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Inactivation of platelet-activating factor by a putative acetylhydrolase from the gastrointestinal nematode parasite *Nippostrongylus brasiliensis*

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SUMMARY

The adult stage of *Nippostrongylus brasiliensis*, a strongylid parasite of the gastrointestinal tract of rats, released a product during *in vitro* culture which functionally inhibited platelet-activating factor (PAF), measured by its ability to mediate platelet aggregation. The extent of inhibition was proportional to the concentration of excretory/secretory (ES) products and the duration of preincubation with PAF prior to the assay of biological activity. The inhibitory activity was heat labile and was specific for PAF, as incubation of ES products with thrombin showed no diminution of platelet aggregation. Experiments using radiolabelled preparations of PAF demonstrated that the acetyl group esterified at the *sn*-2 position of the glycerol backbone was liberated on incubation with ES products, indicative of an acetylhydrolase activity. This activity was susceptible to inhibition by DFP, partial inhibition by eserine, but was resistant to PMSF and TPCK at concentrations which inhibit serine proteases.

INTRODUCTION

Platelet-activating factor (PAF; 1-*O*-alkyl-2-*O*-acetyl-*sn*-glycero-3-phosphorylcholine) is a potent phospholipid mediator of inflammation.[1-3] It is synthesized by many cell types, including neutrophils, monocytes, macrophages, platelets, eosinophils, mast cells, vascular endothelial cells and fibroblasts,[4] and has been shown to mediate a broad spectrum of biological activities. These include chemotaxis, aggregation and secretion in neutrophils and eosinophils, aggregation and secretion for platelets and chemotaxis and induction of the oxidative burst, interleukin-1 (IL-1) production and oncogene expression for macrophages and monocytes.[5-7] It also enhances the production of tumour necrosis factor (TNF) in alveolar macrophages[6] and augments the IgE-mediated functions of eosinophils.[8]

PAF has been implicated as a major contributing factor in the aetiology of asthma and allergic rhinitis, considered to be immunologically synonymous with the symptoms provoked by helminth infection in the lung and gastrointestinal (GI) tissues.[9] It has been found at elevated levels both systemically and in the GI lumen during the expulsion of parasites constituting a primary infection of *N. brasiliensis*,[10] and enhances the cytotoxic effect of eosinophils against larval *Schistosoma mansoni*.[8]

We show here that a component of the secretions from the adult stage of the strongylid nematode parasite *N. brasiliensis* can functionally inhibit PAF in a time- and concentration-dependent manner. Hydrolysis of tritiated PAF suggests that this is due to an acetylhydrolase. This enzyme could play a role in down-regulating immune effector mechanisms which promote expulsion from the GI tract.

Abbreviations: AChE, acetylcholinesterase; ADP, adenosine diphosphate; BSA, bovine serum albumin; DFP, di-isopropyl fluorophosphate; ES, excretory/secretory; GI, gastrointestinal; PAF, platelet-activating factor (1-*O*-alkyl-2-*O*-acetyl-*sn*-glycero-3-phosphorylcholine; 1-3); PC, phosphoryl choline; PMSF, phenylmethylsulphonylfluoride; PRP, platelet-rich plasma; PPP, platelet-poor plasma; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; TxA_2, thromboxane A_2.

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MATERIALS AND METHODS

Parasites

Infections were established by injecting male Sprague Dawley rats with 8000 infective third stage larvae (L_3) subcutaneously through four sites in the flanks. Rats were killed Day 4 post-infection, and the small intestine removed after laparotomy. The anterior half of the intestine was then cut open and placed onto muslin, stretched over a Baermann apparatus filled with warm (37°) saline. Adult stage worms were collected from the bottom of the tube after 60 min.

In vitro culture and collection of excretory/secretory (ES) products

Recovered parasites were washed 10 times in 10 volumes of sterile medium, and cultured at 37° in 5% CO_2. The medium employed was serum-free RPMI-1640 (Gibco, Paisley, Renfrewshire, U.K.) supplemented with L-glutamine to 2 mM,

glucose to 2%, penicillin to 100 U/ml and streptomycin to 100 μg/ml. This was changed daily. Pooled media were concentrated using Centricon 10 microconcentrator filters (Amicon, Upper Mill, Glous., U.K; 4205) as directed by the manufacturers. Once concentrated, the medium was washed into phosphate buffer pH 7·0, and the protein concentration determined.[11] Concentrated ES was stored in aliquots at 4° for up to 8 weeks. Unless stated otherwise, this was used at a concentration of 1 mg/ml.

Platelet aggregation assay

Fifty millilitres of human blood was collected into a 10% volume of 3·8% sodium citrate buffer, mixed gently and spun at 270 ***g*** for 10 min at room temperature. The resulting platelet-rich plasma (PRP) fraction was removed into a polypropylene tube and stored on ice. Typically, platelets stored in this manner lost responsiveness after 3–4 hr. The remaining cell pellet was spun for a further 15 min at 1100 ***g*** at room temperature to give a platelet-poor plasma (PPP) fraction, which was also taken into a polypropylene tube and stored on ice. A Payton Dual Beam Aggregometer (Rotronics, Horley, Surrey, U.K.) was then calibrated using PRP as a zero and PPP as a maximum absorbance blank. This instrument measures the transmission of light through a suspension of platelets (0·5 ml) held at 37° with stirring in siliconized cuvettes; the level of absorbance decreases as the platelets aggregate. The aggregating mediators used in these assays were platelet-activating factor (PAF; Novabiochem, Nottingham, U.K.; 08-74-000) and thrombin from human plasma (Sigma, Poole, Dorset, U.K.; T3010). For each blood sample, a dose–response curve for the aggregating agent under test was obtained and the concentration of mediator used was the last before a submaximal response was observed. Unless stated otherwise, this was 1×10^{-6} M for PAF and 0·2 NIH U/ml for thrombin. Results were recorded on a Rikadenki chart recorder run at 2 cm/min. The subsequent trace was reproduced on a Macintosh graphics package, hence the appearance of time-points which directly mirror the trace.

In this series of experiments the appropriate mediator was preincubated with either a control [0·25% bovine serum albumin (BSA)] solution, or ES for a defined length of time before it was added to the platelet suspension.

PAF hydrolase assay

In order to confirm the presence of an activity which catabolized PAF we used a standard assay for PAF acetylhydrolase described by Stafforini *et al.*[12] This was modified in that three different labelled preparations were used. These were 1-*O*-[acetyl ^{3}H]PAF (New England Nuclear, Boston, MA, NET-910), 1-*O*-[alkyl ^{3}H]PAF (Amersham Co., Amersham, Bucks, U.K.; TRK 741), and [*N*-methyl ^{14}C] PAF (Amersham; CFA 714). Briefly, 5 μCi of labelled PAF was dried down under a stream of nitrogen, resuspended with 4 nM of cold PAF (Novabiochem 08-74-000) in 4 ml of 10 mM phosphate buffer and sonicated on ice for 5 min. The resultant solution was thus 1 μM. Aliquots of 10 μl of ES (0·5 mg/ml) were added to 40 μl of the labelled PAF solution and incubated for 30 min at 37°. The reaction was terminated by the addition of 50 μl of 10 M acetic acid, followed by 1·5 ml of 0·1 M sodium acetate. Background values were determined by stopping the reaction as soon as possible following addition of ES (i.e. 30 seconds). Cleavage products were separated from the substrate by reversed-phase chromatography on octadecylsilica gel cartidges (Waters Sep-Pak; Millipore, Harrow, Middlesex, U.K.; 20805), and the filtrates counted after washing the columns with an additional 1·5 ml of sodium acetate. All assays were done in triplicate.

Inhibitors

A number of inhibitors of serine proteases and serine esterases were incorporated into the platelet aggregation assay. These were added to ES for varying periods of time prior to co-incubation with PAF. Di-isopropylfluorophosphate (DFP) was added at 10^{-5} M, eserine at 10^{-4} M, phenylmethylsulphonylfluoride (PMSF) at 1 mM and *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK) at 0·1 mM.

RESULTS

A component of *N. brasiliensis* ES can inhibit the platelet-aggregating ability of PAF

Different batches of human platelets vary significantly in their responsiveness to PAF, and thus the submaximal aggregating concentration was determined before each experiment. ES was collected from parasites harvested 4 days post-infection, and diluted 1 : 10 into the concentration of PAF required to stimulate submaximal aggregation. This was generally 1 μM. The ES/PAF solution was then preincubated at 37° for a given length of time before assay. A control, in which ES was substituted by 0·25% BSA in phosphate buffer, pH 7·0 was run in parallel on each occasion.

PAF interacts with platelets through a single, high affinity receptor, which is coupled by G proteins to a signal transduction pathway.[13,14] Figure 1 shows that incubation of PAF with ES resulted in a progressive loss of activity. If PAF and ES were added simultaneously ($t=0$), no inhibitory effect was seen. The inhibitor is thus not simply competing for PAF-receptor binding, and is unlikely to be caused by free, or protein-associated phosphorylcholine. At $t=10$ min the maximum change in light transmission was lower than that of the control trace. At $t=40$ min, no aggregation was observed, and the level of transmitted light fell to its original, pre-challenge level after a transient increase.

The level of inhibition of PAF activity after a defined period of preincubation was also dependent on the concentration of ES used. Thus, Fig. 2 illustrates the increased inhibition of PAF by higher concentrations of ES co-incubated for 25 min, and shows that aggregation was completely abolished with 100 μg/ml protein (the results shown in Fig. 1 were generated using ES at a concentration of 100 μg/ml). Collectively, these data show that a component of ES from *N. brasiliensis* prevented PAF from inducing platelet aggregation in a time- and concentration-dependent manner. The PAF-inactivating activity described was stable when ES was stored at 4° for up to 2 months, but was completely abolished by boiling for 10 min (data not shown).

Comparison of Figs 1 and 2 with dose–response traces indicated that these results were consistent with those obtained by progressively decreasing PAF concentrations. The inhibitory activity present in ES could therefore have been operating either via competitive binding or catabolism of PAF, and these possibilities were investigated in the following experiments.

Thrombin-induced platelet aggregation is not inhibited by ES

Studies of PAF-inhibiting activities have shown that some of the inhibitors may interfere downstream of the receptor-binding

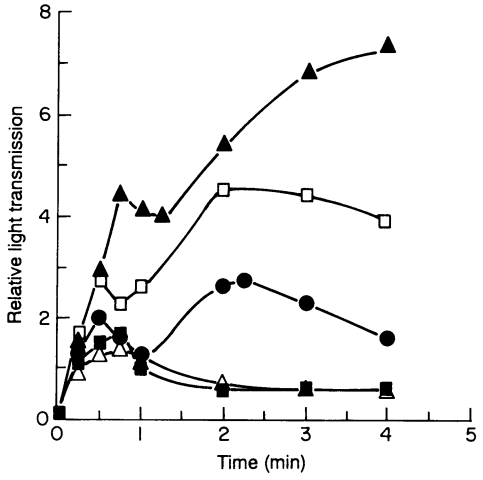

Figure 1. A component of *N. brasiliensis* ES inhibits the platelet-aggregating ability of PAF. ES was diluted 1 : 10 into 1×10^{-6} M PAF to a final concentration of 100 μg/ml. The reaction was then incubated at 37° for 0–40 min (t) before assay on platelets. PAF activity was assayed using an aggregometer (see Materials and Methods). Control (▲); $t = 10$ min (□); $t = 20$ min (●); $t = 30$ min n(■); $t = 40$ min (△).

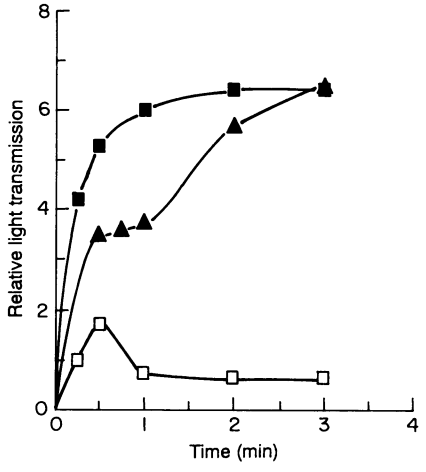

Figure 2. Concentration dependence of PAF inhibition by *N. brasiliensis* ES. 1×10^{-6} M PAF was preincubated in parallel experiments with ES at a final concentration of 50 μg/ml (▲) and 100 μg/ml (□). PAF activity was assayed at various time-points as described in Materials and Methods. The figure shows the results obtained after preincubation for 25 min before assay in each experiment. ■, Control.

event, or may inhibit signalling from the PAF receptor by inducing opposing intracellular signalling pathways.[15-17] The specificity of the inhibitory factor in *N. brasiliensis* ES was thus investigated by utilizing another platelet-aggregating agent—thrombin. Thrombin was chosen because it most closely resembles PAF in the post-receptor signalling events which it provokes.[18,19] The experiments performed were identical to those described in the previous section, except that thrombin was substituted for PAF. The concentration at which thrombin first caused submaximal platelet aggregation was determined, and in each experiment it was found to be 0·1 U in a reaction

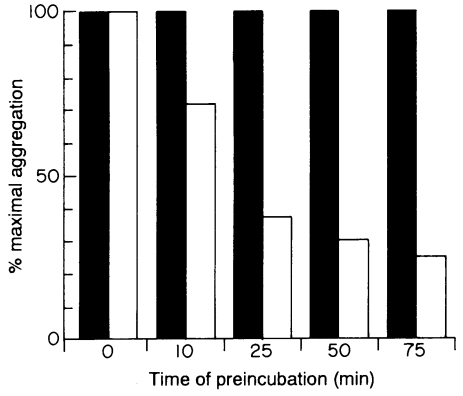

Figure 3. Thrombin-induced platelet aggregation is not inhibited by *N. brasiliensis* ES. The submaximal concentration at which thrombin induced platelet aggregation was determined to be 0·2 U/ml. This concentration of thrombin was preincubated with *N. brasiliensis* ES (■) for up to 75 min before its activity was assayed (see Materials and Methods). In a parallel experiment, ES was preincubated with 1×10^{-6} M PAF (□) for the same time-period. The plot shows the level of platelet aggregation induced by ES/thrombin or ES/PAF plotted as a percentage of the maximal aggregation response measured in a control experiment with each of these mediators.

volume of 0·5 ml. Figure 3 shows the results of preincubating thrombin with *N. brasiliensis* ES before assessing its ability to cause platelet aggregation. Rather than showing the whole trace, we have plotted the maximum level of aggregation attained, presented as a percentage of the control peak at each time-point.

The results show that the ability of thrombin to cause platelet aggregation is not affected in any way by *N. brasiliensis* ES, implying that the inhibitory activity acts directly on PAF rather than causing inhibition of PAF function by interfering with the intracellular signalling process.

Inhibition of PAF by ES is reduced by serine hydrolase inhibitors

In vivo, the immediate (and inactive) catabolite of PAF is lyso-PAF, in which the acetyl group at carbon 2 has been replaced by a hydroxyl group. This reaction is catalysed by a specific acetylhydrolase found in the cytosol of PAF-sensitive cells and systemically in plasma.[20] The cytosolic enzyme can be inhibited by the serine hydrolase inhibitors PMSF and DFP,[21,22] and thus we investigated the sensitivity of the inhibitory factor in *N. brasiliensis* to these agents. In addition, a family of enzymes sensitive to inhibition by DFP and classified as acetylcholinesterases (AChE) have been described in secretions of a number of nematodes, including *N. brasiliensis*.[23] We therefore included a more specific inhibitor of AChE, eserine, in these experiments. Inhibitors were added to ES at concentrations which diminished the AChE activity to less than 20% of normal, determined by a colourimetric assay,[24] and equivalent concentrations were added to the control samples. The inhibitors used were eserine, a reversible inhibitor which binds the anionic site of cholinesterases, and di-isopropylfluorophosphate (DFP), which irreversibly binds the active serine of serine hydrolases. In addition, PMSF and TPCK were used at concentrations which inhibit serine proteases.

The results presented in Fig. 4 again show the level of aggregation induced at each time-point plotted as a percentage

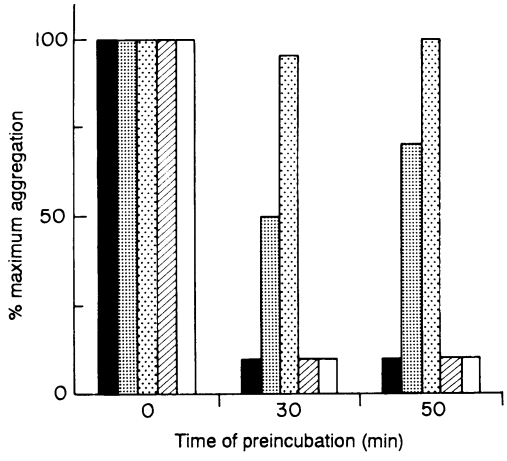

Figure 4. Inhibition of PAF is reduced by serine hydrolase inhibitors. *N. brasiliensis* ES (■) was treated with 0·1 mM TPCK (□), 1 mM PMSF (▨), 0·1 mM eserine (▤) or 10 μM DFP (▩) before preincubation with PAF at 1×10^{-6} M. The effect of these compounds on the ability of *N. brasiliensis* ES to inhibit PAF function was assayed as described in Materials and Methods. PAF activity is expressed as a percentage of the aggregation response achieved by a 'PAF only' control in each case.

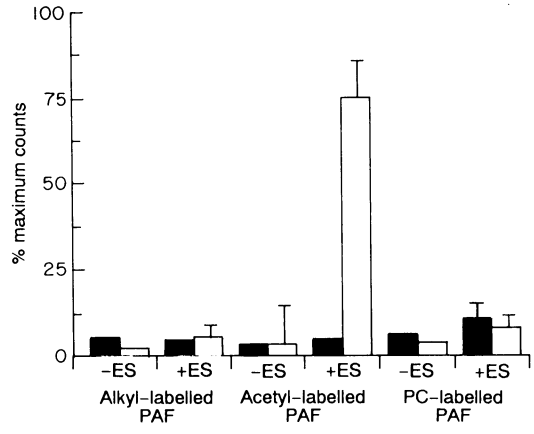

Figure 5. Hydrolysis of [acetyl ^{3}H]PAF. Three different radiolabelled preparations of PAF (see Materials and Methods) were mixed with cold PAF to a concentration of 1 μM. These were incubated with ES at a final concentration of 100 μg/ml, and the reaction stopped after 30 seconds (■) or 30 min (□) incubation at 37°. Cleavage products were separated by reversed-phase chromatography on C_{18} silica gel cartridges and radioactivity determined following addition of scintillation fluid. Results are expressed as a percentage of the total counts eluted from the column. Bars represent 1 SD of triplicate assays.

of that attained by the control. They indicate that the ability of ES to inhibit PAF activity was unaffected by either PMSF or TPCK, but was partially abolished by eserine, and was wholly abolished by DFP. These results are consistent with those that would be expected for competitive (eserine) and irreversible (DFP) inhibitors of a serine hydrolase.

Inactivation of PAF is effected by an acetylhydrolase

To see whether PAF was being cleaved by a parasite-derived enzyme, we utilized an assay developed to determine acetylhydrolase activity.[12,20] This assay uses [acetyl ^{3}H]PAF as substrate, allowing easy separation and quantitation of [^{3}H]acetate liberated by hydrolysis. The assay was also run using PAF labelled (i) on the choline group and (ii) on the alkyl chain. The former derivative was used to address the possibility of hydrolysis of the phosphoryl ester bond, whereas the alkyl-labelled PAF served as an additional control (all the counts should remain bound to the silica gel). The results are shown in Fig. 5, and are expressed as the proportion of total counts eluted from the column. It can be clearly seen that the ^{3}H-labelled acetyl group is liberated from the glycerol backbone, presumably as acetate. In contrast, no significant counts are recovered from PAF labelled on the phosphorylcholine group or the alkyl chain.

DISCUSSION

These studies demonstrate that ES collected from *N. brasiliensis* contains a factor capable of neutralizing the ability of PAF to cause platelet aggregation, and that this activity is abolished by serine esterase inhibitors such as eserine and DFP. Moreover, our assay of PAF hydrolysis suggests that this is attributable to an acetylhydrolase similar to that found in the cytosol of PAF-binding cells and systemically in plasma.[20,22] Hydrolysis was effectively stopped by the addition of acetic acid (see Materials and Methods) and thus, like the PAF acetylhydrolases already described, the nematode enzyme is acid labile. Both the cytosolic and the plasma-derived acetylhydrolases are inhibited by DFP, but only the cytosolic form is sensitive to PMSF, though this difference is thought to be due to glycosylation of the secreted form.[25] These enzymes are distinguished from other acetylhydrolases by their absolute requirement for a 5- to 16-carbon fatty acid chain on carbon 1 of their substrate, and from phospholipase A_2 (PLA_2) by their Ca^{2+} independence.[20]

Numerous studies have described enzymes secreted by *N. brasiliensis* which have been described as acetylcholinesterases. Substrate specificities of cholinesterases are defined by the spatial relationship of the anionic to the active site, and by the size of the acyl pocket in the active site.[26] The substrate specificity defined in this way is not absolute: AChE, for instance, can hydrolyse butyryl or phosphoryl esters (albeit slowly) under certain conditions.[27] Invertebrate AChE show a broader substrate specificity than vertebrate AChE and BChE.[28,29] No convincing *in vivo* function has yet been described for parasite secretory cholinesterases, and as PAF has a phosphorylcholine moiety at the carbon 3 position of the glycerol backbone, we hypothesized that this might be a natural substrate for these enzymes. The choline group could bind into the anionic site of AChE, and the phosphoryl group spatially resembles the transition state formed by the acetyl group of acetylcholine during hydrolysis. Nevertheless, no liberation of ^{14}C phosphorylcholine was observed (Fig. 5), and thus the phosphoryl ester bond of PAF does not seem to be cleaved by the parasite ES products. We are currently purifying both PAF acetylhydrolase and AChE activities from *N. brasiliensis* in order to confirm that they are attributable to distinct enzymes.

A number of endogenous PAF-inhibiting activities have previously been reported in humans and rodents. These do not fall into a single class of compounds, but include fatty acid derivatives such as prostacyclin and prostaglandins,[30,31] various uncharacterized lipid-like molecules[17,32] and a phosphorylcholine (PC)-binding protein (C-reactive protein[15]). It seems clear that the present inhibitory activity is due to direct enzymatic

cleavage of PAF rather than receptor blockade or interference with intracellular signalling processes. It is distinct from that reported for C-reactive protein as there is no effect on thrombin-induced platelet aggregation. C-reactive protein blocks signalling by binding and stabilizing membrane phospholipids, and by inhibiting phospholipase A_2 activity.[15]

Receptor-binding studies with various PAF derivatives have shown that the presence of a phosphate or a phosphonate on carbon 3 is critical for agonist properties, as is the presence of some sort of polar head group.[33] It is thus highly likely that a PC-binding antibody would inhibit PAF function. Interestingly, anti-PC specificities dominate the humoral response in many helminth infections.[34] One might thus speculate that a biological role for these antibodies could be to bind PAF and inhibit the host inflammatory response, and we are currently addressing this possibility.

Liu, Serhan & Weller[35] have recently shown that microfilariae of *Brugia malayi* utilize both exogenous and endogenous arachidonic acid to produce and secrete prostacyclin and prostaglandin E_2, both anti-inflammatory agents and potent inhibitors of platelet aggregation. Co-incubation of microfilariae with platelets inhibited aggregation via a number of agonists. It thus appears that helminth parasites may utilize a number of distinct mechanisms by which to limit or down-regulate inflammatory responses, resulting in the creation of a relatively protected local environment. Other examples include 'taeniaestatin', a proteinase inhibitor secreted by *Taenia taeniaeformis* which inhibits complement activation and neutrophil chemotaxis,[36,37] and a factor released by schistosomes which inhibits mast cell degranulation.[38]

It might be argued that the inhibitory activity described here does not prevent expulsion of adult *N. brasiliensis* from the gastrointestinal tract, but this argument can be countered by two observations. Firstly, adult worms survive long enough to reproduce, and are thus successful in propagating their progeny. Most importantly, however, the infective doses used in the laboratory are unnaturally high: if this is substituted by a regimen of trickle infection (more akin to the natural situation), then the self-cure phenomenon is not observed, and adult worms establish themselves at relatively high levels in the gut.[39,40] It is therefore an attractive proposition that parasite-mediated inactivation of PAF serves to limit or delay the induction of intestinal anaphylaxis. We are investigating this possibility and, more directly, are attempting to purify and fully characterize the putative acetylhydrolase.

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