

Further Characterization of Chickpea Isoflavone 7-O-Glucoside-6''-O-malonate:malonyl-esterase: Evidence for a Highly Specific, Membrane-Bound Enzyme in Roots of *Cicer arietinum* L.

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The specific malonyl-esterase from chickpea (*Cicer arietinum* L.), hydrolyzing biochanin A 7-O-glucoside-6''-O-malonate (BGM), has been purified to apparent homogeneity and characterized recently (Hinderer *et al.*, Arch. Biochem. Biophys. **248**, 570–578 [1986]). Its substrate specificity as well as the high molecular mass of the native enzyme were further investigated. The 5-deoxy-isoflavone conjugate corresponding to BGM, the formononetin 7-O-glucoside-6''-O-malonate (FGM), was shown to be a substrate of the malonyl-esterase essentially as BGM. By contrast, methyl-BGM, a diester of malonic acid was a poor substrate. The purified enzyme completely lacked thioesterase activity with malonyl-CoA as substrate. The inability of the malonyl-esterase to hydrolyze synthetic acetyl or propionyl esters was further demonstrated with a highly sensitive fluorometric assay using esters of 4-methylumbelliferone. The enzyme-catalyzed hydrolysis of BGM was stimulated in the presence of dissociated carboxylic acids like citrate which was most effective at 30 mM and pH 7.5–8.0.

The purified malonyl-esterase as well as the enzyme activity in crude extracts were totally excluded in gelchromatography with Ultrogel AcA 22. The enrichment of the enzyme activity in microsomal fractions gave strong evidence that the malonyl-esterase is membrane-bound *in vivo*. Stimulation of the enzyme activity *in vitro* by detergents indicates the presence of lipid material in the enzyme and the activity profiles obtained after sedimentation analyses suggest that purification of a distinct membrane-protein complex had been achieved.

Introduction

Among the plant esterases only a few have been found to possess a pronounced substrate specificity for endogenous acylated compounds [1–4]. In contrast, the majority of plant acyl hydrolases (esterases, lipases, peptide hydrolases) are considered to be non-specific. These enzymes which occur in multiple forms are often characterized using artificial substrates like α -naphthylacetate (ANA) or 4-nitrophenylacetate. Wheat cells, for example, contain at least 12 of such non-specific, soluble ANA esterases [5]. Some of these esterases may also be active using

malonyl conjugates of plants as substrates. Thus, parsley cells contain four different acylhydrolases which act on flavonoid malonylglucosides. These enzymes were described as three aryl and one acetyl-esterase, because ANA was the preferred substrate [6].

Recently we reported the occurrence of a specific malonyl-esterase in roots of chickpea (*Cicer arietinum* L.) which greatly differs from other known esterases [4]. This enzyme deacylated isoflavone 7-O-glucoside-6''-O-malonates (Fig. 1) and it showed no activity with synthetic esterase substrates such as ANA. The isolation of this malonyl-esterase has only been successful by exclusive use of the natural substrate biochanin A 7-O-glucoside-6''-O-malonate (BGM). This isoflavone conjugate accumulates in large amounts in roots of chickpea. Three aspects of the malonyl-esterase have now been investigated in more detail: I) The substrate specificity with regard to formononetin 7-O-glucoside-6''-O-malonate (FGM) which has a substantial metabolic activity and co-accumulates *in vivo* [7]. II) The high molecular mass of the native enzyme (> 2,000 kDa) with only a sin-

Abbreviations: ANA, α -naphthylacetate; BGM, biochanin A 7-O-glucoside-6''-O-malonate; BGM-CH₃, biochanin A 7-O-glucoside-6''-O-malonic acid methylester; CCR, cytochrome *c* reductase; FGM, formononetin 7-O-glucoside-6''-O-malonate; HPLC, high performance liquid chromatography; 4-MUF, 4-methylumbelliferone.

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gle protein subunit of 32 kDa. III) The *in vitro* stimulation of the enzyme activity by carboxylic acids.

Studies on the enzymatic hydrolysis of malonyl compounds could be of increasing importance with respect to the widespread occurrence of this type of conjugation in the plant kingdom [8].

Materials and Methods

Chemicals

BGM was isolated from roots and FGM from suspension cultured cells of *C. arietinum*. The iso-flavone conjugates were purified as described previously [9]. BGM-CH₃ was prepared by methylation of BGM with diazomethan in 80% methanol. The preparation contained 5% residual BGM. 4-MUF, its esters, ANA, malonyl-CoA, and antimycin A were obtained from Sigma (München, FRG). Cytochrome c and NADH came from Boehringer (Mannheim, FRG), NADPH and Polyclar AT from Serva (Heidelberg, FRG). Ultrogel AcA 22 was a product of LKB (Gräfelfing, FRG).

Plant material

Seeds of *C. arietinum* were purchased from Küper (Oberhausen, FRG) and they were germinated on wet filter paper under gently running tap water at 20 °C. For enzyme preparation 5 d old roots were used.

Buffers

The following buffer systems were used: A) 100 mM KH₂PO₄/K₂HPO₄, pH 7.5, containing 400 mM sucrose; B) 100 mM KH₂PO₄/K₂HPO₄, pH 7.5; C) 100 mM KH₂PO₄/K₂HPO₄, pH 7.5, containing 0.02% NaN₃; D) 20 mM KH₂PO₄/K₂HPO₄, pH 7.5; E) 50 mM KH₂PO₄/K₂HPO₄, pH 7.5.

Enzyme preparations

Crude protein extracts were prepared as follows: A 5 g-sample of roots was pulverized in a mortar in the presence of liquid nitrogen. After thawing in 10 ml of buffer B the cell fragments were further homogenized in a Potter-Elvehjem glass homogenizer. The homogenate was stirred with 0.5 g Polyclar AT for 10 min and centrifuged at 10,000 × g for 15 min. The resulting supernatant was either passed through Sephadex G-25 (coarse) PD-10 columns (Pharmacia,

Freiburg, FRG) using buffer C, and subsequently assayed for enzyme activities, or directly subjected to gelchromatography on Ultrogel AcA 22 (1.6 × 50 cm column). For this chromatography, a 2 ml portion (4–5 mg protein) was separated with a flow rate of 5 ml/h using buffer C, and fractions of 2 ml each were collected. The preparation of microsomes was started with 20 g root material. The root tissue was ground in a mortar with 30 ml of buffer A, 5 g quartz sand, and 2 g Polyclar AT. The homogenate was filtered through a 0.1 mm nylon net and the eluate was centrifuged at 5,000 × g for 10 min. The supernatant was adjusted to 50 mM MgCl₂ using a 1 M MgCl₂ solution, stirred for 10 min, and then centrifuged at 40,000 × g for 30 min. The microsomal pellet was re-suspended in a Potter-Elvehjem glass homogenizer with 3 ml of buffer A. An aliquot of the corresponding supernatant (2.5 ml) was desalted on a PD-10 column before using for enzyme assays. The procedures for enzyme purification and sedimentation analyses were described previously [4].

Enzyme assays

The assay of malonyl-esterase with BGM, FGM, or BGM-CH₃ was performed as previously described [4] using HPLC procedures according to Köster *et al.* [9].

Esterase activity with esters of 4-MUF as substrates was determined by a fluorometric assay. The reaction mixture contained 0.98 ml buffer D, 0.01 ml substrate solution (4-MUF-acetate or -propionate, 20 mM in acetone), and 0.01 ml enzyme preparation. The release of 4-MUF was directly monitored with a ISA spectrofluor JY 3D fluorometer (Jobin, Yvon, France) at 20 °C. The excitation wavelength was 340 nm and the emission was measured at 450 nm. The emission intensity was linear up to 0.2 mM 4-MUF. Quantitation was performed using a standard curve obtained under assay conditions. The enzyme activities were corrected for non-enzymatic hydrolysis determined in controls without enzyme extracts.

The determination of esterase activity with ANA as substrate has already been described [4].

Acyl-CoA thioesterase activity was determined with malonyl-CoA as substrate. The assay mixture contained 0.17 ml buffer D, 0.01 ml enzyme preparation, and 0.02 ml malonyl-CoA (1 mM in 10 mM NaH₂PO₄). The mixture was incubated at 30 °C for 30 min, stopped by adding 0.2 ml 50 mM NaH₂PO₄ (pH shift from 7.5 to 6) and stored on ice until it was

subjected to HPLC analysis. The HPLC method for quantitation of malonyl-CoA and CoA-SH was based on [10]. The separation was carried out with a Latek P 400 chromatograph (Heidelberg, FRG) using a LiChrosorb RP 18 column (250 × 4 mm, Merck, Darmstadt, FRG) and a flow of 0.8 ml/min. A linear gradient of 20% B to 50% B in A within 30 min was applied (solvent A = 40 mM NaH₂PO₄, solvent B = 50 mM NaH₂PO₄:CH₃CN = 4:1 v/v). Quantitation was performed at 260 nm by external standardization.

Cytochrome *c* reductase (CCR) was measured either with NADPH or with NADH in presence of antimycin A. The assay was based on [11] and contained in a final volume of 0.8 ml: 0.025 ml enzyme preparation, 22.5 nmol cytochrome *c*, 75 nmol NADH with 8 nmol antimycin A or 75 nmol NADPH, and 1,250 nmol KCN in buffer E. The reduction of cytochrome *c* was monitored at 550 nm and 25 °C.

Protein determination

Protein concentrations were determined according to Bradford [12] with bovine serum albumin as reference.

Results

Substrate specificity

It was previously shown that the purified malonyl-esterase was not able to hydrolyze acetyl or propionyl esters of α -naphthol or 4-nitrophenol to any significant extent, although the crude protein extract contained appreciable activities for all these substrates [4]. This is further confirmed with a highly sensitive fluorometric assay using 4-MUF-acetate and 4-MUF-propionate as substrates. The activity of the purified enzyme for 4-MUF-acetate was four orders of magnitude lower than for BGM (Table I) and with 4-MUF-propionate as substrate the activity was below the limit of determination. In contrast, the activities obtained with crude extracts were similar for all these esters. Likewise the purified enzyme lacked thioesterase activity with malonyl-CoA as substrate, whereas the crude extract contained considerable hydrolytic activities for malonyl-CoA (Table I).

A second endogenous isoflavone malonylglucoside in chickpea, FGM (Fig. 1), was hydrolyzed with rates equal to BGM (Table I). Assays including both

Table I. Hydrolysis of various esters by crude protein extracts and by purified malonyl-esterase.

Substrate	Relative specific enzyme activity [%]	
	Crude protein extract	Purified malonyl-esterase
BGM	100 ^a	100 ^b
FGM	n.d. ^c	100
BGM-CH ₃	n.d.	7.0
malonyl-CoA	2.7	< 0.01
4-MUF-acetate	22.9	0.03
4-MUF-propionate	35.9	< 0.01

^a 100% = 6.93 mkat/kg.

^b 100% = 4.80 kat/kg.

^c n.d. = not determined.

substrates, FGM and BGM, showed no discrimination for one of the two malonates by the malonyl-esterase. The *K_m* value for FGM was determined to be 0.93 mM, which is somewhat higher than the *K_m* for BGM (0.42 mM). Therefore the malonyl-esterase has no preferential affinity to FGM.

The role of carboxy groups

The natural substrates of the malonyl-esterase, BGM and FGM, are hemiesters of malonic acid and contain a terminal, dissociable carboxyl moiety (Fig. 1). Methylation of the carboxy group of BGM results in the diester BGM-CH₃ (Fig. 1) which is a poor substrate for the malonyl-esterase (Table I). On the other hand, the enzymatic hydrolysis of BGM was stimulated *in vitro* in presence of di- or tricarboxylic acids of which citrate was most effective [4].

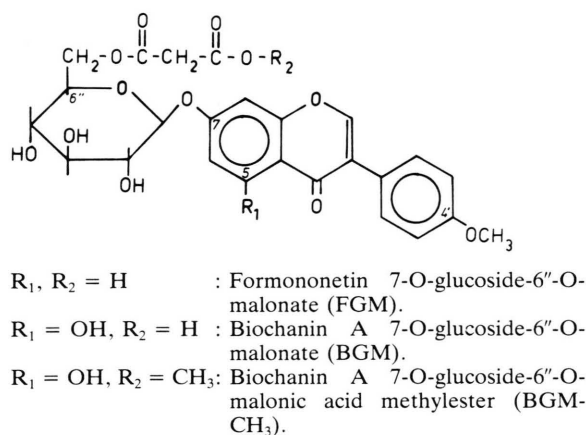


Fig. 1. Natural (BGM, FGM) and synthetic (BGM-CH₃) substrates of chickpea malonyl-esterase.

Fig. 2 shows the dependence of this stimulation on the concentration of citrate (Fig. 2a) and on pH (Fig. 2b). Maximum increase of enzyme activity was observed at about 30 mM citrate (at pH 7.5). The stimulation clearly depended on the pH value of the assay. Thus, no effect was observed at pH 7 and below, whereas between pH 7 and pH 8.5 a marked enhancement of the enzyme activity was measured under the influence of 10 mM citric acid. The pH optimum of the enzyme also shifted from 7.5 to 8.0. The activity of the malonyl-esterase at pH 6 and below was practically zero (Fig. 2b, controls). In conclusion, these data suggest that dissociated (charged) carboxy groups, including the carboxy group of the substrate, play an important role in the hydrolysis of malonic acid hemiesters catalyzed by chickpea malonyl-esterase.

Structure of the malonyl-esterase

As already mentioned [4] the purified malonyl-esterase has an unusually high molecular mass of at least 2,000 kDa as observed in gelchromatography and ultracentrifugation. In contrast, gelelectrophoresis under denaturing conditions revealed one single protein of 32 kDa. One explanation for this discrepancy could be a polymerization process of the enzyme during purification, possibly induced by ammonium sulfate precipitation, or concentration by ultrafiltration. Experiments were now carried out to solve this problem. Exclusion from Ultrogel AcA 22 (exclusion limit 2,000 kDa) should clearly indicate

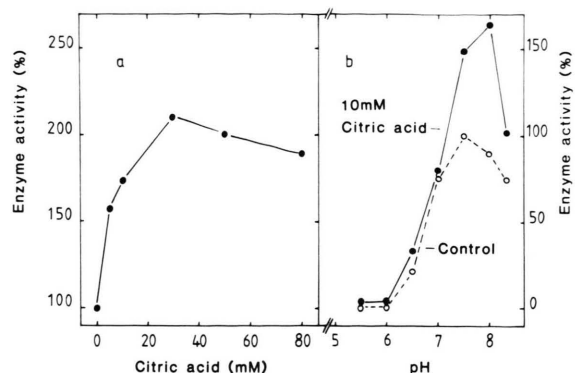


Fig. 2. Effect of citric acid on malonyl-esterase activity *in vitro*. a) Dependence on the concentration of citric acid at pH 7.5. b) pH-Dependence of the stimulation by 10 mM citric acid.

the high molecular structure of the enzyme. Fig. 3 demonstrates that the malonyl-esterase as found in the crude protein extracts was totally excluded from the gel. No activity appeared within the fractionation range of the gel. ANA esterase activity, however, was detected within the fractionation range and not in the void volume (Fig. 3). When different homogenization methods for preparation of crude protein extracts were applied the same result as shown in Fig. 3 was obtained.

Analyses of microsomes, precipitated with $MgCl_2$ indicated that the BGM malonyl-esterase is membrane-bound. The increase of the specific enzyme activity in the membrane fraction (Table II) and the distribution of total enzyme activity between pellet

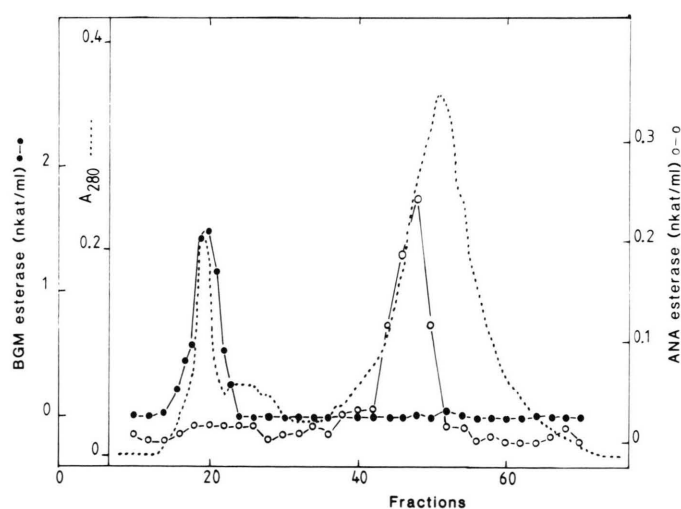


Fig. 3. Exclusion of malonyl-esterase activity of the crude protein extract in Ultrogel AcA 22 chromatography and separation from ANA esterase activity.

Table II. Enzyme activities in microsomal fractions and corresponding supernatants.

Enzyme	Specific activity [mkat/kg]		Distribution [%] ^a	
	Microsomes	Supernatant	Microsomes	Supernatant
BGM-esterase	24.1	2.9	69	31
ANA-esterase	3.4	1.1	9	91
CCR (NADH) ^b	8.6	1.2	67	33
CCR (NADPH)	1.7	0.3	58	42

^a 100% = Sum of activities in microsomes and supernatant.^b Part of activity insensitive to antimycin A.

and supernatant was similar to CCR (NADH). The latter enzyme has been chosen as a general marker for membranes of the endoplasmic reticulum [11]. On the contrary, the bulk of the ANA esterase activity, remained in the soluble fraction (Table II). The relative distribution of malonylcoesterase during differential centrifugation parallels those of CCRs (NADH and NADPH) except for a lower amount of activity of the malonylcoesterase in the final 100,000 × *g* supernatant (data not shown).

Finally, investigations with the purified enzyme suggested that purification of a membrane-protein complex had been achieved. The pronounced stimulation of the enzyme activity by Triton X-100 and digitonin (Fig. 4) is best explained by presence of lipid material. The activity profiles obtained in sucrose gradients after ultracentrifugation showed the rapid migration of the enzyme and point to a relatively distinct particle size (Fig. 5).

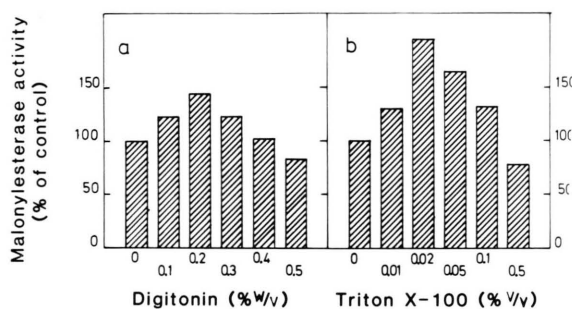


Fig. 4. Effect of increasing detergent concentrations on the activity of purified malonylcoesterase.

Discussion

According to our recent report, roots from young chickpea plants contain one specific malonylcoesterase besides various non-specific (ANA active) esterases

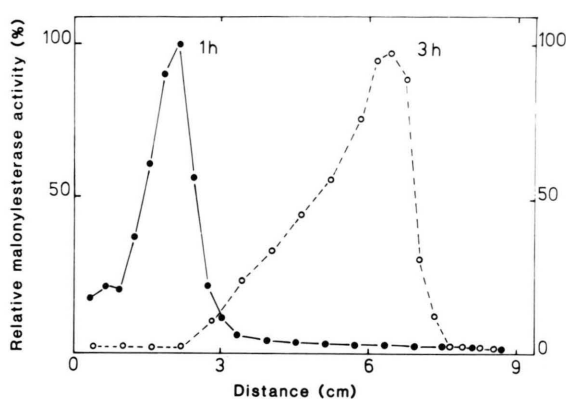


Fig. 5. Sedimentation analysis of purified malonylcoesterase. Migration in isokinetic sucrose gradients (5–20%) during ultracentrifugation.

[4]. This specific enzyme was isolated and purified like a soluble enzyme without solubilization, *e.g.* ammonium sulfate precipitation and ionexchange chromatography were the initial steps. The purification yielded a preparation which consisted of one single protein subunit. We now present additional results which strongly suggest that the high molecular mass of the enzyme is due to the presence of membranes. As outlined in this paper the malonylcoesterase appears to be membrane-bound *in vivo* so that a membrane-protein complex had been isolated. Membrane-bound plant esterases are rarely observed. Red radish contains a sinapoylcoesterase reported to be membrane-bound [13] which also belongs to phenylpropanoid metabolism.

The chickpea malonylcoesterase showed a distribution behaviour between microsomes and supernatant like CCR (NADH), which was used as an indicator for membranes. In a simplified purification procedure, *i.e.* ammonium sulfate fractionation (0–35%) and subsequent Ultrogel Aca 22 chromatography,

CCR parallels malonyl-esterase activity and was thus enriched about 30-fold. As suggested previously the 32 kDa protein of the malonyl-esterase occurs in relatively high amounts in the roots. Nevertheless, this cannot explain the isolation of a complex of this large size containing only one type of protein. Although traces of other proteins had been visualized by silver staining in polyacrylamide gels [4], we do not consider this to be of any significance.

Using detergents the enzyme activity *in vitro* could be enhanced by 50–100% at low concentrations though the effect was reversed at higher concentrations. Chromatography of partially purified esterase enzyme after treatment with Triton X-100 resulted in a complete loss of activity most likely due to a then decreased stability of the enzyme. So far, we have no data on the structural organization of the malonyl-esterase complex and its localization *in vivo*.

With monoaryl malonates as model substances it was shown that hydrolysis of such compounds involves intramolecular catalysis by the ionised carboxy group. Such data are possibly of significance to explain hydrolysis involved in enzyme catalysis [14].

The pH-dependence of the malonyl-esterase with BGM as substrate suggests that the anion is the true substrate. Uncharged substrates, such as the diester BGM-CH₃, are converted with much lower rates. Interestingly, the enzyme catalyzed the hydrolysis of BGM at a remarkably increased rate in the presence of dissociated carboxy groups, for example provided by citric acid. Non-enzymatic hydrolysis was however, not significantly influenced. The increase of V_{\max} was accompanied by a decrease of K_m . This effect occurs in a concentration range of 10–100 mM (Fig. 2a) and therefore it seems questionable if this phenomenon can play a role *in vivo*. But it should be mentioned that the malonyl-esterase would be stimulated by one of its products, malonate, if the substrate concentration were sufficiently high. Thus, quantitative conversion of malonyl-esters to the glucosides even at very high substrate concentrations appears to be possible.

BGM-CH₃ was found to be a poor substrate, and therefore it is not surprising that uncharged acetyl or

propionyl esters of various alcohols are no substrates of the malonyl-esterase. On the other hand, charged esters are poor substrates for acetyl-, carboxyl- or arylesterases [15].

In parsley cell cultures flavonoid malonylglucosides are exclusively located in vacuoles [16]. It was recently shown that malonylation is a prerequisite for the transport of these compounds into the vacuole. The glucosides themselves were not transported [17]. However, conjugation must not be a one way reaction. In chickpea roots FGM exhibited substantial metabolic activity *in vivo* and it is turned over with significant rates, whereas BGM appears to be metabolically rather inert [6]. This differential turnover cannot be explained at the level of the malonyl-esterase because the enzyme possesses similar kinetic data *in vitro* for both endogenous substrates. Future investigations on this differential turnover should especially deal with both the pattern of cellular compartmentation of biosynthetic and catabolic reactions and the localization of the enzymes involved in metabolism of isoflavone conjugates in chickpea.

Undoubtedly, the malonyl-esterase catalyzes the first step in the degradation of the chickpea malonylglucosides followed by hydrolysis of the glucosides caused by specific β -glucosidases [18]. Both catabolic reactions also occurred during the degradation of BGM by the fungus *Fusarium javanicum*, which transiently accumulated the aglycone [19]. In plants a further example of a demalonylation step has recently been demonstrated in the recycling of the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) from its widely occurring N-malonyl conjugate (MACC) [20]. In addition to a metabolism of MACC *in vivo* [20] also MACC hydrolase (amidase) was detected in peanut [21] and watercress [20].

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