Biogenic-amine acetylation: an additional function of the *N*-acetyltransferase from *Fasciola hepatica*

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The previously described polyamine *N*-acetyltransferase from *Fasciola hepatica* has been observed to have an additional function, the acetylation of biogenic amines. The activities for biogenic amines, diamines and polyamines were in a constant ratio throughout the purification process. Biogenic amines found to be substrates for the enzyme included tyramine, tryptamine, β-phenylethylamine and histamine, with K_m values of 0.12 mM, 0.26 mM, 0.30 mM and 0.76 mM respectively. Octopamine, 5-hydroxytryptamine and α-phenylethylamine were also acceptable as substrates, though to a lesser degree. The optimum pH for biogenic-amine acetylation was 7.5, and CoA was inhibitory to the process, with a K_i of 5.5 μM. *N*-Acetylation appears to play a major role in the amine metabolism of this trematode. We presume that acetylation represents the process by which the parasite inactivates excess amines.

INTRODUCTION

In some parasitic helminths, enzyme preparations capable of acetylation of diamine (Wittich and Walter, 1989, 1990), biogenic amines (Isaac et al., 1990, 1991), diamine and polyamine (Aisien and Walter, 1992) have been reported. These enzymes are thought to have the role of inactivating excess amines before excretion. Until now, it was generally presumed that biogenic-amine and polyamine acetylation in parasitic helminths, like in mammalian tissues, was catalysed by two different enzymes, namely an arylalkylamine *N*-acetyltransferase and a polyamine *N*-acetyltransferase respectively. Results from our present study indicate otherwise. In this paper, we present data showing that the previously reported polyamine *N*-acetyltransferase from *Fasciola hepatica* (Aisien and Walter, 1992) is also responsible for biogenic-amine acetylation in this trematode.

EXPERIMENTAL

Materials

[1-^{14}C]Acetyl-CoA (60 mCi/mmol) was purchased from Amersham–Buchler, Braunschweig, Germany. 3,3'-Diamino-dipropylamine (sym-norspermidine) was obtained from Aldrich, Steinheim, Germany. Blue Sepharose CL 6B was a product of Pharmacia, Freiburg, Germany. DE-52 DEAE-cellulose was obtained from Whatman, Maidstone, Kent, U.K. 3-Methyl-butan-1-ol and Brij 35 were from Merck, Darmstadt, Germany. 6-Aminohexanoic acid–Sepharose 4B, biogenic amines and other biochemicals were purchased from Sigma, Deisenhofen, Germany. *N*-Acetyltryptamine was kindly provided by Dr. Cyril Usifo, of the Institute for Pharmaceutical Chemistry, University of Münster, Münster, Germany.

Parasites

Adult *Fasciola hepatica* were collected from naturally infected liver of cattle obtained from local slaughterhouses. The worms were thoroughly washed in several changes of normal saline (0.9% NaCl) and then rinsed in sterile saline before storage at -79 °C.

Purification of the *N*-acetyltransferase from *Fasciola hepatica*

The preparation of the crude extract, ion-exchange chromatography on DEAE-cellulose and affinity chromatography on norspermidine–Sepharose were as previously described (Aisien and Walter, 1992), except that the crude extract was extensively dialysed against 50 mM Tris/HCl, pH 8.3, containing 1 mM dithiothreitol, 1 mM EDTA, 0.02% Brij 35 and 0.1 mM phenylmethanesulphonyl fluoride (buffer A) before application to the DEAE-cellulose column. The eluate from the norspermidine–Sepharose column was adsorbed on a column of Blue Sepharose (1 cm × 4 cm) equilibrated with 20 mM Tris/HCl, pH 8.7, containing 1 mM dithiothreitol, 1 mM EDTA, 0.02% Brij 35 and 0.1 mM phenylmethanesulphonyl fluoride (buffer B). The column was washed successively with buffer B, 0.5 M NaCl and 0.85 M NaCl in the same buffer and then eluted with 0.85 M NaCl containing 1 mM CoA in buffer B. Activity in the 3 ml fractions collected was assayed after they had been freed from CoA by successive concentration and dilution with buffer B using a Centricon-10 microconcentrator (Amicon). The molecular mass of the enzyme was determined by f.p.l.c. on a calibrated HiLoad Superdex 200 column (1.6 cm × 60 cm) as previously described (Aisien and Walter, 1992).

Assay for biogenic-amine and polyamine acetylation

Biogenic-amine acetylation was assayed by the method described by Weissbach et al. (1961) with modification. A standard 60 μl incubation mixture contained 50 mM Tris/HCl, pH 7.5, 5 mM tyramine or the mentioned biogenic amine, 4.2 μM (25 nCi, 60 mCi/mmol) of [1-^{14}C]acetyl-CoA, 1 mM dithiothreitol and 1 mM EDTA. The mixture was incubated for 5 min at 37 °C, and the reaction was terminated by addition of 0.1 ml of NaCl-saturated 0.5 M sodium borate buffer (pH 10) and cooling on ice. The acetylated products were extracted with 0.6 ml of 3-methylbutan-1-ol by vortex-mixing, followed by centrifugation at 10000 *g* for 3 min. A 300 μl portion of the organic phase was transferred to a scintillation vial containing 3 ml of scintillation cocktail and assayed for radioactivity. Assay for polyamine acetylation was performed as previously described (Aisien and

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Table 1   Purification of N-acetyltransferase from Fasciola hepatica

One unit of enzyme represents 1 nmol of product/min at 37 °C.

Purification step	Total protein (mg)	Total activity (units)	Sp. activity (units/mg)	Purification (fold)	Yield (%)
Crude extract	213.5				
Tyramine		241.3	1.13	1	100
Spermidine		170.8	0.80	1	100
Putrescine		47.0	0.22	1	100
DEAE-cellulose column	87.77				
Tyramine		218.6	2.49	2.2	91
Spermidine		136.9	1.56	2.0	80
Putrescine		32.5	0.37	1.7	69
Norspermidine–Sepharose	1.97				
Tyramine		102.4	51.98	46	42
Spermidine		98.5	50.00	63	57
Putrescine		19.1	9.70	44	41
Blue Sepharose	0.36				
Tyramine		79.5	220.83	195	33
Spermidine		62.6	173.89	217	37
Putrescine		12.9	35.83	163	28
Superdex 200	0.12				
Tyramine		47.9	399.17	353	20
Spermidine		42.3	352.50	441	25
Putrescine		8.7	72.50	330	19

Walter, 1992). A unit of enzyme activity represents 1 nmol of product/min at 37 °C.

Identification of the reaction products

Approx. 1.5 ng of enzyme protein from the Blue Sepharose column was incubated with 5 mM of biogenic amine, 50 mM Tris/HCl, pH 7.5, and 16.8 μM of [1-^{14}C]acetyl-CoA (100 nCi) in a final volume of 240 μl at 37 °C for 30 min. The reaction was terminated with 24 μl of 2M $HClO_4$ and the reaction mixture placed on ice. The $HClO_4$-precipitated protein was sedimented by centrifugation at 10000 g for 10 min. Reverse-phase h.p.l.c. analysis of the resulting supernatant was performed on the equipment previously described by Wittich et al. (1987). Separation was achieved on a 250 mm × 4 mm steel column filled with 5 μm Spherisorb ODS II connected to a diode array detector (Kontron Instruments) set at 280 nm. The labelled products were detected with a radioactive monitor (LB 506, Berthold). The mobile phase consisted of: solvent A (90 % 0.1 M sodium acetate solution containing 10 mM 1-octanesulphonic acid, pH 4.5, and 10 % methanol); solvent B (90 % of solution B1 and 10 % methanol), solution B1 consisting of 10 parts (v/v) of 0.2 M sodium acetate containing 10 mM 1-octanesulphonic acid, pH 4.5, and 3 parts (v/v) of acetonitrile. The gradient program, which was controlled by a Data Systems 450 instrument (Kontron Instruments) was as follows: 0–15 min, 4–10 % B; 15–35 min, 10–100 % B; 35–48 min, 100 % B; 48–52 min, 100–4 % B; 52–58 min, 4 % B.

Protein determination

Protein concentration was determined by the method of Bradford (1976), with BSA as standard.

RESULTS

Purification of the N-acetyltransferase

In our previous paper (Aisien and Walter, 1992), we mentioned the occurrence of some inhibitory factors interfering with the activity in the crude extract. In the present study, we have established that these substances could be removed by subjecting the crude extract to extensive dialysis, followed by centrifugation to remove precipitates formed in the process. As also previously reported, the enzyme did not bind to DEAE-cellulose, hence the flow-through fractions containing acetylase activity were pooled and purified further. Chromatography of the enzyme on Blue Sepharose proved to be an effective step in the purification procedure. The enzyme was so strongly bound to this matrix that washings with up to 0.85 M NaCl failed to elute the enzyme from the column. However, a trace amount of activity was detected in washings with higher salt concentrations. As a result, the column was washed with up to 0.85 M NaCl and then eluted with 0.85 M NaCl containing 1 mM CoA. Elution of the enzyme with CoA in association with NaCl was imperative, since attempts to elute the enzyme solely with CoA proved unsuccessful. The enzyme material from this purification step, when subjected to gel filtration, yielded a highly purified acetylase with a specific activity of approx. 400 units/mg of protein with respect to tyramine. With the purification steps summarized in Table 1, an overall purification factor of 300 and a yield of approx. 20 % was achieved. The amine-acetylating activities for the three substrates remained in constant proportions throughout the purification procedure. Chromatography of the enzyme on a calibrated Superdex 200 column yielded an elution pattern with similar activity profile for tyramine, spermidine and putrescine (Figure 1). The molecular mass of the enzyme was approx. 50 kDa.
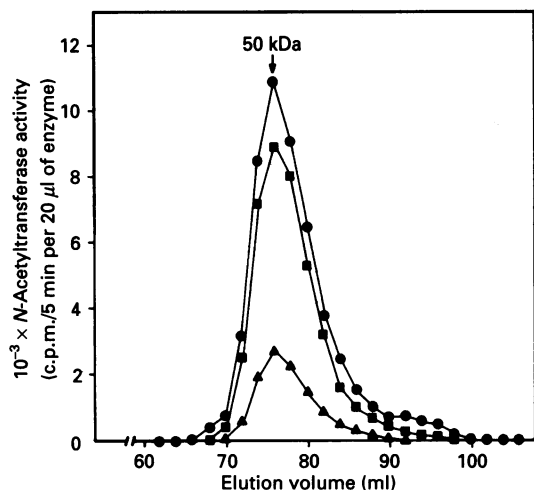
Figure 1   Gel filtration of the *N*-acetyltransferase from *F. hepatica*

The concentrated eluate from the Blue Sepharose column was chromatographed on Hiload Superdex 200. The individual 2 ml fractions were assayed for *N*-acetyltransferase activity with tyramine (●), spermidine (■) and putrescine (▲).
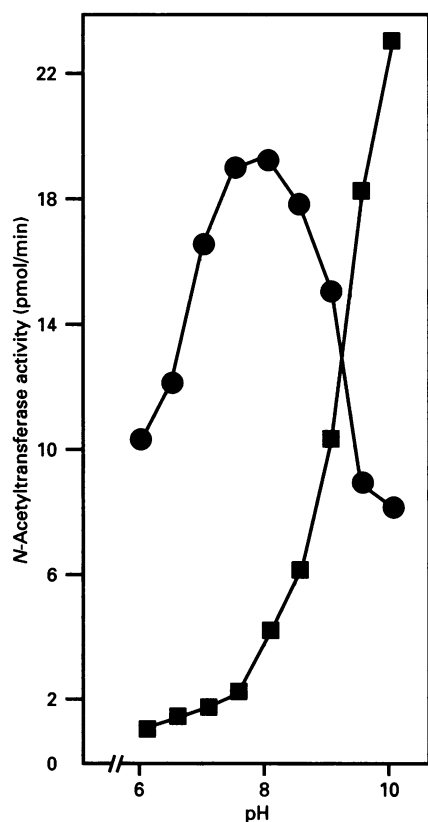
Table 2   Substrate specificity of the *N*-acetyltransferase from *Fasciola hepatica* for biogenic amines

The relative rates of acetylation of the biogenic amines (5 mM), expressed as percentages of the values obtained for tyramine, and apparent K_m values (in the presence of 4.2 μM acetyl-CoA) are shown. Results are means of three replicate determinations (n.d., not determined).

Substrate	Relative reaction rate (%)	Apparent K_m (mM)
Tyramine	100	0.12
β-Phenylethylamine	98	0.26
α-Phenylethylamine	15	n.d.
Tryptamine	96	0.30
Histamine	54	0.76
Octopamine	39	n.d.
5-Hydroxytryptamine	28	10
Adrenaline	< 5	n.d.
Noradrenaline	0	n.d.
Dopamine	0	n.d.
Butylamine	< 5	n.d.
Ethylamine	< 5	n.d.

Figure 2   Effect of pH on biogenic-amine acetylation

The assays were performed with 50 mM potassium phosphate buffer (pH < 7), 50 mM Tris/HCl (pH 7–9) and 50 mM glycine/NaOH (pH > 9). Results are presented for non-enzymic acetylation (■) and for enzymic acetylation minus the non-enzymic acetylation (●).
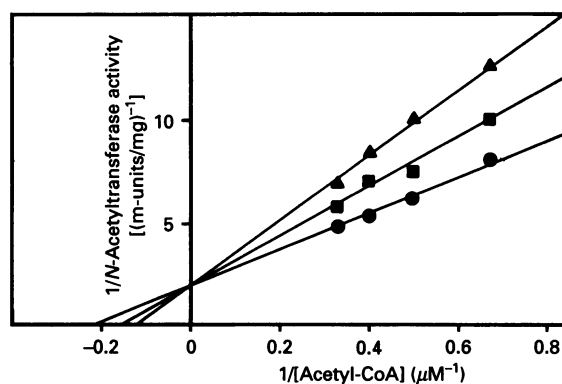

Figure 3   Inhibition of the *N*-acetyltransferase activity by CoA

The assays were carried out in the absence (●) and in the presence of 2 μM (■) or 4 μM (▲) CoA, and the reciprocal of initial reaction velocity (v) was plotted against the reciprocal of acetyl-CoA concentration shown.

pH 7.5, because the level of non-enzymic acetylation increased drastically from pH 8, exceeding the enzymically catalysed reactions at 9.5 and 10. Enzyme activity measured with tyramine at pH 7.5 was linear over 5 min at 37 °C.

Substrate specificity

The *N*-acetyltransferase from *Fasciola hepatica* was observed to catalyse the acetylation of a number of naturally occurring biogenic amines (Table 2). Among these, the best substrate was tyramine, with an apparent K_m of 0.12 mM. β-Phenylethylamine and tryptamine were equally acceptable substrates for the enzyme. Unlike the β-isomer, α-phenylethylamine had very little affinity for the acetylase. Histamine, octopamine and 5-hydroxytryptamine were also acceptable as substrates, though to a lesser degree. Dopamine and noradrenaline were inactive, whereas adrenaline, ethylamine and butylamine had reaction rates of less than 5%. The K_m for acetyl-CoA was 4.5 μM. CoA was found to be inhibitory to biogenic-amine acetylation, with an inhibition constant of 5.5 μM (Figure 3). Reverse-phase h.p.l.c. analysis of the reaction products of tryptamine and 5-hydroxytryptamine

Optimal pH for biogenic-amine acetylation

As shown in Figure 2, the optimal pH for biogenic-amine acetylation was 7.5–8. Assays were, however, performed at
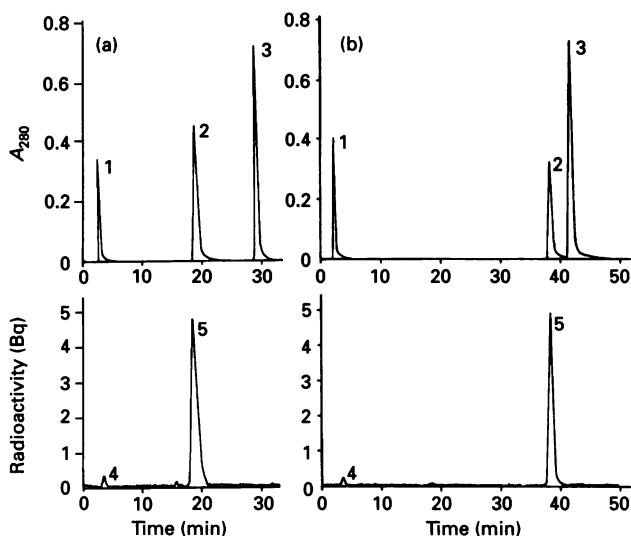
Figure 4   Separation of the acetylated products of 5-hydroxytryptamine and tryptamine by reverse-phase h.p.l.c.

The purified N-acetyltransferase was incubated with the biogenic amines as detailed in the Experimental section. Portions (25 μM) of standard acetylated products were co-analysed with 20 μl of the clarified reaction mixtures. (a) 5-Hydroxytryptamine: 1, solvent front; 2, N-acetyl-5-hydroxytryptamine standard; 3, 5-hydroxytryptamine; 4, acetyl-CoA; 5, radiolabelled N-acetyl-5-hydroxytryptamine. (b) Tryptamine: 1, solvent front; 2, N-acetyltryptamine standard; 3, tryptamine; 4, acetyl-CoA; 5, radiolabelled N-acetyltryptamine.

showed a single labelled product for each substrate. These products were identified as N-acetyltryptamine and N-acetyl-5-hydroxytryptamine respectively, by comparing their retention times with those of authentic standards (Figure 4). A single labelled product was similarly obtained for tyramine (result not shown). This product could not be conclusively identified, for the lack of an authentic standard for N-acetyltyramine.

DISCUSSION

In our previous paper we demonstrated the presence of an acetylase capable of acetylation of polyamines and diamines in *F. hepatica* (Aisien and Walter, 1992). Results from our present study clearly indicate that the same enzyme is responsible for biogenic-amine acetylation in this trematode. The fact that we were unable to achieve a separation of the biogenic amine-, polyamine- and diamine-acetylating activities after purification on column matrices using different adsorption principles is indicative of the fact that this amine-acetylating activity is resident in a single enzyme protein. This result is supported by the constant proportions observed among the acetylation rates of tyramine, spermidine and putrescine during purification, and the fact that a single peak of activity was obtained for these substrates on Superdex 200. This activity peak, which corresponded to 50 kDa, is in agreement with the previously determined value for the polyamine- and diamine-acetylating activities in *F. hepatica* (Aisien and Walter, 1992). It is noteworthy that the K_m value of 4.5 μM for acetyl-CoA and a K_i value of 5.5 μM for CoA obtained with tyramine were also the values obtained with spermidine as acetyl acceptor (Aisien and Walter, 1992).

The enzyme readily acetylated tyramine, β-phenylethylamine, tryptamine and histamine, with K_m values in the range 0.1–0.7 mM. It is therefore doubtful if 5-hydroxytryptamine constitutes a physiological substrate for the enzyme, in view of

the very high K_m value of 10 mM obtained for this compound. Whereas the K_m values obtained for the aforementioned biogenic amines fall between those obtained for putrescine/spermidine and spermine, respectively (Aisien and Walter, 1992), these values are, however, in the range of results obtained for these compounds with the pigeon liver arylamine-N-acetyltransferase (Andres et al., 1983). There seems to exist some relationship between the structure and activity of some of the substrates. For example, tyramine and tryptamine were very good substrates for the enzyme. Hydroxylation of these compounds drastically decreased their suitability as substrates. This becomes evident when the activities of these amines are compared with those of their hydroxylated derivatives, namely dopamine and octopamine from tyramine and 5-hydroxytryptamine from tryptamine. Similarly, the decreased activity of the α-isomer of phenylethylamine is probably due to the steric hindrance resulting from the methyl group attached to the primary carbon atom.

The trematode acetylase is clearly different from the mammalian liver polyamine N-acetyltransferase and the isofunctional enzyme from the chicken duodenum (Della Ragione and Pegg, 1983; Shinki and Suda, 1989). Whereas these other acetylases catalysed only polyamine acetylation, the N-acetyltransferase from *F. hepatica* accepts a rather broad range of substrates. It is in this regard similar to the arylalkylamine N-acetyltransferase of mammalian liver, which, although reported to have a broad substrate specificity (Weber, 1971), is however not known to catalyse polyamine acetylation. In addition, the trematode enzyme also differs from the arylalkylamine N-acetyltransferase reported in some parasitic nematodes (Isaac et al., 1990, 1991), in that it is incapable of dopamine acetylation. Unlike the nuclear N-acetyltransferase of mammalian cells, it is incapable of histone acetylation (Libby, 1978; Blankenship and Walle, 1977, 1978).

The role of N-acetylation in parasitic helminths is still unclear and therefore needs elucidation. In the polyamine metabolism of mammalian cells, N-acetylation is the rate-limiting step of the interconversion pathway (Bolkenius and Seiler, 1981; Pegg et al., 1981; Seiler et al., 1981a,b). Cells are also known to inactivate excess amines by this process, thereby enhancing their transport and excretion (Weber, 1971; Seiler, 1981, 1987). Circumstantial evidence gives reason to believe that N-acetylation plays the latter role in parasitic helminths. In *Ascaris suum* and *Onchocerca volvulus*, a novel acetylase involved in the degradation of polyamines, releasing N-acetylputrescine as excretory product, has been described by Wittich and Walter (1990). Further evidence supporting this finding was recently provided by Müller and Walter (1992). These investigators observed that, in contrast with the situation in mammalian cells, the polyamine oxidase in *A. suum* was solely responsible for polyamine interconversion, with the putrescine N-acetyltransferase serving an inactivation and excretory function. We therefore presume that N-acetylation serves the same purpose in *F. hepatica* and other trematodes.

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