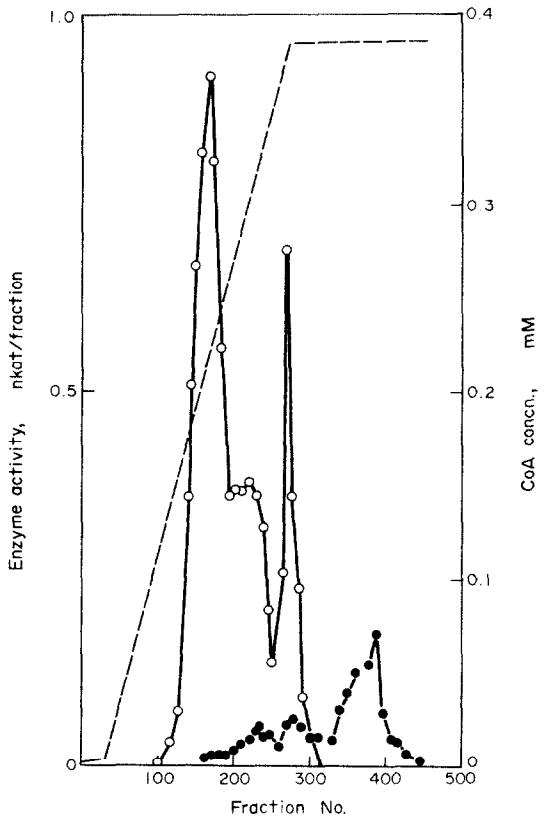

Fig. 1. Separation of CQT on a column of Blue Sepharose. The separation of CQT prepared from potatoes stored at 0° for 103 days is shown as open circles while that from potatoes stored at 10° is shown as solid circles. The CoA gradient measured by the absorbance of the various fractions at 260 nm is shown as a dashed line.

The purity of the 3 peaks after Blue Sepharose chromatography was tested by discontinuous PAGE using the buffer system Tris–HCl–glycine, pH 8.9 [12]. As a preliminary to electrophoresis it was necessry to devise a method for concentrating the fractions from the Blue Sepharose column which had protein contents as low as 3 μg/ml. This method of concentration had to avoid loss, inactivation or fractionation of the protein. Several methods including ultra-filtration, lyophilisation, dialysis against saturated solutions of polyethyleneglycol, and dehydration with dry Sephadex G25 were tried and discarded. Eventually the method used was to absorb and desorb the protein from small columns of DEAE-cellulose (see Experimental) and this was achieved with relatively little loss of activity or protein and an increase in protein concentration by up to 10-fold. A minimum of 6 μg of protein was applied to each gel in the analysis of the fractions and the gels after electrophoresis were stained by a method capable of detecting bands containing 1 μg of protein [13]. After electrophoresis, peak I of CQT revealed only a single band ($Rm = 0.42 \pm 0.03$ relative to bromophenol blue as reference marker) staining with Coomassie blue which corresponded in position on the gel with the CQT activity. Estimation of the area under the densitometer tracing of the Coomassie blue stained gel suggests that up to 90% of the protein in peak I is associated with CQT activity. Peaks II and III also gave protein bands ($Rm = 0.42 \pm 0.03$, and 0.42 ± 0.02, respectively) which correspond with the position of the CQT activity in the gel. However, both these fractions showed additional bands running ahead of the CQT activity. These bands had values of $Rm = 0.48$ in Peak II and a band at $Rm = 0.49$ and a diffuse area of $Rm = 0.51$ in the case of peak III. In both cases these bands of higher Rm were less intense than that of the band at $Rm = 0.42$. The gel electrophoresis was carried out using both 7 and 10% gels with similar results although the mobilities of the bands were somewhat modified. These results suggest that in peak I nearly 90% of the protein in the fraction is associated with CQT activity. In peaks II and III, CQT is the principal component of the fraction although other subsidiary bands are present.

Properties of the purified enzyme

CQT eluted as a single symmetrical peak on Ultrogel columns and the estimate of its MW determined by its

elution volume from a calibrated column of Ultrogel AcA 54 was 41500 ± 2400. This compares with an estimate of 40000 ± 1600 for the enzyme from the tomato. SDS-PAGE of peak I from Blue Sepharose revealed a single polypeptide species with a MW of 14900 ± 550 indicating that the CQT protein may consist of 3 polypeptide chains. The SDS-PAGE was carried out at two pHs with similar estimates of the MW of the polypeptide unit.

The general properties of the purified enzyme from potato tubers are similar to those described for the partially purified enzyme from tomato fruits [2]. However, the pH optimum for each of the 3 peaks was at 6.25 compared with the tomato enzyme at pH 7 and the activity of the enzyme from potato, but not that from tomato, was stimulated by the inclusion of BSA in the assay medium. This requirement for BSA was shown at all stages of purification even though the degree of stimulation with a given level of BSA decreased as the purification increased. For instance, in crude extracts 1–2 mg/ml BSA gave a 2- to 4-fold stimulation in activity while the fractions after Blue Sepharose chromatography was stimulated by only 35%. A similar situation occurs in the stimulation by BSA of another enzyme involved in phenolic biosynthesis, hydroxycinnamate CoA ligase, when again the degree of stimulation decreased as the enzyme is purified [14].

The potato enzyme is heat labile; at 50° the enzyme in Tris buffer is inactivated by 65% after 5 min. The heat stability is affected by the buffer used, for instance phosphate buffer protects the enzyme from inactivation and after 5 min at 50° the degree of inactivation only reaches 12%. As with the tomato enzyme [2] concentrations of Tris above 10 mM cause inhibition of the CQT activity in potato; at 100 mM the inhibition reaches 50% and this occurs with each of the 3 peaks of activity.

Study of the effect of temperature on the enzyme shows that Arrhenius plots (Fig. 2) of the CQT activity show a discontinuity at 6.5° with apparent energies of activation of 13840 and 16850 kcal/mol above and below the break point. Triton X100 at 0.1% had no significant effect on either the break point or the activation energies above and below the break. In membrane-bound enzymes, biphasic Arrhenius plots have been related to a change in the lipid environment of the enzyme at the critical break temperature [15, 16]. The presence of a breakpoint in the Arrhenius plot of membrane-bound enzymes isolated from certain plant tissues has been correlated with the sensitivity of these tissues to chilling injury [16]. There are some examples of apparently soluble enzymes which also give biphasic Arrhenius plots, the best known example of which is fumarase [17], and in this case, the discontinuity is related to changes in the configuration of the enzyme at a critical temperature. In the plant kingdom, soluble starch synthetase from maize, avocado and sweet potato [18, 19] shows a discontinuity in its Arrhenius plot at 12°. *t*-Butanol but not Triton X-100 abolishes the discontinuity and this has been correlated with the presence of lipid material associated with the soluble enzyme. Tamaka and Uritani [20] showed a discontinuity at 17° in the Arrhenius plot of activity of PAL purified from aged sweet potato disks and it was suggested that the discontinuity probably represented a change in the sub-unit interactions at the critical temperature. The significance in the biphasic nature of the Arrhenius plot of CQT from potatoes is uncertain but it is interesting that two enzymes of phenylpropanoid metabolism show the same phenomenon even though the temperature at the break point is very different for the two enzymes. With CQT the K_m values were 151 μM at 30°, 104 μM at 20°, 94 μM at 10° and 73.5 μM at 3°, respectively and if these data are plotted in the form of an Arrhenius plot, there is no evidence for a discontinuity. This situation differs both from the observation with PAL [20] and fumarase [17], in which Arrhenius plots

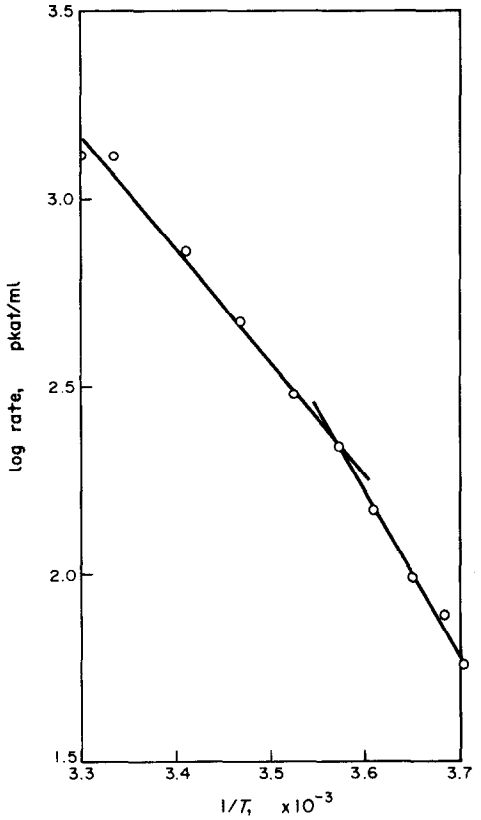

Fig. 2. Arrhenius plot of the variation of activity of peak I of CQT with temperature.

Table 2. Substrate specificity of the purified CQT fractions

Substrate		Peak I	Peak II (nkat/fraction)	Peak III
Chlorogenic acid	+ CoA	18.0	8.6	10.2
p-Coumaroyl CoA	+ quinic acid (2 mM)	9.8	6.9	6.7
	+ shikimic acid (2 mM)	0	0.24	0
Caffeoyl CoA	+ quinic acid (2 mM)	9.10	nm	nm
	+ shikimic acid (2 mM)	0	nm	nm
Feruloyl CoA	+ quinic acid (2 mM)	1.13	1.57	1.50
	+ shikimic acid (2 mM)	0	0	0

nm = Not measured.

of the K_m show an abrupt decrease in slope corresponding to the similar changes in slope of the velocity data plotted in the same manner.

In a previous paper [2] using a partially purified (32-fold) CQT fraction from tomatoes, it was shown that this fraction showed small activities towards shikimic acid and towards feruloyl CoA as substrates. It was not clear whether these small activities represented a lack of absolute specificity towards the normal CQT substrates or whether there were separate transferases specific for feruloyl residues or for the formation of shikimate esters. More recently Zenk [21] has reported the presence of a separate shikimate esterifying enzyme (CST) in *Nicotiana*. This led us to re-investigate the specificity of CQT using highly purified enzyme and the results obtained are shown in Table 2. Crude extracts of potato tissue show activity towards both quinate and shikimate but the activity towards shikimate is only *ca* 5% of that towards quinate. After DEAE-cellulose chromatography there is shikimate dependent activity underlying both peaks of CQT. However, the peaks I and III of CQT activity after Blue Sepharose chromatography show no detectable activity towards shikimate. Analysis of the fractions from Blue Sepharose for shikimate dependent activity suggests the presence of a small diffuse peak corresponding in elution position to peak II of CQT. These results are in agreement with the presence of separate enzymes involved in the esterification of quinic and shikimic acids. Each of the peaks of CQT activity shows activity towards feruloyl CoA in the synthetic reaction as well as *p*-coumaroyl and caffeoyl CoA. This activity is between 10 and 20% of the activity with *p*-coumaroyl CoA and it is thus likely that CQT is involved in the metabolism of feruloylquinic acid as well as *p*-coumaroyl quinic and chlorogenic acids.

Table 3 shows the apparent K_m values for the 3 peaks of CQT after chromatography on Blue Sepharose. These values are of the same order as the previously published values for the tomato enzyme [2]. For all substrates the K_m value is highest with peak I and decreases in peaks II and III but the overall differences in K_m are only *ca* 2- to 3-fold. The potato enzyme is competitively inhibited (Table 4) by a range of cinnamic acids. The most potent inhibitors in each case are *p*-coumaric and caffeic acids while ferulic and cinnamic acids are less inhibitory. In each case, however, peak I is more sensitive to inhibition than either peaks II or III. Since in all cases the K_i value is close to 1 mM, it is unlikely that such inhibition is of physiological significance. CQT measured in the direction of chlorogenic acid cleavage is inhibited by quinic acid in a competitive manner with a K_i value of 1.1 mM while shikimate has no inhibitory effect. The enzyme is also inhibited by divalent ions such as Hg^{2+} and Zn^{2+}.

Table 3. Apparent K_m values for the three fractions of CQT activity separated on Blue Sepharose

	Peak I	Peak II	Peak III
Concentration of CoA for elution (μM)	220	290	390
K_m values for			
Chlorogenic acid	151	72	90
CoA	21	5.1	6.6
Caffeoyl CoA	73	46	41

Table 4. Inhibitors of CQT of potato tubers

Inhibitor	Peak I	Peak II K_i (mM)	Peak III
p-Coumaric acid	0.70 c	0.89 c	0.95 c
Caffeic acid	0.82 c	0.87 c	1.07 c
Cinnamic acid	0.94 c	1.28 c	1.33 c
Ferulic acid	1.17 c	1.35 c	1.50 c
Quinic acid	1.10 c	nd	nd
Shikimic acid		No inhibition	
Zn^{2+}	0.074 nc	0.057 nc	0.042 nc

nc = Non-competitive; c = competitive; nd = not determined.

With Zn^{2+} the inhibition is potent with a K_i value of between 42 and 74 μM. The inhibition is non-competitive in nature and is most marked in the peak III fraction. Mg^{2+}, Ca^{2+}, Cd^{2+} and Be^{2+} cause very little inhibition at 1 mM concentrations. The inhibition by Zn^{2+} is completely overcome by EDTA at 1 mM. Zn^{2+} inhibits the cleavage of both chlorogenic and *p*-coumaroylquinic acids. This suggests that Zn^{2+} does not act solely by forming a complex with dihydroxyphenol substrates but that rather the effect is a direct one on the enzyme. The non-competitive nature of the Zn^{2+} inhibition suggests that the metal binds to a site other than the active site of the enzyme and this leads to the modification of the activity.

The 3 peaks of CQT activity after Blue Sepharose differ in the CoA concentration required for their elution but appear to be similar in a number of other properties. For instance they have the same MW and the same mobility under two different conditions of PAGE. They show small differences (of the order of 2- to 3-fold) in their apparent K_ms for substrates and cofactors but are inhibited to similar extents by both cinnamic acids and Zn^{2+}. Thus the significance of the 3 forms of CQT is at present difficult to assess. However, when the purification procedure was applied to extracts of potatoes which had been stored at non-chilling temperatures (10°) since harvest, a different pattern of elution was observed after Blue Sepharose chromatography (see Fig. 1). The total activity in the extract of potatoes stored at 10° was 10.2 nkat/100 g fr. wt compared with an activity of 104 nkat/100 g fr. wt in the extract of potatoes stored at 0° (see [8]). On Blue Sepharose the extract of non-chilled potatoes was resolved into 3 peaks, two of which corresponded in elution position with peaks II and III of the chilled samples and the third peak appeared only after prolonged washing of the column with 0.4 mM CoA in 5 mM phosphate pH 7 (see Fig. 1). In the potatoes stored at 10° throughout there was no peak of activity corresponding with the major peak of the chilled sample (peak I). The significance of this change in elution pattern is unclear but the development of a rapid and simple procedure for isolation of CQT in a high degree of purity is an essential step in our studies of the mechanism of the increase in activity of this enzyme during low temperature storage [7, 8].

EXPERIMENTAL

The potatoes used in these experiments were of the variety Homeguard grown at Easton, Norfolk. After harvest the tubers were washed and stored at 10° for 2 weeks and then were either

maintained at 10° or were transferred to 0°. CQT activity was measured as previously described [2].

Purification of CQT. Potato tissue (100–200 g) was homogenized in 2 vol. of a medium containing 0.25 M NaPi pH 7.5, 1 mM EDTA, 2 mM DTE with 0.5 g Polyclar AT/10 g of tissue. The resulting homogenate, after clarification, was fractioned with $(NH_4)_2SO_4$. The 30–85% $(NH_4)_2SO_4$ fraction was desalted on a column of Sephadex G25 [2] and applied to a column of DEAE-cellulose (DE52, 1.5 × 14 cm). The column was eluted with a linear gradient of KCl in 5 mM Tris, pH 7.45–1 mM DTE between 0 and 0.4 M in a total vol. of 650 ml. The active fractions were bulked, dialysed against 5 mM NaPi–1 mM DTE, pH 7 and applied to a column of Blue Sepharose (Pharmacia, 1.5 × 18 cm) equilibrated with 5 mM NaPi–1 mM DTE, pH 7. After washing, the column was eluted with a linear gradient of CoA in 5 mM NaPi, 1 mM DTE, pH 7 between 0 and 0.4 mM.

PAGE electrophoresis was carried out using Tris–HCl–glycine, pH 8.9 as the buffer system with 7 and 10% acrylamide gels [12]. 0.2 ml of the purified fractions was applied to gels 55 × 5 mm and electrophoresis was carried out at 2 mA for 10 min followed by 4 mA/gel for 35 min. Gels were stained for protein using Coomassie blue [13] or were sliced into 1 mm sections for spectrophotometric assay of CQT activity. Gels stained for protein were recorded photographically or by densitometry using a wavelength of 690 nm and a slit width of 0.25 mm. SDS-polyacrylamide gel electrophoresis was carried out by the method of ref. [22] using standard proteins as markers (Sigma MW-SDS-70 kit). For either type of electrophoresis it was essential to concentrate the protein fractions after Blue Sepharose chromatography. To achieve this the fractions were applied to micro-columns (80 × 3 mm) of DEAE-cellulose (DE-52). The columns were washed and eluted with 0.2 M KCl in 5 mM Tris, pH 7. 0.5 ml fractions were collected and the peak of eluted CQT activity was bulked and dialysed prior to electrophoresis. A minimum of 6 μg of protein was applied for the analytical PAGE.

MW determination. 5 ml of the 30–85% $(NH_4)_2SO_4$ fraction were applied to a column of Ultrogel AcA 54 (LKB, 38 × 2.5 cm) equilibrated with 0.2 M KH_2PO_4–0.5 mM DTE, pH 7. The column was eluted with the same buffer at a flow rate of 17 ml/hr and 1.9 ml fractions were collected and assayed for CQT activity. The column was calibrated with a range of proteins of known MW and an estimate of the MW of CQT was made on the basis of its elution vol.

The protein content of the various extracts was determined by a modification of the dye binding method of ref. [23] using the commercially available reagent (Bio-rad). The conc reagent (1 ml) was mixed with up to 4.1 ml of test soln in a final vol. of 5.1 ml. After mixing and standing for 5 min the mixture was measured at 595 nm against a reagent blank. Standard solns of BSA treated in the same manner were used for calibration. In some of our initial preparations the estimation of protein was carried out by both the Bradford [23] and Lowry [24] methods. The latter method was applied after TCA precipitation and re-solution of the protein in 0.1 N NaOH. The Lowry method consistently gave a value for protein slightly higher than that by the dye binding method but the relative values given by the two methods at the various stages of purification were quite similar. The fractions after Blue Sepharose chromatography did not give visible ppts. on adding TCA and only barely measurable colours if treated directly by the Lowry reagent. This low response led us to apply the more sensitive modified Bradford procedure.

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