Pergamon

Bioorganic & Medicinal Chemistry 8 (2000) 233–237

BIOORGANIC & MEDICINAL CHEMISTRY

# Mechanism of Biochemical Action of Substituted 4-Methylbenzopyran-2-ones. Part 6: Hydrolysis of 7,8-Diacetoxy-4-methylcoumarin by a Novel Deacetylase in Rat Liver Microsomes — a Simple Method for Assay and Characterisation

Hanumantha Rao G. Raj,[a] Virinder S. Parmar,[b,*] Subhash C. Jain,[b] Sanjay Goel,[a] Yogesh K. Tyagi,[a] Sunil K. Sharma,[b] Carl E. Olsen[c] and Jesper Wengel[d]

[a]*Department of Biochemistry, V.P. Chest Institute, University of Delhi, Delhi-110 007, India*
[b]*Department of Chemistry, University of Delhi, Delhi-110 007, India*
[c]*Chemistry Department, Royal Veterinary & Agricultural University, 40 Thorvaldsensvej, Frederiksberg C, DK-1871 Copenhagen, Denmark*
[d]*Department of Chemistry, University of Copenhagen, Universitetsparken 5, DK 2100 Copenhagen, Denmark*

Received 2 August 1999; accepted 21 September 1999

**Abstract**—The existence of a novel microsomal deacetylase in rat liver catalysing deacetylation of diacetoxy 4-methylcoumarins has been reported. A simple method is outlined for the enzyme assay based upon the quantification of the dihydroxy derivative by measuring the UV absorption of its complex with ADP and $Fe^{3+}$ at 600 nm. The enzyme can be routinely assayed using 7,8-diacetoxy-4-methylcoumarin (DAMC) as the substrate and demonstrated hyperbolic kinetics and yielded $K_m$ and $\nu_{max}$ values of 1250 μM and 500 units, respectively. The pH optima was found to be 7.5 for the enzyme. No DAMC deacetylase activity was found in hepatic cytosol and the enzyme activity was not discernible in extrahepatic tissues. © 2000 Elsevier Science Ltd. All rights reserved.

## Introduction

As a part of our ongoing research programme towards screening of various lipases/esterases for carrying out selective protection/deprotection of polyphenolics, we explored the possibility of using tissue esterases for selective protection/deprotection of coumarins. A very large number of esterases are known to occur in human tissues.[1] However, the knowledge on tissue microsomal esterases is rather incomplete and complex, as analysis and characterisation of tissue esterases has not only been complicated in terms of purification but also due to their existence in multiple molecular forms. Mostly the pattern of substrate specificity and sensitivity to various inhibitors have been the basis for characterisation of the esterases. Hammer and Ladu[2] employed extraction of human liver microsomes by glycerol, followed by gel filteration for the isolation of esterases. Cardona and King[3] demonstrated that *O*-glucuronide of *N*-hydroxy-*N*-2-fluorenylacetamide was deacetylated by liver microsomes of guinea pigs. Deacetylation of *O*-glycoside of *N*-hydroxyacetanilide was found to be localized in liver microsomes of guinea pigs.[4]

We herein report the deacetylation of diacetoxy 4-methylcoumarins using rat liver microsomes (Fig. 1(A)). The assay and characterisation of the enzyme is carried out principally using 7,8-diacetoxy-4-methylcoumarin (DAMC) (**1**) as the substrate and the method is extended to assay the deacetylase with other diacetoxycoumarins as substrates.

## Materials and Methods

ADP was purchased from Sigma Chemical Co., St. Louis (MO, USA). *p*-Hydroxymercuribenzoate (PHMB) was procured from E-Merck, Germany. $FeCl_3$, Tris and DMSO were purchased from local suppliers and were of high purity. DAMC (**1**), 7,8-dihydroxy-4-methylcoumarin

*Corresponding author. Tel.: +91-11-725-6555/7206; fax: +91-11-725-7206; e-mail: vparmar.duchem@axcess.net.in

0968-0896/00/$ - see front matter © 2000 Elsevier Science Ltd. All rights reserved.
PII: S0968-0896(99)00272-2

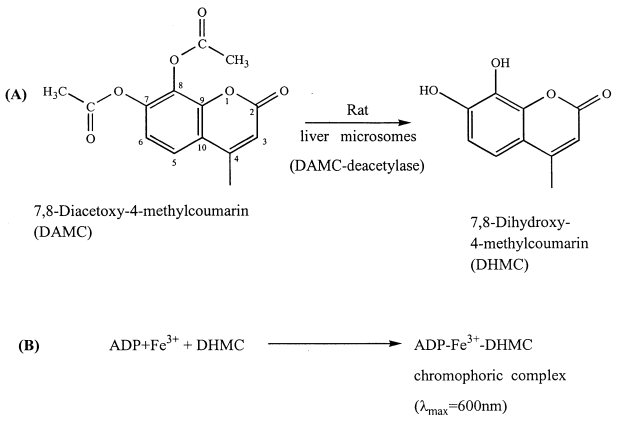



**Figure 1.** (A) Enzymatic deactylation of diacetoxy 4-methylcoumarin; (B) formation of mixed ligand complex.

(DHMC), 6,7-diacetoxy-4-methylcoumarin (**2**) and 7,8-diacetoxycoumarin (**3**) were synthesized in our laboratory.

## Quantitation of DHMC

Standard solution (2 mM) of DHMC was prepared in DMSO. Aliquots of the standard solution (10 to 300 μL) were pipetted out into different tubes taking care that each concentration was taken in triplicate, 0.2 mL of 0.25 M Tris–HCl (pH 7.5) was added to all the tubes followed by the addition of 0.2 mL of 30 mM ADP and 0.2 mL of 1.5 mM $FeCl_3$. The total volume of the reaction mixture was ensured to be 2.0 mL by the addition of appropriate amount of water. The contents of the tubes were mixed thoroughly on a vortex mixer and intensity of the green colour developed was measured at 600 nm using Beckman spectrophotometer (Model DU 64).

## Preparation of rat liver microsomes

Male albino rats of Wistar strain weighing 190–200 g fed on rat chow supplied by Hindustan Lever Ltd., Mumbai (India) were sacrificed, liver excised and microsomes prepared as described earlier.[5] Protein was estimated by the method of Lowry et al.[6]

## Assay of rat liver microsomal DAMC deacetylase activity

The assay mixture consisting of Tris–HCl (0.025 M, pH 7.5), microsomes (1 mg protein), DAMC (50 to 600 μM) and water to make total volume of 1.6 mL was incubated for 10 min at 37 °C. The tubes were covered later with glass marbles and placed in a boiling water bath for 10 min, cooled, ADP (3 mM) and $FeCl_3$ (0.15 mM) added, the final volume of reaction mixture was adjusted to 2.0 mL and the concentration of DHMC was calculated by measuring the absorption at 600 nm. One unit of deacetylase catalysed the hydrolysis of 1 μmol *ortho* diacetoxycoumarin derivatives under the conditions of the assay. The conditions for the assay of liver microsomal DAMC deacetylase were chosen to ensure that the initial rate was linear with respect to time of incubation and enzyme concentration.

## Kinetics of rat liver microsomal DAMC deacetylase

**Initial velocity as a function of substrate concentration.** Several concentrations of DAMC (25 to 250 μM) were separately incubated with microsomes (1 mg protein), Tris–HCl (0.025 M, pH 7.5) and water in a total volume of 1.6 mL for 10 min at 37 °C. The progress of the reaction was assayed by the quantification of DHMC as described above. The reciprocal of initial rate (1/v) was plotted against that of substrate concentration (l/[S]) in order to calculate $K_m$ and $v_{max}$ values.

**Effect of pH.** The reaction mixture consisting of DAMC (125 μM), liver microsomes (1 mg protein), different buffers (0.025 M) of varying pH (4.0 to 11.0) and water in a total volume of 1.6 mL was incubated for 10 min at 37 °C. The enzyme was inactivated by heat exposure as described above and pH adjusted to 7.5 before the development of colour by the addition of ADP and $Fe^{3+}$ as described above.

## Substrate specificity

Several structural analogues of DAMC were separately incubated at several concentrations (25–250 μM) with microsomes (1 mg protein), Tris–HCl (0.025 M, pH 7.5) and water in a total volume of 1.6 mL. The activity of the enzyme was quantified as described earlier.

## Results and Discussion

The existence of microsomal deacetylase that catalyses the deacetylation of diacetoxy 4-methylcoumarins has been reported here for the first time. A simple method for the assay of this deacetylase is described which enables preliminary characterisation of the enzyme.

The enzyme assay is based upon the quantification of the deacetylated product of DAMC (or any other *ortho* diacetoxycoumarin) resulting in the formation of DHMC (or corresponding *ortho* dihydroxy derivatives)

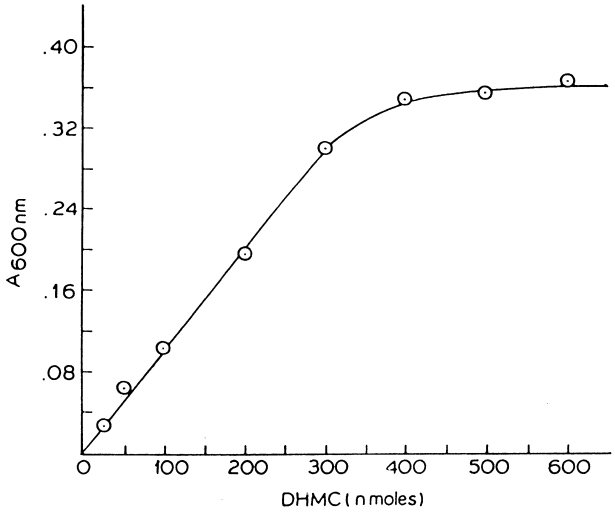



**Figure 2.** Quantification of DHMC.

(Fig. 1(A)). We earlier demonstrated that DHMC forms a stable mixed ligand complex of ADP-$Fe^{3+}$–DHMC (Fig. 1(B)) with ADP and $Fe^{3+}$ which had intense UV absorption at 600 nm.[5] The data depicted in Fig. 2 reveals linearity of absorption at 600 nm with respect to the concentration of ADP-$Fe^{3+}$–DHMC complex which served to quantify the concentration of DHMC.

The method afforded an accuracy of estimation up to a minimum of 25 nmols of DHMC (4.8 μg). The molar extinction coefficient of DHMC worked out to be $2\times10^3$ $M^{-1}$ $cm^{-1}$ under the conditions of the assay procedure. It is worth mentioning that 6,7-dihydroxy-4-methylcoumarin exhibited the same extinction as DHMC. Hence DHMC can be employed as a standard when different *ortho* diacetoxy derivatives are used as the substrates for the deacetylase described here. DAMC deacetylase was assayed by incubating DAMC with rat liver microsomes under the specified conditions and the progress of the reaction was followed by quantifying the product DHMC as described earlier. The linearity observed with respect to time of incubation and enzyme concentration (Figs 3 and 4) confirm the enzymatic hydrolysis of DAMC by rat liver microsomes. DAMC deacetylase exhibited hyperbolic kinetics and application of double reciprocal plot to rat liver

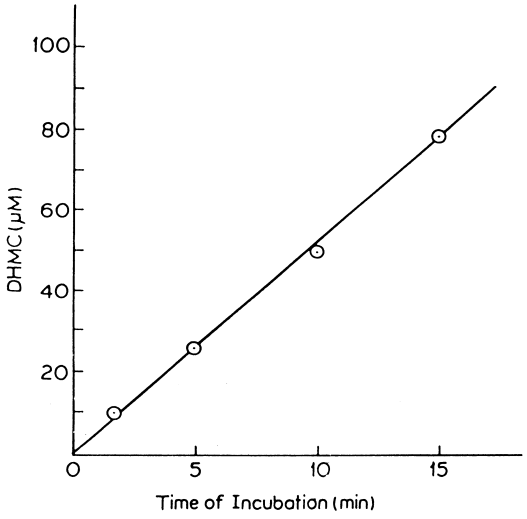



**Figure 3.** Effect of time of incubation on DAMC deacetylase activity. Rat liver microsomes (1 mg protein) was incubated with DAMC (125 μM). DAMC deacetylase was assayed as described under Materials and Methods.

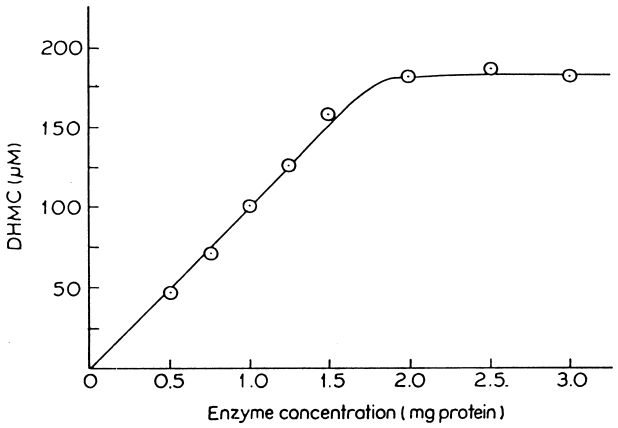



**Figure 4.** Effect of enzyme concentration on DAMC deacetylase activity. Rat liver microsomes (0.5–3.0 mg protein) was separately incubated with DAMC (250 μM) for 10 min and DAMC deacetylase was assayed as described under Materials and Methods.

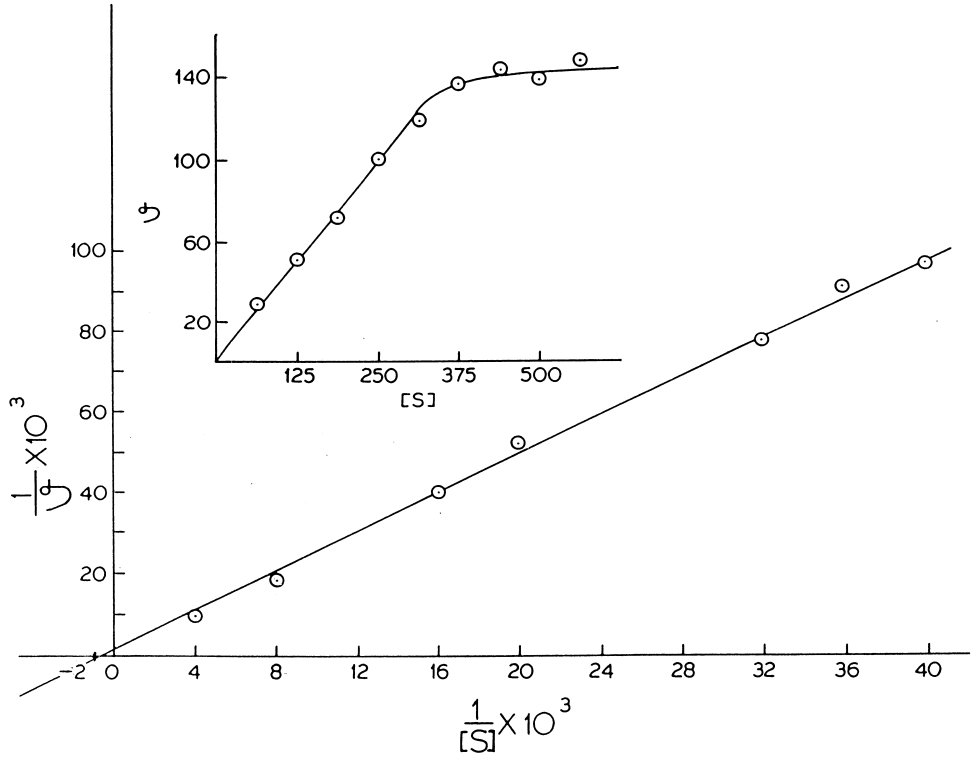



**Figure 5.** Effect of DAMC concentration on DAMC deacetylase activity.

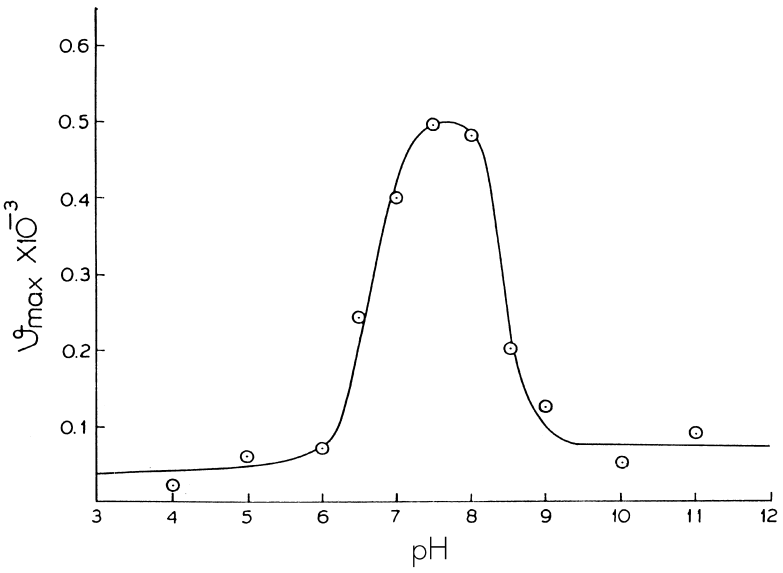



**Figure 6.** Effect of pH on DAMC deacetylase activity.

**Table 1.** Substrate specificity for rat liver microsomal diacetoxy 4-methylcoumarin deacetylase

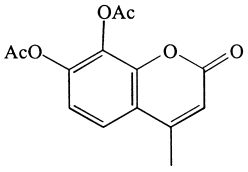
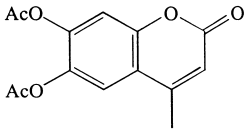
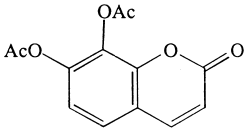
| Compound | Kinetic constants | |
|---|---|---|
| | $K_m$ (μM) | $\nu_{max}$ (units) |
|  **1** | 1250 | 500 |
|  **2** | 1437 | 450 |
|  **3** | 825 | 670 |

**Table 2.** Tissue specificity of microsomal diacetoxy 4-methylcoumarin deacetylase of rat tissues

| Tissue | Diacetoxy 4-methylcoumarin deacetylase |
|---|---|
| Liver | + + + + |
| Lung | 0 |
| Kidney | 0 |
| Intestine | 0 |

microsomal DAMC deacetylase (Fig. 5) yielded $K_m$ and $\nu_{max}$ values of 1250 μM and 500 units, respectively. Since the chromophoric complex ADP-$Fe^{3+}$–DHMC is decolorised at the acidic pH, care was taken to readjust the pH of different buffers used to 7.5 during the study of the effect of pH on the DAMC deacetylase activity profile which revealed pH optima of 7.5 (Fig. 6). An effort was made to assess the substrate specificity for the rat liver microsome-catalysed deacetylation of diacetoxycoumarins (Table 1). In this investigation, *ortho* diacetoxy derivatives were taken as the substrates with a view that the *ortho* dihydroxy moiety generated upon deacetylase action can impart green colour with ADP and $Fe^{3+}$. 7,8-Diacetoxycoumarin (**3**) was found to be a better substrate as compared to DAMC for the deacetylase (Table 1). An effort was made to examine the effect of the thiol binding agent PHMB on deacetylase by including the mercurial compound at a concentration of $10^{-2}$ M in the enzyme assay mixture. The result demonstrated that PHMB had no effect on the enzyme activity (data not shown) indicating that no thiol group is involved at the active centre of DAMC deacetylase. The DAMC deacetylase reported here was found to be very specifically present in the rat liver, other extrahepatic tissues such as lung, kidney and intestine revealed no measurable deacetylase activity (Table 2). It is worth noting that no deacetylase activity towards DAMC was detected in rat liver cytosol although the cytosol has been shown to catalyse a variety of deacetylation reactions.[1] Hepatic microsomes have been reported to hydrolyse carboxy ester groups,[7–10] DAMC deacetylase can possibly belong to the family of aryl carboxyl esterases. Purification of DAMC deacetylase is being undertaken to confirm such possibilities and to help further characterisation.

## Acknowledgements

The financial assistance of the Danish International Development Agency (DANIDA) is gratefully acknowledged. The authors acknowledge the help and cooperation of Dr V. K. Vijayan, Director, VP Chest Institute.

## References

1. Ladu, B. N.; Snady, H. *Handbook Exp. Pharmacol.* **1971**, *28*, 477.
2. Hammer, G. S.; Ladu, B. N. *Pharmacologist* **1968**, *10*, 202.
3. Cardona, R. A.; King, C. M. *Biochem. Pharmacol.* **1976**, *25*, 1051.
4. Suzuki-Kurasaki, M.; Yoshioka, T.; Hematsu, T. *Biochem. J* **1997**, *325*, 155.
5. Raj, H. G.; Sharma, R. K.; Garg, B. S.; Goel, S.; Parmar, V. S.; Jain, S. C.; Tyagi, Y.; Singh, A.; Olsen, C. E.; Wengel, J. *Bioorg. Med. Chem.* **1998**, *6*, 2205.
6. Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. *J. Biol. Chem.* **1951**, *193*, 265–275.
7. Satoh, T.; Morol, K. *Biochem. Pharmacol.* **1972**, *2*, 3111.
8. Heymann, E.; Mentiein, R.; Rix, H. *Methods Enzymol.* **1981**, *77*, 405.
9. Dueker, S. R.; Lame, M. W.; Segall, H. J. *Toxicol Appl. Pharmacol.* **1992**, *117*, 116.
10. Pastra-Landis, S. C.; Evans, D. R.; Lipscomb, W. N. *J. Biol. Chem.* **1978**, *253*, 4624.