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Mechanism of Biochemical Action of Substituted 4-Methylbenzopyran-2-ones. Part 7: Assay and Characterization of 7,8-Diacetoxy-4-methylcoumarin:Protein Transacetylase from Rat Liver Microsomes Based on the Irreversible Inhibition of Cytosolic Glutathione S-Transferase

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Abstract—The enzymatic transfer of acetyl groups from acetylated xenobiotics to specific proteins is a relatively grey area in the evergreen field of biotransformation of foreign compounds. In this paper, we have documented evidence for the existence of a transacetylase in liver microsomes that catalyses the transfer of acetyl groups from 7,8-diacetoxy-4-methylcoumarin (DAMC) to glutathione S-transferase (GST), either purified or present in cytosol leading to the irreversible inhibition of GST. A simple procedure is described for the assay of transacetylase by preincubation of DAMC with liver microsomes and pure GST/liver cytosol, followed by the addition of 1-chloro-2, 4-dinitrobenzene (CDNB) and reduced glutathione (GSH) in order to quantify GST activity by the conventional procedure. The extent of inhibition of GST by DAMC under the conditions of the assay is indicative of DAMC:protein transacetylase activity. Following the assay procedure described here, the transacetylase was shown to exhibit hyperbolic kinetics. The bimolecular nature of the transacetylase reaction was apparent by the demonstration of K_m and v_{max} values. 7,8-Dihydroxy-4-methylcoumarin (DHMC), one of the products of transacetylase reaction was identified and quantified using the partially purified enzyme. The fact that *p*-hydroxymercuribenzoate (PHMB) and iodoacetamide abolished irreversible inhibition of GST upon the action of transacetylase on DAMC strongly characterized transacetylase as a protein containing thiol group at the active site. In addition, the relative specificities of acetoxy 4-methylcoumarins to transacetylase have been demonstrated.

Introduction

Acetylation of foreign compounds catalysed by acetyl coenzyme A-linked acetyl transferases has been widely studied.[1–5] However, the enzymatic transfer of acetyl groups of acetylated xenobiotics to the cellular targets viz. specific proteins, enzymes, etc. has received little attention. John Vane[6] discovered that aspirin (acetyl salicylate) acetylates cyclo-oxygenase leading to inhibition of biosynthesis of prostaglandins and thus exerts the anti-inflammatory effects. Aspirin mediated acetylation of protein described above is non-enzymatic in nature. During the course of our investigations on the mechanism of biochemical action of DAMC, we have earlier observed that the activities of several enzymes and related biological effects[7] were modulated by DAMC while its deacetylated product 7,8-dihydroxy-4-methylcoumarin (DHMC) was devoid of such effects. These observations gave an indication of the potential of DAMC to exert biological acetylation catalysed by transacetylase localized in liver microsomes.[8,9] In this paper, we describe the mechanism-based inhibition of liver cytosolic GST by DAMC catalysed by hepatic microsomes as a measure of DAMC:protein transacetylase action.

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Materials and Methods

7,8-Diacetoxy-4-methylcoumarin, 7,8-dihydroxy-4-methylcoumarin 6,7-diacetoxy-4-methylcoumarin, 5,7-diacetoxy-4-methylcoumarin, 7-acetoxy-4-methylcoumarin and 7,8-diacetoxy-4-(3,4-diacetoxyphenyl)coumarin were synthesized in our laboratory. Reduced glutathione (GSH) and 1-chloro-2, 4-dinitrobenzene (CDNB) and mixture of purified glutathione S-transferase from bovine liver catalogue No: G8386 were procured from Sigma Chemical Co., St. Louis, MO (USA). Sodium *p*-hydroxymercuribenzoate (PHMB) was obtained from E. Merck (Germany). Iodoacetamide was the product of Lancaster Synthesis; DEAE-sepharose was the product of Pharmacia. All other chemicals used were of high purity and were obtained from local suppliers.

Male albino rats of Wistar strain weighing around 190–200 g were used.

Preparation of liver microsomes and cytosol

Rats were killed by decapitation, liver removed and 30% homogenate (w/v) was prepared in 10 mM phosphate buffer containing 0.25 M sucrose and 1.4 mM β-mercaptoethanol and the pH adjusted to 7. The homogenate was centrifuged at 10,000×*g* for 30 min and the supernatant was spun at 100,000×*g* for 1 h using Backman Ultracentrifuge Model L7. The cytosolic fraction was set aside at −20 °C. The microsomal pellet was resuspended in 1.15% KCl and microsomes were resedimented and suspended in 0.25 M sucrose. Protein content of microsomes and cytosol were assayed by the method of Lowry et al.[10]

Partial purification of rat liver DAMC:protein transacetylase

(a) Solubilization of the microsomal pellet. The procedure of Dey et al.[11] was followed. Microsomes were thawed and resuspended by brief homogenization in 1 M potassium phosphate buffer, pH 7.4 (4 mL/mg protein). The mixture was stirred on a magnetic stirrer for 30 min at 4 °C and then centrifuged at 100,000×*g* for 1.25 h. The clear supernatant was decanted and used as a source of transacetylase. The clear supernatant so obtained was dialysed against 10 mM potassium phosphate buffer containing 1.4 mM β-mercaptoethanol (pH 7.2) (loading buffer).

(b) Ion exchange chromatography. The dialyzed supernatant was loaded onto 3 mL DEAE-sepharose column at 4 °C and unbound proteins were eluted with loading buffer, collected in 3 mL fractions and read at 280 nm. The fractions with appreciable absorption at 280 nm were assayed for transacetylase activity, if any.

The enzyme was eluted with increasing molar concentration of NaCl in loading buffer. The fractions of 1 mL volume were read at 280 nm, followed by assay of transacetylase. The enzyme was found to elute optimally with 0.4 M NaCl, fractions were collected and dialyzed against loading buffer.

The dialyzed sample (12 mL) was loaded on to a 1 mL DEAE-sepharose column and enzyme eluted (3 mL) with 0.4 M NaCl in loading buffer. The enzyme preparation was again dialysed against loading buffer.

Glutathione S-transferase (GST) assay

The method of Habig et al.[12] was followed using GSH and CDNB as the substrates. The assay was carried out in 1.0 mL spectrophotometric cuvette (1 cm light path). The reaction mixture consisted of 0.25 M phosphate buffer (pH 6.5), cytosol (5–25 μg protein), 1 mM CDNB (added in 50 μL ethanol), 1 mM GSH in a total volume of 1.0 mL. The contents were mixed and progress of the reaction was followed at 340 nm in Beckman Model DU-64 spectrophotometer using the kinetic software and ensured that the reaction was linear with respect to enzyme concentration.

Liver microsome-catalyzed irreversible inhibition of cytosolic GST by DAMC

Rat liver microsomes (25 μg protein) were mixed with a fixed concentration of DAMC (50–100 μM, in 0.05 mL DMSO), 0.25 M phosphate buffer (pH 6.5), purified GST or liver cytosol (12.5 μg protein) and water added to make total volume of 0.8 mL. The contents (scaled up as per requirement) were preincubated at 37 °C. The aliquots (0.8 mL portion) were removed periodically into a spectrophotometer cuvette containing GSH and CDNB to make their final concentration 1 mM in a total volume of 1 mL and GST was assayed as mentioned earlier.

The procedure outlined above without the addition of DAMC served as a control (taken as 100%). The effect of DAMC on preincubation with microsomes mediating the inhibition of GST was represented by plotting the inhibition of GST (as percentage of control) against the time of preincubation.

Assay of liver microsomal acetoxycoumarin:protein transacetylase

The transacetylase in rat liver microsomes was assayed routinely using DAMC as a substrate unless otherwise mentioned and cytosolic GST as the second substrate. The assay mixture in a total volume of 0.8 mL consisted of 0.25 M phosphate buffer (pH 6.5), rat liver microsomes (25 μg protein), cytosol (12.5 μg protein) and DAMC (50 μM in 50 μL DMSO) and preincubated for 10 min, followed by the addition of GSH and CDNB for the assay of GST as described above.

The unit of transacetylase activity was expressed in terms of% inhibition of GST under the conditions of the assay.

Effect of PHMB on liver microsome-catalyzed irreversible inhibition of GST by DAMC

PHMB at a concentration of 10^{-2} M was included in the preincubation reaction mixture and the assay was carried out as described above.

Kinetics of rat liver microsomal acetoxycoumarin: protein transacetylase

(a) Effect of enzyme concentration. The transacetylase assay described above with the addition of varying amounts of liver microsomes was carried out. The transacetylase assay was routinely carried out under the conditions, where enzyme activity was linear with respect to enzyme concentration.

(b) Effect of varying substrate concentration on liver microsomal transacetylase. Rat liver microsomes (25 μg protein) were mixed separately with a fixed concentration of DAMC (10 to 100 μM, in 0.05 mL DMSO), 0.25 M phosphate buffer (pH 6.5), liver cytosol (12.5 μg protein) and water to make a total volume of 0.8 mL. The contents of the tube were incubated for 10 min, followed by the addition of GSH and CDNB required for the assay of cytosolic GST as described earlier. The % inhibition of cytosolic GST was plotted against the concentration of DAMC to compute the kinetic parameters K_m and v_{max}.

Since DAMC:protein transacetylase catalysed reaction is bimolecular in nature, the effect of varying the concentration of the second substrate GST (cytosolic protein) was quantified by performing the assay using a fixed concentration of the substrate (DAMC).

Identification and quantitation of the products of transacetylase reaction

The preincubation mixture (in duplicate tubes) consisted of Tris–HCl (0.025 M, pH 7.0), partially purified transacetylase (75 μg protein), DAMC (100 μM), purified GST (0.095 units) and iodoacetamide (10^{-2} M) (wherever included), the volume was adjusted to 1.6 mL with water. The mixture was incubated for 10 min at 37 °C. The tubes were covered with glass marbles and placed in boiling water bath for 10 min and cooled.

To one set of tubes, 2 mL of ethyl acetate was added and the contents mixed using a Vortex shaker in order to extract the metabolites. Each tube was centrifuged at 2000 rpm for 10 min and the clear organic layer was transferred into another tube. The aqueous mixture was re-extracted twice, the organic layers pooled and evaporated to dryness by blowing N_2 gas. The products were dissolved in 1 mL methanol. The metabolites were separated by HPLC using Beckman high pressure liquid chromatograph (Model Beckman Gold) fitted with ODS column, 20 μL of methanol extract prepared as described earlier was injected and isocratically eluted with methanol: H_2O (60:40 v/v). The flow rate was adjusted to 1 mL/min and the metabolites were detected using UV–Vis detector (NEC) at the wavelength 254 nm. Authentic samples of DAMC and DHMC were also subjected to HPLC to identify them in the test sample.

To the other set of tubes were added ADP (3 mM), $FeCl_3$ (0.15 mM) and water to make up the volume to 2 mL. The contents of the tube were mixed thoroughly on a Vortex shaker. The tubes were centrifuged at 2000 rpm for 10 min. The clear supernatant was read at 600 nm on a Beckman spectrophotometer (Model 64). The concentration of DHMC was calculated[13] using the molar extinction coefficient $2\times10^3\ M^{-1}\ cm^{-1}$.

Substrate specificity for acetoxycoumarin:protein transacetylase

Several acetoxycoumarins (synthesised in our Laboratory), such as 6,7-diacetoxy-4-methylcoumarin; 5,7-diacetoxy-4-methylcoumarin; 7-acetoxy-4-methylcoumarin and 7,8-diacetoxy-4- (3,4-diacetoxyphenyl) coumarin and DAMC were used as substrates for the transacetylase at a concentration of 10–100 μM for the measurement of kinetic parameters.

Results and Discussion

Our early studies demonstrated the inhibitory action of DAMC on several liver microsome P-450-linked mixed function oxidases, such as liver microsome-mediated AFB_1-epoxidation, dealkylation of alkylated resorufins[8] and toxicokinetics of benzene.[14] DAMC mediated irreversible inhibition of microsome P-450-linked MFO activities[8] was described as pseudomechanism based inhibition differing from the classical effect of suicide inhibitors of P-450, such as chloramphenicol,[15] secobarbital,[16] 21-chlorinated steroids,[17] and *N*-alkylaminobenzotriazoles.[18] The action of liver microsomal transacetylase on DAMC leading to the transfer of acetyl group to the active site of lysine residue of apoprotein P-450 resulting in the inhibition of MFO activity was postulated.[8]

Further studies on the mechanism of action of DAMC revealed the possible role of microsomal transacetylase in the modification of other enzyme proteins resulting in the alteration of the catalytic activity. Accordingly, DAMC was shown to irreversibly activate liver microsomal NADPH cytochrome C reductase[9] and irreversibly inhibit the cytosolic glutathione transferase as reported in this paper. For this purpose, purified GST/rat liver cytosol was preincubated with DAMC in the

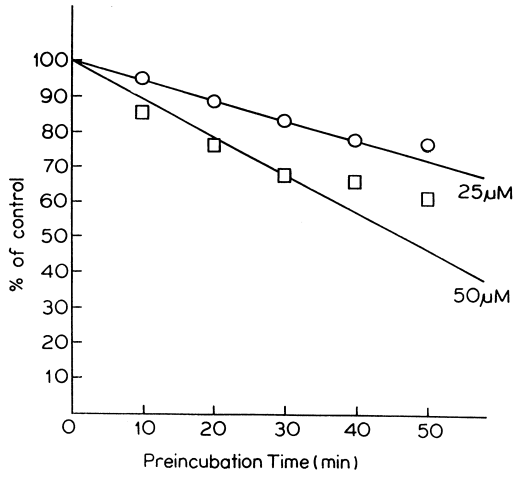


Figure 1. Mechanism based inhibition of purified GST by DAMC upon incubation with rat liver microsomes.

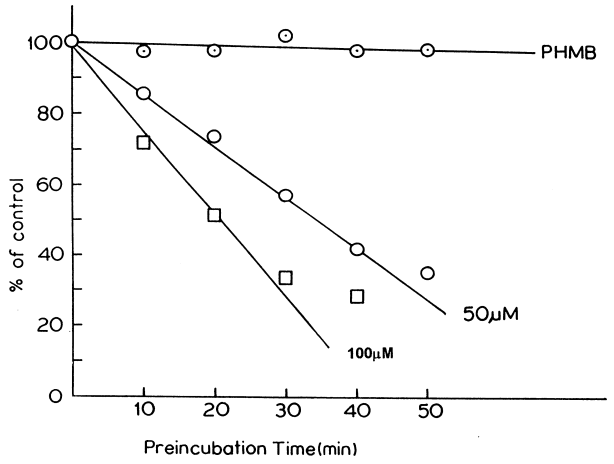


Figure 2. Mechanism based inhibition of cytosolic GST by DAMC upon incubation with liver microsomes.

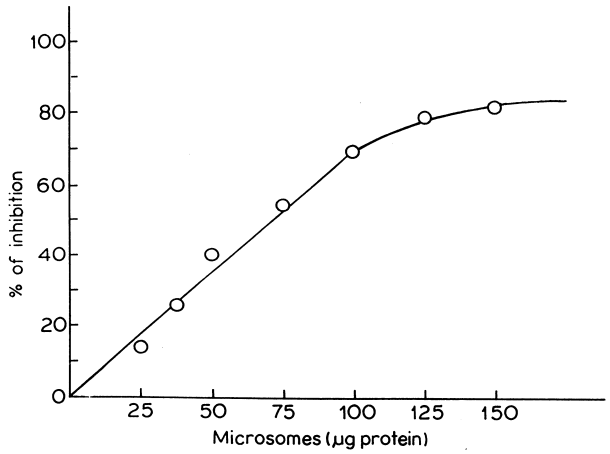
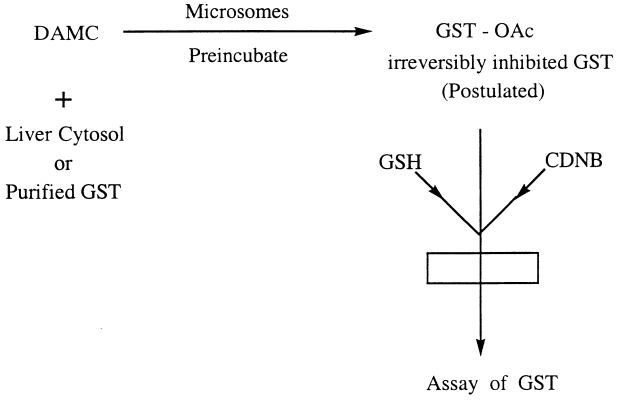


Figure 3. Effect of enzyme concentration on microsomal DAMC:protein transacetylase.

presence of microsomes, followed by the assay of GST. The data included in Figures 1 and 2 clearly indicate time dependent inhibition of GST. Since the irreversible inhibitory action of DAMC on GST was apparent either with the use of rat liver cytosol or purified GST preparation (Figs 1 and 2), the former was routinely used in the studies reported here. GST was found to be inhibited up to 30% by 100 μM concentration of DAMC at 10 min (Fig. 2). DHMC, the deacetylated product of DAMC was devoid of the potential to elicit time dependent inhibition of GST. Hence, it is clear that the mechanism-based inhibition of GST can be attributed to the acetyl groups of DAMC transferred by transacetylase localized in liver microsomes to GST of liver cytosol, resulting in the possible acetylation of GST protein. The irreversible inhibition of GST due to the action of transacetylase was found to be abolished by inclusion of PHMB in the reaction mixture (Fig. 2), while GST per se was unaffected by PHMB. These results indicate that transacetylase is a protein containing thiol group at the active site. A fixed concentration of rat liver cytosol (12.5 μg protein) along with 50 μM DAMC was separately preincubated with varying concentration of microsomes for 10 min at 37 °C, followed by the assay of GST. The results demonstrated in Figure 3 revealed that liver microsomes-catalysed inhibition of GST by DAMC was dependent on the enzyme viz. transacetylase localised in liver microsomes. The extent of inhibition of GST by DAMC under the conditions described earlier is indicative of transacetylase activity (Fig. 4). Hence, inhibition of cytosolic GST as described above was routinely used for the assay of transacetylase. Although we have not demonstrated per se the acetylation of GST protein by DAMC under conditions described here, we believe on the basis of evidences cited here that the action of transacetylase in protein acetylation is quite plausible.

An attempt was made to characterise acetoxycoumarin: protein transacetylase activity by measuring the kinetic



GST : Glutathione S-transferase
Samples containing DMSO in place of DAMC served as the Control. Percent inhibition of GST due to DAMC is representative of transacetylase activity.
□ : indicative of inhibition of GST compared to control

Figure 4. Principle governing the assay of DAMC:protein transacetylase in tissue microsomes.

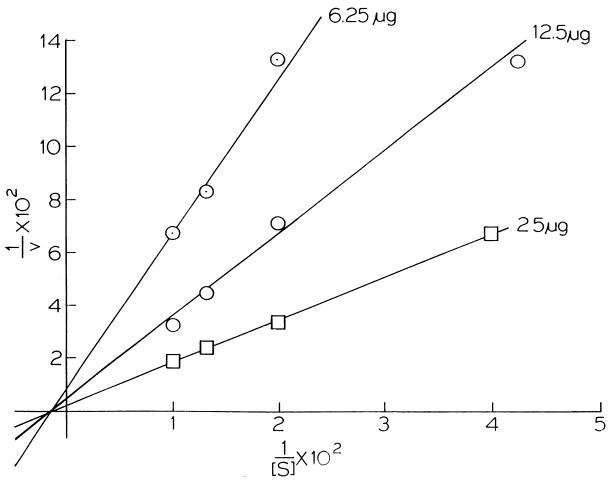


Figure 5. Effect of varying DAMC concentration on microsomal DAMC:protein transacetylase at fixed concentration of cytosolic protein (GST) (6.25–25 µg). [S] = DAMC (µ M); v = % inhibition of GST.

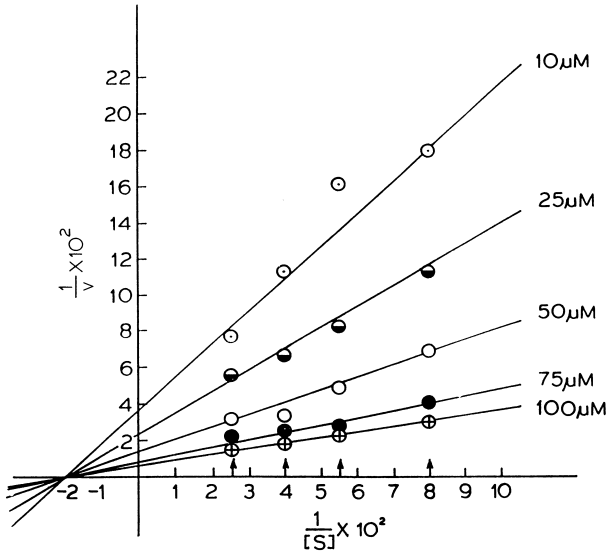


Figure 6. Effect of varying cytosol (GST) protein concentration on DAMC:protein transacetylase at fixed concentration of DAMC (10–100 µM). [S] = Protein concentration (µg); v = % inhibition of cytosolic GST.

constants. For this purpose, the effect of the concentration of the substrate DAMC as well as GST (used as liver cytosol) on transacetylase was separately studied. The data revealed that the enzyme demonstrated hyperbolic kinetics. K_m and v_{max} for the transacetylase at varying DAMC concentrations (concentration of cytosolic protein kept constant) as derived from the double reciprocal plot (Fig. 5) were found to be 1 mM and 364 units (12.5 µg cytosolic protein). Similarly K_m and v_{max} values for transacetylase at varying concentration of cytosolic protein (DAMC concentration kept constant) were found to be 50 µg microsomal protein and 12.5 unit (50 µM DAMC), respectively under the conditions of the assay (Fig. 6). These data establish the bimolecular nature of transacetylase reaction connoting the protein (in this case GST serving as acetyl group acceptor) as one of the substrates and the donor of acetyl group (acetoxy 4-methylcoumarin) as the other substrate. The results tabulated in Table 1 were obtained employing partially purified transacetylase preparation from rat liver microsomes. Although the transacetylase preparation has the contaminating presence of DAMC deacetylase,[13] the addition of GST in the transacetylase reaction mixture resulted in the enhanced formation of DHMC (Table 1) with the concomittant absence of the peak with retention time of 156 sec. (appearing between DAMC and DHMC peaks), tentatively assigned to monoacetoxy, monohydroxy derivative of 7,8-dioxygenated-4-methylcoumarin (MAMHC). The inclusion of iodoacetamide in the transacetylase reaction mixture resulted in the drastic reduction of DHMC (Table 1, Fig. 7), thereby confirming the inhibitory action of thiol blocking agent on transacetylase as described earlier. Thus the direct evidence for the authenticity of DAMC: protein transacetylase was provided by the identification and quantification of one of the products of transacetylase action viz. DHMC as described above.

Substrate specificity of acetoxycoumarin:protein transacetylase was studied using a number of acetoxy derivatives of 4-methylcoumarins (Table 2). The results tabulated in Table 2 highlighted that monoacetoxycoumarin is a much poorer substrate compared to diacetoxy derivatives. It is obvious that the greater the number of acetoxy groups on the coumarin nucleus, greater is the probability of acetyl group transfer by the enzyme to GST and hence superior the substrate. Moreover, 6-acetoxy-4-methylcoumarin when used as the substrate yielded less transacetylase activity (data not shown), while 7-acetoxy-4-methylcoumarin was a much better substrate. It is clear from these observations that the acetoxy groups farther from the heteroatom are less prone to enzymatic transfer. 7,8-Diacetoxy-4-methylcoumarin when used as a substrate

Table 1. Product profile of the DAMC:protein transacetylase reaction

No.	Reaction mixture	Product formed[e]		Transacetylase[d]
		DHMC	MAMHC[a]	µ mol/min/mg protein DHMC formed
1.	Enz[b] + DAMC	+	+	0.056
2.	Enz + DAMC + GST	+ + +	—	0.137
3.	Enz + DAMC + GST + IA[c]	+	+ + +	0.035

[a]MAMHC: monoacetoxy, monohydroxy-4-methylcoumarin.
[b]Enz: partially purified rat liver transacetylase.
[c]IA: iodoacetamide.
[d]The values are average of three experiments with variation < 5%.
[e]The detailed procedure is described under Materials and Methods.

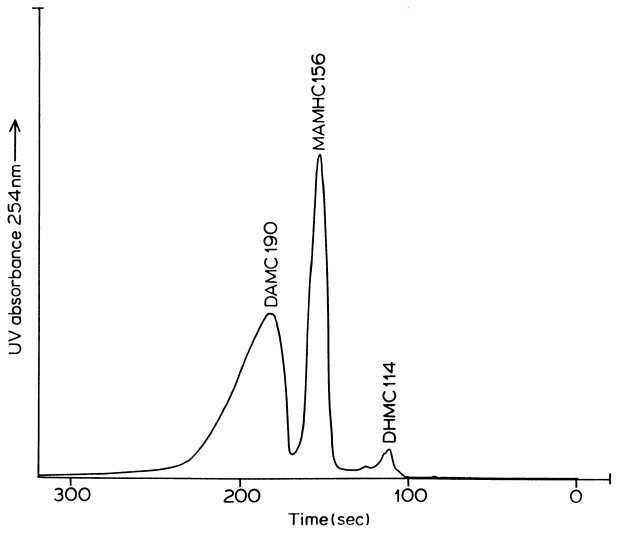


Figure 7. Identification of the products of transacetylase reaction by HPLC. (Refers to reaction mixture no. 3 in Table 1).

Table 2. Substrate specificity for DAMC:protein transacetylase

Compound	K_m (μM)	v_{max} (units)
7,8-Diacetoxy-4-methylcoumatrin	1000	364
6,7-Diacetoxy-4-methylcoumarin	1250	333
7,8-Diacetoxy-4-(3,4-diacetoxyphenyl)coumarin	1420	333
5,7-Diacetoxy-4-methylcoumarin	1470	250
7-Acetoxy-4-methylcoumarin	1540	200

for transacetylase yielded slightly higher activity as compared to 6,7-diacetoxy-4-methylcoumarin (Table 2). Further 5,7-diacetoxy derivative gave lower activity of transacetylase as compared to 6,7-diacetoxy and 7,8-diacetoxy derivatives, thereby indicating that enzyme is more effective in the transfer of acetoxy groups present at *ortho* positions to each other as compared to those present at *meta* positions.

The aforementioned studies have given a strong evidence for the existence of transacetylase in liver microsomes catalysing the transfer of acetyl group from acetylated xenobiotics (acetoxy-4-methylcoumarins) to a protein (cytosolic GST) leading to mechanism-based inhibition of the catalytic activity of the acceptor protein. A simple and elegant method for the assay and characterisation of transacetylase based on irreversible inhibition of cytosolic GST has been described in this paper. The method was conveniently employed for partial purification of the enzyme.

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