Specificity and localization of lipolytic activity in adult *Drosophila melanogaster*

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The triacylglycerol lipases present in adult *Drosophila melanogaster* have been investigated. Different lipase activities are present in various tissues in the fly. In particular, an abundant lipase activity is present in the male accessory gland. An *esterase* null mutant was used to confirm that the enzyme activity was due to a distinct lipase and not non-specific activity from esterase 6 which is also abundant in accessory glands. The properties of the accessory-gland lipase were investigated, and pH optima and substrate utilization suggest that it has some similarities to vertebrate bile-salt-stimulated lipase. Lipase activity is significantly reduced in males and increased in females shortly after mating. This finding suggests that lipase activity is transferred to the female and may be important in mating and reproduction in *Drosophila*.

INTRODUCTION

Lipids are the major energy stores in higher organisms. They are either digested from the food or synthesized *de novo* and stored in tissues, from which they are released when required and transported to target tissues for utilization. Generally lipids are transported in the blood by lipoproteins, with non-esterified fatty acids being transported complexed to albumin. To complement this process a variety of lipases are required in order to utilize the diacyl- and triacyl-glycerol-rich lipoproteins, for the transfer of cholesterol esters and for the breakdown of intracellular stores. Much is known about the transport and metabolism of lipids in mammalian systems but little is known of this process in insects although knowledge is increasing. The main storage tissue for lipids in insects is the fat-body (Rizki, 1978), a tissue thought to be functionally analogous to vertebrate liver [for recent evidence see Abel et al. (1992), Falb and Maniatis (1992) and Søndergaard (1993)] and adipose tissue. In the adult insect, stored diacylglycerols are transported to the flight muscle, when required and in response to hormonal signals, using lipoprotein carriers synthesized in the fat-body (Shapiro and Law, 1983; Wells et al., 1987). In *Drosophila*, lipid levels increase during larval development, then fall dramatically during metamorphosis to quite low levels in adults (Church and Robertson, 1966). Thus in adults presumably there is a balance between dietary lipid intake and lipid utilization for flight and reproduction.

A family of lipases has been identified in vertebrates with related but distinct functions (Hide et al., 1992). Little is known about lipases in insects, although their presence and specificity in the *Locusta* flight muscle and other tissues has been investigated (Wheeler and Goldsworthy, 1985; Wheeler et al., 1986). The study of metabolism and transport of lipid in insects is of interest, but, to make a thorough study, the enzymes and genes involved must be characterized. Although progress has been rapid in mammalian systems, the genes are much more difficult to clone in the large insects currently being studied, as they have large genomes and are often difficult subjects for genetic and molecular approaches. The ideal organism from this viewpoint is *Drosophila*, but this is a poorly studied insect for physiological work. Some progress has been made with identification and cloning of apolipoproteins in *Drosophila*, but nothing is known about lipases. Before attempting to isolate lipase genes from *Drosophila*, as a step towards unravelling the physiology of lipid transport and metabolism, it is necessary to know more about the tissue distribution and substrate specificity of the lipase(s) present.

In this paper we present data on lipase activity in a number of adult *Drosophila* tissues. We report evidence for a number of lipases in *Drosophila* which are located in various body parts. Somewhat surprisingly there is a very high level of lipase activity in the male accessory glands, which is due to a different enzyme from that found in other tissues. We present evidence that this lipase activity is transferred to the female during copulation.

MATERIALS AND METHODS

Biochemical techniques

Radiolabelled lipid substrates were supplied by Professor P. Belfrage (University of Lund, Sweden). Di-[1,3-^{3}H]isopropyl fluorophosphate ([^{3}H]DFP) was obtained from New England Nuclear, non-radioactive DFP was from Sigma and tetrahydrolipstatin (THL) was a generous gift from Professor A. Fischli (F. Hoffman-La Roche Ltd., Basel, Switzerland).

Lipolytic activity was assayed by measuring the release of [^{3}H]oleic acid from tri[^{3}H]oleoylglycerol, cholesterol [^{3}H]oleate and 1(3)-mono[^{3}H]oleoyl-2-*O*-oleoylglycerol (MOME) using an ethanol suspension assay based on the method of Khoo et al. (1976). Stock solution in ethanol (50 μl) containing the various substrates (40 mM, specific radioactivity approx.

Abbreviations used: DMSO, dimethyl sulphoxide; DFP, di-isopropyl fluorophosphate; MOME, 1(3)-mono-oleoyl-2-*O*-oleoylglycerol; THL, tetrahydrolipstatin.

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5.15×10^4 d.p.m./nmol) was added with swirling to 3.45 ml of 58 mM sodium phosphate buffer at the indicated pH, containing 100 mg of BSA, to form the working substrate. Extract (25 μl) was then assayed against 0.175 ml of the substrate which gave a final substrate concentration of 0.5 mM. Assays were routinely performed in duplicate for 60 min at 37 °C, conditions in which less than 5% of the substrate was hydrolysed. The reaction was terminated and the free [^{3}H]oleic acid was measured as described in Khoo et al. (1976). When lipase activity was measured in the pH range 4–5, which is outside the buffering capacity of sodium phosphate buffer, this was replaced by sodium acetate/acetic acid buffer at the same concentration.

DFP was dissolved in propylene glycol and THL in dimethyl sulphoxide (DMSO). Then 0.1 vol. was added to extracts of *Drosophila* testes or accessory glands to give the final concentrations indicated. After incubation at 30 °C for 30 min, triacylglycerol lipase activity was determined at the pH indicated. Control incubations were performed with 0.1 vol. of propylene glycol or DMSO alone, which had a negligible effect on enzyme activity.

One unit of enzyme activity catalyses the release of 1 μmol of oleic acid/min. Protein was determined by the method of Bradford (1976).

Maintenance of stocks

OrR wild-type stock and the *Esterase 6*^{0} (*Est 6*^{0}) flies were maintained on a standard yeast, agar, sugar and cornmeal medium at 25 °C.

Dissection of tissues

Flies aged 3 days were dissected in *Drosophila* Ringer's solution, transferred to buffer and homogenized. The debris was removed by centrifugation at 10000 *g* and samples were assayed within 36 h.

To determine optimum storage conditions, flies and tissues were homogenized and stored in a variety of buffers. Optimal activity was obtained using 10 mM Tris/HCl, pH 7.4, 250 mM sucrose, 1 mM EDTA and 0.1% (v/v) 2-mercaptoethanol as the buffer and storing the samples at 4 °C rather than frozen before assaying.

Western blotting

Twenty male and 20 female OrR and *Est 6*^{0} flies were homogenized in 10 μl of Laemmli sample buffer and separated by SDS/PAGE using 10% (w/v) polyacrylamide in the running gel. The proteins were transferred electrophoretically to nitrocellulose, and esterase 6 protein (esterase 6) was detected using anti-esterase primary antibody (courtesy of Professor R. Richmond, Department of Biology, The University, Bloomington, IN 47405, U.S.A.). The secondary antibody for use as a detection system was anti-rabbit IgG conjugated to horseradish peroxidase.

Mating experiment

Pupae of OrR were placed into individual vials containing standard fly media and allowed to hatch and age for 3 days. Individual pair matings were then set up and the flies observed. Those that copulated were selected and, after they had separated naturally, the mated males and females were collected into sample buffer and treated as above. For each replicate, 20 flies were used. Control unmated males and females were collected and maintained in individual vials in the same way. Flies that did not mate during the period of observation were not used as controls in case of transfer of enzymes during courtship.

RESULTS

Preliminary results obtained by measuring triacylglycerol lipase activity in extracts of wild-type (OrR) whole flies at pH 4.5, 7.0 and 8.5 indicated highest activity at pH 7.0, although significant activity was also present at pH 4.5 and 8.5. Furthermore, when triacylglycerol lipase activity was measured at pH 7.0, it was found that the activity in males was approximately 5-fold higher than that in females. To locate this activity, lipase was measured in heads, thoraces, guts, abdominal carcasses and gonads. In non-gonadal parts of the body, triacylglycerol lipase activity at pH 7.0 was detected in all tissues examined, with highest levels being present in the gut. No differences were found between male and female flies in these tissues (Table 1).

Comparison of lipase activity in the gonads of males and females revealed that the ovaries had very low neutral triacylglycerol lipase activity. In contrast, male gonads were rich in lipase activity, with levels approximately 20-fold higher than in female gonads (Table 1). To determine the location of lipase activity within the male gonad, gonads were dissected into testicular tissue and accessory glands. Neutral lipase activity was localized predominantly in the accessory gland, with the specific activity in the gland being approximately 12-fold higher than in the testes (Table 1).

It has been reported previously that the accessory glands contain high levels of a non-specific esterase (esterase 6) which has some activity against triacylglycerol substrates (Mane et al., 1983a; White et al., 1988). To investigate the possibility that this esterase was responsible for the high lipase activity found in accessory glands, neutral lipase activity was determined in various parts of *Est 6*^{0} flies, which are defective in the gene encoding the non-specific esterase. To confirm that the *Est 6*^{0} stock was indeed deficient in the esterase 6 enzyme, Western blotting was carried out using an anti-(esterase 6) antibody. Figure 1(a) shows clearly that both male and female mutant flies completely lack this protein. Furthermore, incubation of accessory-gland extract with

Table 1 Lipase activity in different *Drosophila* extracts

Lipase activity was determined in each *Drosophila* extract by measuring triacylglycerol hydrolase activity at pH 7.0 as described in the Materials and methods section. Values are means ± S.E.M. [$n = 2$ for all samples except *Est 6*^{0} male and female gut samples as well as OrR male abdomen, female gut and female abdomen samples ($n = 3$). In addition $n = 4$ for OrR male gut samples].

	Specific activity (munits/mg)	
Drosophila extract	OrR	*Est 6*^{0}
Male		
Gut	22.2 ± 2.4	31.2 ± 3.0
Abdominal carcass	6.9 ± 1.3	8.8 ± 1.5
Head	7.4 ± 1.6	5.3 ± 2.8
Thorax	3.1 ± 0.8	2.8 ± 0.7
Whole gonad	38.9 ± 4.5	42.9 ± 2.4
Testes	9.0 ± 5.6	4.6 ± 2.3
Accessory gland	109.6 ± 31.0	90.0 ± 15.4
Female		
Gut	21.5 ± 4.7	23.8 ± 3.9
Abdomen	6.9 ± 1.4	8.0 ± 3.7
Head	7.8 ± 2.1	11.5 ± 5.5
Thorax	3.0 ± 0.7	3.3 ± 1.6
Ovary	2.9 ± 0.3	3.4 ± 0.7

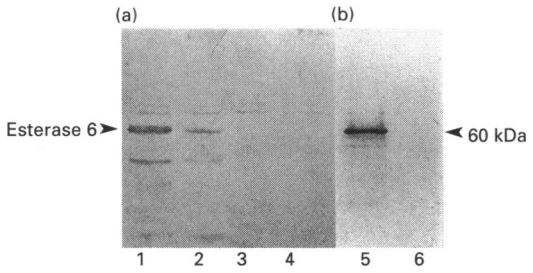

Figure 1 Demonstration of the absence of esterase 6 in the mutant *Est 6⁰*

(**a**) Homogenates of whole male and female flies from both OrR and *Est 6⁰* stains were subjected to Western blotting using anti-(esterase 6) as the primary antibody as described in the Materials and methods section. Lane 1, male OrR flies; lane 2, female OrR flies; lane 3, male *Est 6⁰* flies; lane 4, female *Est 6⁰* flies. (**b**) OrR and *Est 6⁰* accessory-gland extracts were incubated with 0.175 mM [^{3}H]DFP (1×10^6 d.p.m./nmol, added in 0.1 vol. of propylene glycol) for 30 min at 30 °C. The reaction was terminated by precipitation of the protein with 20% (w/v) trichloroacetic acid and the recovered protein was analysed by SDS/PAGE, electroblotting and direct autoradiography. Lane 5, OrR accessory-gland extract; lane 6, *Est 6⁰* accessory-gland extract.

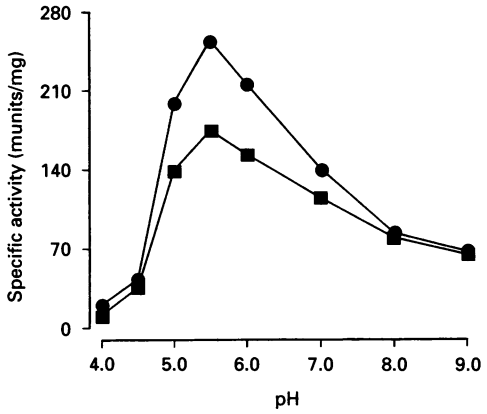

Figure 2 pH–activity profile of accessory-gland trioleoylglycerol lipase from wild-type and mutant flies

Trioleoylglycerol lipase activity of accessory-gland extract of wild-type OrR (●) and *Est 6⁰* mutant (■) flies was determined at the pH indicated as described in the Materials and methods section.

[^{3}H]DFP, a serine hydrolase inhibitor, led to radiolabelling of a 60 kDa protein in the wild-type OrR extracts which was absent from the *Est 6⁰* mutants (Figure 1b). This corresponds to the reported molecular mass of esterase 6 (62–65 kDa) and so confirms the absence of this enzyme in the mutant strain.

Lipase activity (at pH 7.0) in extracts of different body regions of female and male flies was essentially identical in the wild-type OrR and the mutant *Est 6⁰* strain (Table 1). Furthermore, when lipase activity was determined in the gonads of these flies, a very high level of activity was again localized to the accessory glands in the *Est 6⁰* mutants. The level of activity was slightly higher in OrR than in *Est 6⁰* flies, probably because of a contribution from the triacylglycerol-hydrolysing activity of the non-specific esterase 6 enzyme.

Properties of accessory-gland lipase

The existence of high lipase activity in the accessory glands of male flies was unexpected. To help to establish its function, further characterization of its basic enzyme properties was carried out. Lipases can be broadly categorized both in terms of pH optima and substrate specificity, in particular their ability to hydrolyse cholesterol esters.

Preliminary observations of the activity of accessory-gland lipase at pH 4.5, 7.0 and 8.5 revealed highest activity at pH 7.0, indicating it to be a neutral enzyme, although significant activity was also present at pH 4.5 and 8.5 (results not shown). Subsequent detailed analysis of the pH–activity profile indicated that the lipase has a rather broad pH–activity range; maximum activity was observed at pH 5.5, but there was significant activity in the pH range 4.5–9.0 (Figure 2). Similar profiles were observed for accessory-gland extracts from wild-type and mutant flies, with a slightly higher activity observed in the wild-type flies, again presumably because of a contribution from the esterase 6 enzyme.

Drosophila accessory-gland extract was able to hydrolyse triacylglycerol and MOME (a diacylglycerol analogue) at approximately equal rates at pH 5.5, whereas activity against cholesterol oleate was only approximately 4% of this activity for the *Est 6⁰* mutant enzyme (results not shown).

Another characteristic of certain lipases is their susceptibility to inhibition by DFP and THL. When accessory-gland extracts from both strains were incubated with increasing amounts of DFP, inhibition of the lipolytic activity at pH 5.5 was observed (Table 2). Approx. 50% inhibition was brought about by 10 μM DFP with over 90% inhibition by 1 mM. The inhibition profiles were similar for the wild-type and mutant enzymes. The absence of any detectable [^{3}H]DFP-labelled band in the mutant accessory-gland extracts (Figure 1b) indicates that, in common with other known lipases, the accessory-gland enzyme has a high specific activity and hence the enzyme polypeptide is present in undetectable amounts. THL had no effect on lipase activity even when present at concentrations of up to 1 mM (a concentration that inhibited the triacylglycerol hydrolase activity of purified bovine hormone-sensitive lipase by 85%) (results not shown).

In order to investigate whether the activity seen in testes was due to contamination with accessory-gland lipase or was an independent lipolytic activity, a pH–activity profile was obtained for an extract of this tissue. A completely different profile was

Table 2 Inhibition of accessory-gland triacylglycerol lipase activity by DFP and THL

Accessory-gland extracts from wild-type and mutant flies were preincubated with 0.1 vol. of DFP (in propylene glycol) or THL (in DMSO) to give the final inhibitor concentration shown. Residual triacylglycerol lipase activity was determined at pH 5.5 as described in the Materials and methods section. Results are expressed as a percentage of the activity seen in the presence of propylene glycol or DMSO alone and are the means of duplicate values obtained from a single representative experiment.

Inhibitor	Final inhibitor concentration (mM)	Triacylglycerol lipase activity (% of control)	
		OrR	*Est 6⁰*
DFP	0	100.0	100.0
	0.01	50.0	45.0
	0.1	7.0	14.5
	1.0	3.0	11.0
THL	0	100.0	100.0
	0.01	96.0	95.0
	0.1	96.0	93.0
	1.0	94.0	96.0

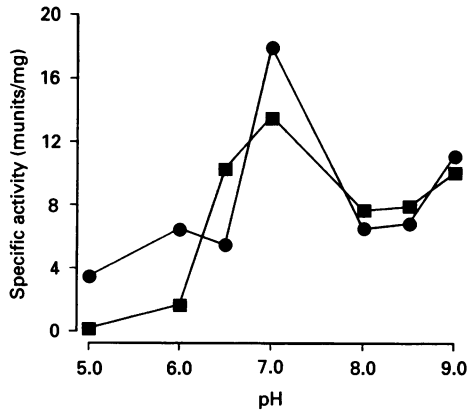

Figure 3 pH–activity profile of testes trioleoylglycerol lipase from wild-type and mutant flies

Trioleoylglycerol lipase activity in testes extract from wild-type OrR (●) and *Est 6*^{0} mutant (■) flies was determined at the pH indicated as described in the Materials and methods section.

Table 3 Lipase activity in whole extracts of mated and unmated *Drosophila*

Lipase activity was determined in extracts of whole flies from the OrR strain of *Drosophila* both before and after mating, by measuring triacylglycerol lipase activity at pH 6.0 as described in the Materials and methods section. Assays were performed at pH 6.0 to select for accessory-gland lipase activity. Values are means ± S.E.M. ($n = 5$): $^*P < 0.01$, $^{**}P < 0.001$ compared with unmated (paired Student's t test).

	Lipase activity (munits/mg)	
	Unmated	Mated
Male	43.60 ± 6.00	22.40 ± 1.64*
Female	2.24 ± 0.26	7.11 ± 0.87**

observed with a peak of activity for both strains at pH 7.0 and high activity at pH 9.0 (Figure 3). In particular, there was minimal activity at pH 6.0 and below, indicating that this is a different lipase from that detected in the accessory glands. In contrast with the accessory-gland lipase, triacylglycerol lipase activity at pH 7.0 in testes was relatively insensitive to DFP with only approximately 25% inhibition observed with 1 mM DFP. Furthermore, this activity was sensitive to inhibition by THL (80% inhibition by 1 mM) (results not shown).

Function of the accessory-gland lipase

The function of the accessory-gland lipase is unknown but we investigated the possibility that it is transferred to the female during copulation and might therefore have a role in mating.

Individual OrR males and females (separated as pupae and aged for 3–4 days) were placed together and copulation observed. Lipase activity at pH 6.0, the optimum pH for the accessory-gland enzyme, was then measured in groups of 20 mated males and females and in unmated males and females of the same age and from the same stock. As can be seen in Table 3, lipase activity in the male decreased twofold after mating, and there was a dramatic increase in the mated female. However, the transfer of lipase activity was not quantitative, consistent with other evidence that there is significant leakage and loss of accessory-gland fluid during copulation (Bownes and Partridge, 1987).

DISCUSSION

We have shown here that significant triacylglycerol lipase activity is present in all body parts of both male and female adult *Drosophila* flies. Within the head, thorax and abdomen are fat-body cells which store triacylglycerol and for which lipases may be needed for mobilization. Furthermore, the thorax also contains major flight muscles which have been shown to be a major source of lipase activity in active and adipokinetic-hormone-induced locusts, in which lipases have a critical role in using the diacylglycerols delivered on the adipokinetic-hormone-induced lipoprotein present in the haemolymph (Wheeler and Goldsworthy, 1985; Wheeler et al., 1986). Perhaps not surprisingly the highest level of lipase activity outside of the gonad is observed in the gut, where it presumably functions in the utilization of dietary triacylglycerol.

There are very low lipase levels in the ovary. The largest cells of the ovary are the developing oocytes and the major protein components of these are the yolk proteins. These have been shown to have significant sequence similarity to certain vertebrate lipases but lack the active-site serine (Bownes et al., 1988). The low lipase activity in ovaries confirms our observations that yolk proteins do not have a major lipolytic role in oogenesis or early embryogenesis (Bownes, 1992).

The highest lipase activity is in the accessory gland of the male gonads. This is particularly intriguing given the observations that there is also high esterase 6 enzyme activity in this tissue (Sheehan et al., 1979). The accessory glands secrete fluids which are transferred to the female along with the sperm during mating. These contain components such as the sex peptide, which inhibits females from remating and promotes egg production (Chen et al., 1988; Aigaki et al., 1991), a peptide with serine-protease-inhibitory activity (Schmidt et al., 1989) and a product resembling a peptide pheromone precursor (Mosma and Wolfner, 1988). There is even an antibacterial peptide produced by the male which may protect the female from bacteria transferred during mating (Samakovlis et al., 1991). We here present evidence that the accessory-gland lipase is also transferred to the female during copulation.

The esterase 6 enzyme is found in the ejaculatory duct and is transferred to the female within 2–3 min of mating (Richmond et al., 1980; Richmond and Senior, 1981). A male lipid, *cis*-vaccenyl acetate, is also transferred to the female reproductive tract during mating (Butterworth, 1969; Brieger and Butterworth, 1970; Jallon et al., 1981). The esterase 6 enzyme is able to use *cis*-vaccenyl acetate as a substrate *in vitro* (Mane et al., 1983b). The lipid is transferred during the first 3–6 min of mating and levels fall sharply during the first 4–6 h after mating (Jallon et al., 1981; Vander Meer et al., 1986). This means that *cis*-vaccenyl acetate is unlikely to be acting to make females unattractive to males [although it can do this when topically applied (Jallon et al., 1981; Zawistowski and Richmond, 1986)], as this response occurs much sooner after mating begins. There is good evidence that the male lipid is not converted into *cis*-vaccenol or transferred from the reproductive tract to the cuticle of the mated female, again suggesting it is not used for signalling to inhibit further mating (Scott and Richmond, 1987). Thus the function of this male lipid is unclear, but, as accessory-gland lipase is also transferred to the female during copulation, the function of this enzyme may be related to the utilization of the male lipid.

Many molecules that are important for female reproductive behaviour are transferred in the ejaculate. It seems possible that

enzymes also involved in this behaviour are also transferred. It should be noted that the pH in both the accessory glands and testes is approximately 6.8. This is consistent with the pH optimum for the activity of the testes enzyme, but not for that of the accessory-gland enzyme which is optimal below pH 6.0, although it still has significant activity at pH 6.8. It is possible that pH conditions are different in the female reproductive tract and this could activate the lipase.

Comparison of the characteristics of the accessory-gland lipase described here with those of other known mammalian lipases reveals a number of features. Almost all known lipases are inhibited by DFP, suggesting that they possess an essential serine residue (Maraganore and Heinrikson, 1986); accessory-gland lipase is consistent with this. A pH optimum of 5.5 is unusual amongst mammalian lipases which generally display optimum activity around pH 7.0–8.0. There are, however, some mammalian lipases with lower pH optima including lingual lipase (pH 4.4–5.4) (Hamosh, 1984) and bile-salt-stimulated lipase (pH 5.5–7.4) (Rudd and Brockman, 1984). A further characteristic of lipases is their ability to hydrolyse a range of substrates. The accessory-gland extract was found to possess high lipolytic activity against tri- and di-acylglycerol and a low but significant activity against cholesterol ester. It will be essential to purify the lipase to homogeneity in order to confirm that all three activities are catalysed by the same polypeptide. The ability to hydrolyse cholesterol esters is rare among lipases; hormone-sensitive lipase possesses a high cholesterol ester hydrolase activity (150% of that towards triacylglycerol) (Fredrikson et al., 1981) whereas bile-salt-stimulated lipase has a low activity (1% of that towards triacylglycerol) (Bläckberg and Hernell, 1981). The basic enzyme properties of the accessory-gland lipase examined suggest that it shares most characteristics, namely a similar pH optimum (pH 5.5) and substrate specificity, with bile-salt-stimulated lipase.

Ideally accessory-gland lipase should be purified. The small size of this gland, along with the finding that, although high lipase activity is observed because of the high specific activity of lipases in general, very little lipase protein is likely to be present, make conventional biochemical purification methods unsuitable. However, knowledge of the characteristics of accessory-gland lipase may help in isolation of the gene from a *Drosophila melanogaster* accessory-gland cDNA library, for example by selecting sequences from around the active site of bile-salt-stimulated lipase as a possible probe. This would enable expression of the protein in *Escherichia coli*, yeast or baculovirus cells. Purified protein would then be available for study of its biochemical properties and ultimately genetic manipulation in order to determine its function *in vivo*.

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