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Aldehyde reductase activity in the antennae of *Helicoverpa armigera*

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Abstract

In the present study, we identified two aldehyde reductase activities in the antennae of *Helicoverpa* species, NADH and NADPH-dependent activity. We expressed one of these proteins of *H. armigera*, aldo-keto reductase (AKR), which bears 56% identity to bovine aldose reductase, displays a NADPH-dependent activity and is mainly expressed in the antennae of adults. Whole-mount immunostaining showed that the enzyme is concentrated in the cells at the base of chemosensilla and in the nerves. The enzyme activity of *H. armigera* AKR is markedly different from those of mammalian enzymes. The best substrates are linear aliphatic aldehydes of 8–10 carbon atoms, but not hydroxyaldehydes. Both pheromone components of *H. armigera*, which are unsaturated aldehydes of 16 carbons, are very poor substrates. Unlike mammalian AKRs, the *H. armigera* enzyme is weakly affected by common inhibitors and exhibits a different behaviour from the action of thiols. A model of the enzyme suggests that the four cysteines are in their reduced form, as are the seven cysteines of mammalian enzymes. The occurrence of orthologous proteins in other insect species, that do not use aldehydes as pheromones, excludes the possibility of classifying this enzyme among the pheromone-degrading enzymes, as has been previously described in other insect species.

Keywords: aldo-keto reductase, *Helicoverpa armigera*, reductase inhibitors, nonanal, immunostaining, Western-blot.

Introduction

Aldo-keto reductases (AKRs) represent a large family of NAD(P)H-dependent enzymes catalysing the reduction of aldehydes and ketones to their corresponding alcohols, and widely studied in mammals. Based on sequence similarities, they have been grouped into 15 families, with no clear relationship to enzyme activity or phylogenetic distance. Members of several subfamilies have been characterized in the same species, with a special focus on human enzymes. By contrast, insect AKRs have received little attention and none of them has been completely characterized. Few studies have been published, and these have been mostly on members of the steroid hormone family (Salvucci *et al.*, 1998; Takeuchi *et al.*, 2000; Sieglaff *et al.*, 2005; Yang *et al.*, 2011), but, thanks to genomic information, a large number of sequences homologous to mammalian AKRs are available in the databases.

Aldose reductase (AR), an NADPH-dependent aldehyde reductase (Del Corso *et al.*, 2000) belonging to the AKR superfamily (Barski *et al.*, 2008), has been widely studied and characterized in mammals (Del Corso *et al.*, 2008). The human (Bohrani *et al.*, 1992; Wilson *et al.*, 1992) and the pig (Rondeau *et al.*, 1992) AR structures, resolved by x-ray crystallographic analysis, show that the single peptide chain is organized as an eight-stranded β/α barrel with a small β-sheet capping the N-terminal end and a C-terminal extension. The pyridine cofactor, whose release appears to be the limiting step for the enzyme-catalysed reaction, appears to be tightly bound to the enzyme (Del Corso *et al.*,

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1990) in a cavity close to the C-terminal end of the β barrel (Bohrani *et al*., 1992; Rondeau *et al*., 1992; Wilson *et al*., 1992).

The enzyme, which is able to reduce hydrophilic compounds such as glyceraldehyde and different aldo sugars (Grimshaw, 1992; Cappiello *et al*., 1994), is considered to be the first enzyme of the so-called polyol pathway. This route allows the conversion of glucose to fructose through the AR-dependent formation of sorbitol, which is then oxidized to fructose by an NAD^+-dependent sorbitol dehydrogenase (Kinoshita, 1974). AR has been acknowledged as a detoxifying enzyme able to reduce hydrophobic toxic aldehydes generated from lipid peroxidation (Srivastava *et al*., 1995; Vander Jagt *et al*., 1995). In this regard, AR is able to efficiently reduce 4-hydroxy-2-nonenal (HNE), a common endproduct of radical-induced degradation of lipids, and a number of long-chain hydrophobic alkenals and alkanals (Srivastava *et al*., 1999). More recently, the efficient reducing activity of AR on the Michael adduct between glutathione and HNE (GS-HNE) suggested the involvement of AKR in the antioxidant cell signalling pathway through the pro-inflammatory action of 3-glutathionyl-1,4-nonanediol, the product of GS-HNE reduction (Srivastava *et al*., 1999; Ramana *et al*., 2006; Ramana & Srivastava, 2010). Because of the deleterious effects associated with AR activity in hyperglycaemic conditions in inflammation and in a number of different pathologies (Shoeb *et al*., 2011), AR is actively investigated as a target enzyme for drug development (Del Corso *et al.,* 2013). Despite the above-mentioned 15 classes of AKRs, their amino acid sequences appear to be well conserved across mammals, with identity values of ~80%. In addition, enzyme activity seems to be similar for members belonging to different subclasses and across species. Such similarities are also in apparent contrast to a variable number of cysteines, from 4 to 7, all of them in their reduced form. The accessibility to solvent also presents some variability. The human and the bovine enzymes, for instance, exhibit 3–4 solvent-accessible cysteines (Liu *et al*., 1989; Bresson *et al*., 2011) of the seven residues present in the structure (Cappiello *et al*., 1994; Bresson *et al*., 2011). Among them, Cys298, which is located close to the active site, is susceptible to covalent modification by both hydrophobic and hydrophilic reagents (Bohren *et al*., 1989; Liu *et al*., 1989; Cappiello *et al*., 1996). The S-thiolation of the enzyme has been shown to affect the enzyme activity depending on the nature of the thiolating agent; by contrast, all the thiolated forms appear less susceptible than the native enzyme to inhibition (Del Corso *et al*., 1998; Cappiello *et al*., 2001).

In the present study, we report the identification, bacterial expression and characterization of a member of the AKR family in the lepidopteran *Helicoverpa armigera* (*Harm*AKR). To the best of our knowledge, this is the first description of the properties of an AKR in insects.

Results and discussion

Detection of aldehyde reductase activity

Both *H. armigera* and *Helicoverpa assulta* use mixtures of two aldehydes as their sex pheromone blend, (*Z*)-9-hexadecenal and (*Z*)-11-hexadecenal (Wang *et al*., 2005). As reported for other insect species, we suspected the presence of pheromone-metabolizing enzymes in the antennae of adults; therefore, we incubated crude antennal extracts with both aldehyde components in the absence or in the presence of either NADH or NADPH and identified the products by gas chromatography (GC)/mass spectrometry (MS) after extraction of the incubation mixtures with dichloromethane. In the presence of NADH or NADPH, both aldehydes were almost converted into their alcohols. Figure 1 shows the GC analyses of crude dichloromethane extracts of antennal extracts from both species incubated with the aldehydes and NADH, as described in the experimental procedures section. Similar results were obtained when we replaced NADH with NADPH. These results indicate the presence of either one enzyme able to use both pyridine cofactors or, most likely, two enzymes with aldehyde reductase activity, one NADH- and the other NADPH-dependent.

An aldehyde-keto reductase in Helicoverpa armigera

In an attempt to characterize the enzyme(s) responsible for such activities, we performed a search of the literature and databases. Although, as expected, a large amount of work on aldehyde and ARs in mammals is available, we could not find characterization of similar enzymes in insects; however, protein and nucleotide databases contain sequences similar to mammalian AKRs in several insect species. Among them, a single entry [National Center for Biotechnology Information (NCBI) acc. no. JF417982] reports a sequence of the AKR family in the moth *H. armigera*. We decided to focus our attention on this enzyme, although its subsequent characterization indicated a role of this protein in detoxification rather than in removing pheromone molecules from the sensory area, as reported for the so-called 'pheromone-degrading enzymes'.

To put this enzyme in a wider context, we first performed a BLAST search, using its sequence as the query, and obtained a large number of protein sequences with significant similarity. Figure S1 reports the alignment of representative insect AKRs, selected among the most similar to *Harm*AKR and relative to species, whose genomes have been sequenced. Identities between members range from

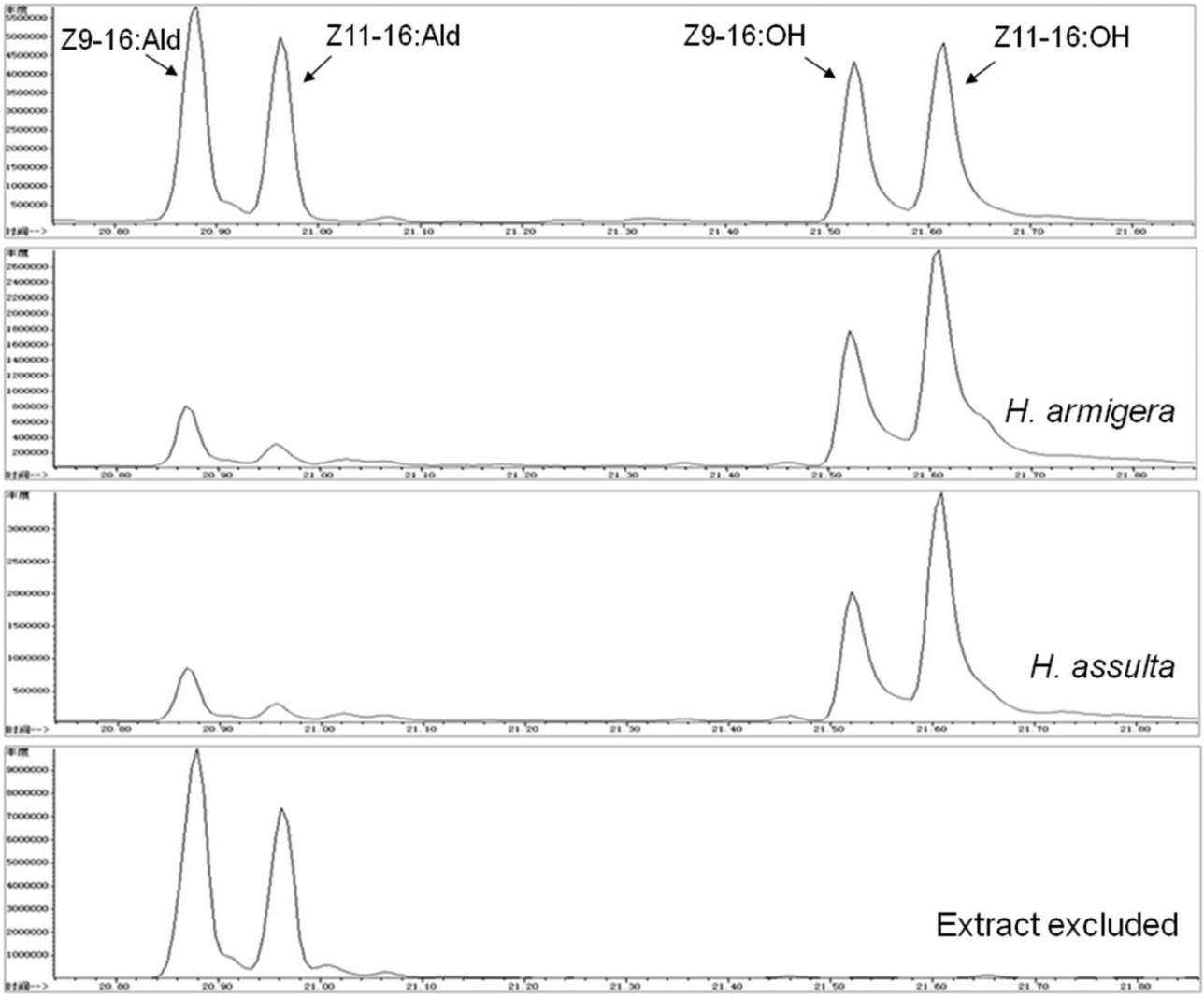

Figure 1. Top panel. Gas chromatography (GC) profile of an equimolar mixture of the two pheromone components, (*Z*)-9-hexadecenal and (*Z*)-11-hexadecenal, and their corresponding alcohols. Middle two panels. GC profiles of extracts obtained after incubation of the two aldehydes with crude antennal extracts from male *Helicoverpa armigera* or *Helicoverpa assulta* and NADH for 1 h. With both species, most of the aldehydes have been converted to their alcohols. Similar results were obtained in parallel experiments when NADH was replaced with NADPH. Lower panel. GC profile of incubation solution excluding extracts. Without the effect of reduction in antennae, the two pheromone components were not reduced to the corresponding alcohols. Z9-16:Ald, (*Z*)9-hexadecenal; Z11-16:Ald, (*Z*)11-hexadecenal; Z9-16:OH, (*Z*)9-hexadecen-1-ol; Z11-16:OH, (*Z*)11-hexadecen-1-ol.

58 to 84%, without a clear correlation with orders and species, indicating a wide diversity among insect AKRs. As reported for vertebrate AKRs, those of insects are also likely to be representative of several subgroups, although such classification has not yet been proposed for insect AKRs. This classification, however, is well supported by the variable number of cysteines, occurring in insects' sequences, between 4 and 7, as in vertebrate AKRs.

A wider comparison of AKRs from arthropods to mammals is shown in the similarity tree of Fig. S2. Again, sequences have been selected among those which produced the highest scores in a BLAST search, and include proteins with different numbers of cysteines. Insects and vertebrate AKRs are segregated into two well-defined groups, with ~50% of identical residues, on average, between members of the two groups. Next, we decided to produce the AKR of *H. armigera* in bacteria in order to understand for which of the two reductase activities observed in the crude extract it was responsible, to study its enzymatic activity and map its expression in the insect tissues.

Bacterial expression and purification of Harm*AKR*

*Harm*AKR was successfully obtained in high yields (20 mg/l of culture) and entirely in soluble form. The

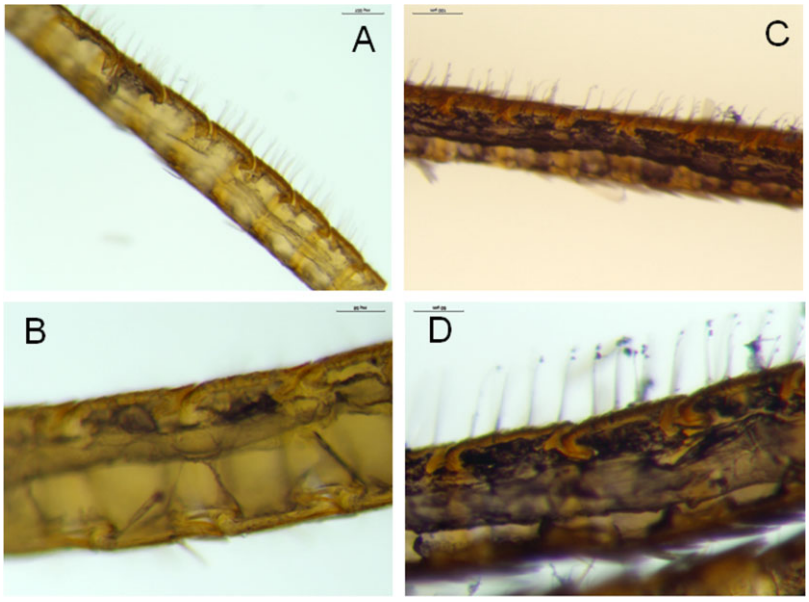

Figure 2. Whole mount immunostaining of *Helicoverpa armigera* antennae with a polyclonal antiserum prepared against the recombinant aldo-keto reductase of *H. armigera* (*Harm*AKR). (A) and (B): control using a pre-immune serum as primary antiserum; (C) and (D): stained with the *Harm*AKR antiserum. No staining was observed either when the primary antiserum was omitted.

construct included a segment of six histidine residues at the N-terminus, that we used to purify the protein by affinity chromatography. Figure S3 reports the sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analyses of expression products and purification steps, as well as Western blot identification of the purified band, using antibodies against the His-tag segment.

Tissue and temporal expression of Harm*AKR*

The expression of the enzyme in different parts of the body and during developmental stages were monitored both by PCR with specific primers and by Western blot experiments, using a polyclonal antiserum raised against the recombinant *Harm*AKR. The results, reported in Fig. S4, indicate a strong antennae-rich expression without significant differences between sexes. Such specificity is most evident in the PCR experiments, but also significant in Western blot. The weak staining observed in all tissues with the polyclonal antiserum might also be attributable to cross-reactivity with other proteins similar in their sequences to *Harm*AKR (Fig. S4).

During development, we did not observe significant variations from the first instar of larvae to pupae. The expression of this enzyme all through the insect developmental stages seems to rule out any relationship with the aldehyde pheromones, that are only produced and utilized during adult life (Fig. S5).

Immunostaining of whole-mount longitudinal sections of antennae (Fig. 2) showed expression of the enzyme in the cells at the base of sensilla and in the nerves. This omnipresence of *Harm*AKR also helps us to rule out the possibility of this enzyme having a role in termination of the olfactory signal through pheromone degradation.

Enzyme activity

We characterized the recombinant enzyme in terms of (a) reductase activity towards different aldehydes; (b) effect of different inhibitors; (c) modification of the activity by thiols, and compared its properties with those of a mammalian AKR (NCBI GenPep acc. no. NP_001012537) abundantly expressed in bovine lens (Del Corso *et al.*, 2008), which may be considered a classic AKR reference model. The bovine lens AR (*bov*AR) and *Harm*AKR share 56% of their amino acids, but differ by presenting seven and four cysteines, respectively.

The NADPH-dependent activity of *Harm*AKR towards a series of aldehydes of different size and structure is reported in Table 1 and compared with that of *bov*AR. No activity could be detected for *Harm*AKR and, as expected, for the *bov*AR when NADH instead of NADPH was used as cofactor. Among those tested, very good substrates for both enzymes are the linear aldehydes of 8–10 carbon atoms. Hydroxyaldehydes, instead, such as glyceraldehyde and HNE, present specificity constants [apparent catalytic constants (k_{cat})/apparent Michaelis constants (K_M)] 5- and 10-fold lower, respectively, for *Harm*AKR than for the bovine enzyme. This result is mainly attributable to the much larger increase of K_M in *Harm*AKR, despite the high values of k_{cat} measured for both substrates.

When linear alkanals are used as substrates, both *Harm*AKR and *bov*AR exhibit, a progressive increase in the affinity with the length hydrocarbon chain. In fact, the apparent K_M of *bov*AR for dodecanal can be only

Table 1. Activity of recombinant aldo-keto reductase (AKR) of *Helicoverpa armigera* compared with that of AKR isolated from bovine lens (Del Corso *et al*., 2008). All measurements were performed at 37 °C

	Helicoverpa armigera			Bovine lens		
Substrate	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M
D,L-Glyceraldehyde	1.130 ± 0.057	4.220 ± 0.160	3.700 ± 0.200	0.039 ± 0.011	0.790 ± 0.090	20.300 ± 6.300
HNE	0.719 ± 0.127	1.490 ± 0.250	2.100 ± 0.500	0.037 ± 0.002	0.770 ± 0.020	20.800 ± 1.200
Octanal	0.046 ± 0.005	3.390 ± 0.330	73.700 ± 10.800	0.017 ± 0.008	1.810 ± 0.790	106.500 ± 70.500
Nonanal	0.053 ± 0.005	3.890 ± 0.330	73.400 ± 9.300	0.027 ± 0.002	1.550 ± 0.090	57.400 ± 5.400
Decanal	0.062 ± 0.014	1.900 ± 0.390	30.600 ± 9.400	0.005 ± 0.002	0.850 ± 0.110	170.000 ± 74.200
Dodecanal	0.007 ± 0.001	0.400 ± 0.010	57.100 ± 8.300	<0.005†	0.580†	
NADPH*	0.069 ± 0.008	5.510 ± 0.410	79.800 ± 11.000	0.032 ± 0.002	0.790 ± 0.070	24.700 ± 2.700

HNE, 4-hydroxy-2-nonenal; K_M, apparent Michaelis constant; k_{cat}, apparent catalytic constant.
*Using 4.7 mM D,L- glyceraldehyde as substrate.
†Values were estimated on admitting a pseudo saturation of the enzyme at 5 μM of substrate; no differences in the enzyme activity (approx. 1 U/mg) were observed varying the concentration of dodecanal in the range 5–24 μM;
For both enzymes k_{cat} values were calculated on the basis of a molecular weight of 36 KDa.

estimated (<5 μM); no differences in enzyme activity (~1.0 U/mg of protein) were observed when the dodecanal concentration was varied in the range from ~5 to 24 μM. With regard to the k_{cat} values, for both *Harm*AKR and *bov*AR, a decrease of the apparent k_{cat} was observed with the length of the hydrocarbon chain. Based on this obesrvation, we understand why any attempt to measure the activity of *Harm*AKR spectrophotometrically on the longer aldehyde components of the pheromone failed. In this regard, the apparent discrepancy with the results of GC/MS experiments showing a NADPH-dependent reductase activity on both pheromone components by *H. armigera* crude extracts (Fig. 1), is clarified by the evidence of the reduction of the pheromone components when *Harm*AKR and cofactor concentrations in the assay mixture, 2.8 μM and 0.4 mM, respectively, were significantly increased with respect to the spectrophotometric assay conditions (Fig. S6). Combining these data, we can conclude that *Harm*AKR, even though able to reduce long-chain aldehydes as the pheromone components, does not seem to recognize them as the substrates of choice, preferring shorter (8–10 carbon atoms) linear aldehydes.

These results, together with other characteristics of this enzyme, such as the absence of a signal peptide (predicted by the programme SignalP 3.0 and in agreement with all other enzymes of the same family), its intracellular expression and the detection of the protein all through the developmental stages, help to exclude the hypothesis that *Harm*AKR could act as a pheromone-degrading enzyme, similar in function to those reported in the literature.

As a further comparison between *Harm*AKR and the *bov*AR, their susceptibility to inhibition by sorbinil and epalrestat, two potent and specific AR inhibitors, was tested in the same assay conditions. Both compounds have a much weaker effect on the activity of *Harm*AKR: the concentrations required to achieve the same effect as in the bovine enzyme are one order of magnitude higher for sorbinil and about two orders of magnitude higher for epalrestat (Fig. 3). This evidence and the kinetic data indicate that the *Harm*AKR is similar, in certain aspects, in its activity and selectivity to the *bov*AR, while the two enzymes present different behaviours mainly towards hydroxyaldehydes and inhibitors.

It is known that thiol agents, including physiological compounds such as cysteine and glutathione, may interfere with the cysteine redox status of the *bov*AR, affecting its activity and susceptibility to inhibition. Thus, to conclude the comparative analysis between *Harm*AKR and the *bov*AR, the effects of oxidized glutathione (GSSG) and reduced glutathione (GSH) were evaluated on the two enzymes (Fig. 4). The *bov*AR slowly loses activity when incubated in buffer, but is deactivated much faster in the presence of GSSG. The activity of *Harm*AKR, by contrast, decreases very rapidly and this process is not accelerated by the presence of GSSG. Nevertheless, the presence of GSH protects the enzyme very efficiently, preserving its activity for the entire duration of the experiment. Both the deactivated *Harm*AKR and the *bov*AR can be almost completely recovered by the addition of 5 mM dithiothreitol (DTT). Even though these results would require a deeper investigation to univocally interpret the functional differences of the two enzymes, it is clear that *Harm*AKR may also undergo thiol/disulphide interconversion, being more prone than the bovine enzyme to thiols oxidation. The observed protection exerted by GSH on the *Harm*AKR activity may be relevant in determining the *in vivo* redox status of the enzyme.

Molecular modelling

To put our results, and in particular the comparison between the two enzymes, into a structural context, we

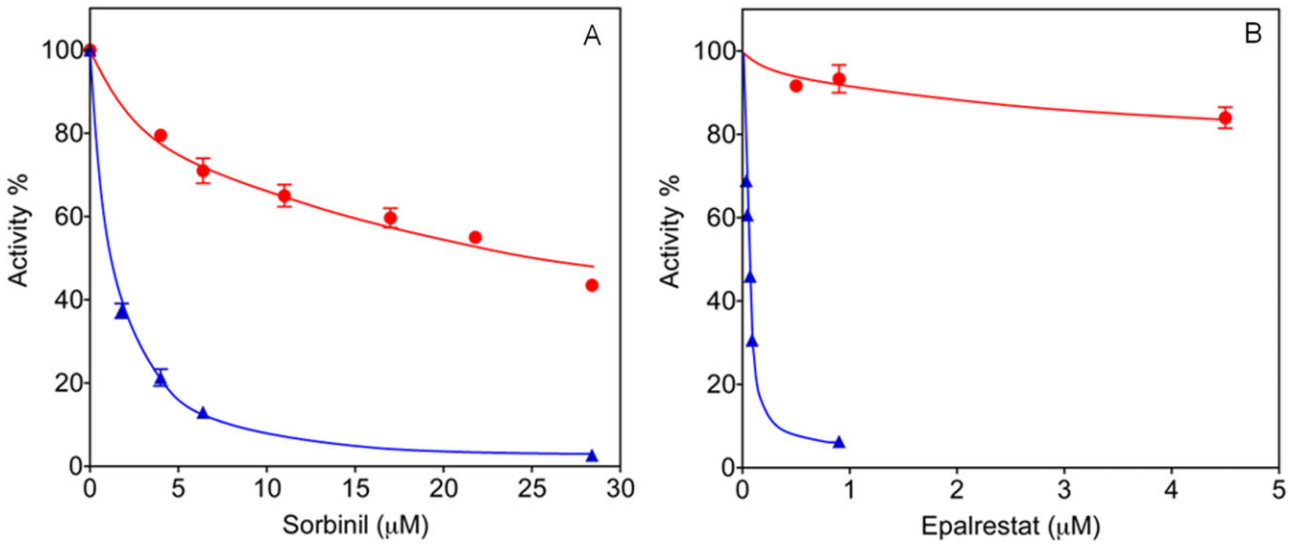

Figure 3. Susceptibility to inhibition of aldo-keto reductase of *Helicoverpa armigera* (*Harm*AKR), by classic aldose reductase (AR) inhibitors. *Harm*AKR (circles) and bovine lens AR (triangles) were assayed for enzyme activity in standard assay conditions using glyceraldehyde as substrate in the presence of the indicated concentrations of sorbinil (panel A) and epalrestat (panel B).

built a model of *Harm*AKR on the crystal structure of the human AKR. Although the two proteins are only 54% identical at the amino acid level, their three-dimensional foldings appear extraordinarily similar (Fig. 5). The most remarkable difference is in the number of cysteines. In fact the human AR presents seven cysteines, in positions 44, 80, 92, 186, 199, 298, 303, all in their reduced form despite the distances between some of them (Cys186–Cys199: 10.9 Å; Cys186–Cys298: 12.8 Å) might be compatible with the establishment of disulphide bridges. Only four cysteines are found in the *H. armigera* enzyme, all in conserved positions (48, 187, 200, 294) in which the close proximity of Cys 187 with Cys 200 (11.2 Å) and Cys 294 (12.3 Å) strongly resembles the disposition of corresponding residues on the human enzyme.

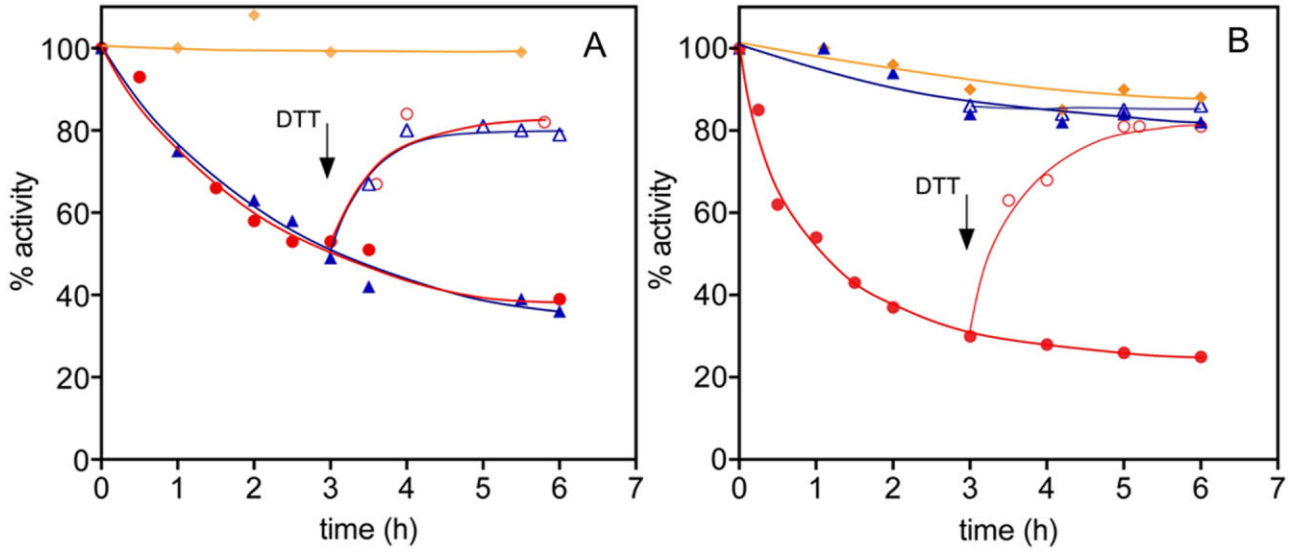

Figure 4. Effect of thiol agents on the activity of aldo-keto reductases (AKRs). AKR of *Helicoverpa amigera* (*Harm*AKR; panel A), and bovine lens AR (panel B) were incubated (3.5 μM) at 37 °C in 10 mM sodium phosphate buffer pH 7.0 in the presence of the following effectors: none (▲), 1.5 mM oxidized glutathione (●), 1.5 mM reduced glutathione (◆). At the time indicated by the arrow 5 mM dithiothreitol was added to aliquots of the mixtures containing no effectors (△) and GSSG (○). The activity (expressed as percent of that measured at zero time of incubation) was assayed using 4.7 mM D,L-glyceraldehyde as substrate. DTT, dithiothreitol.

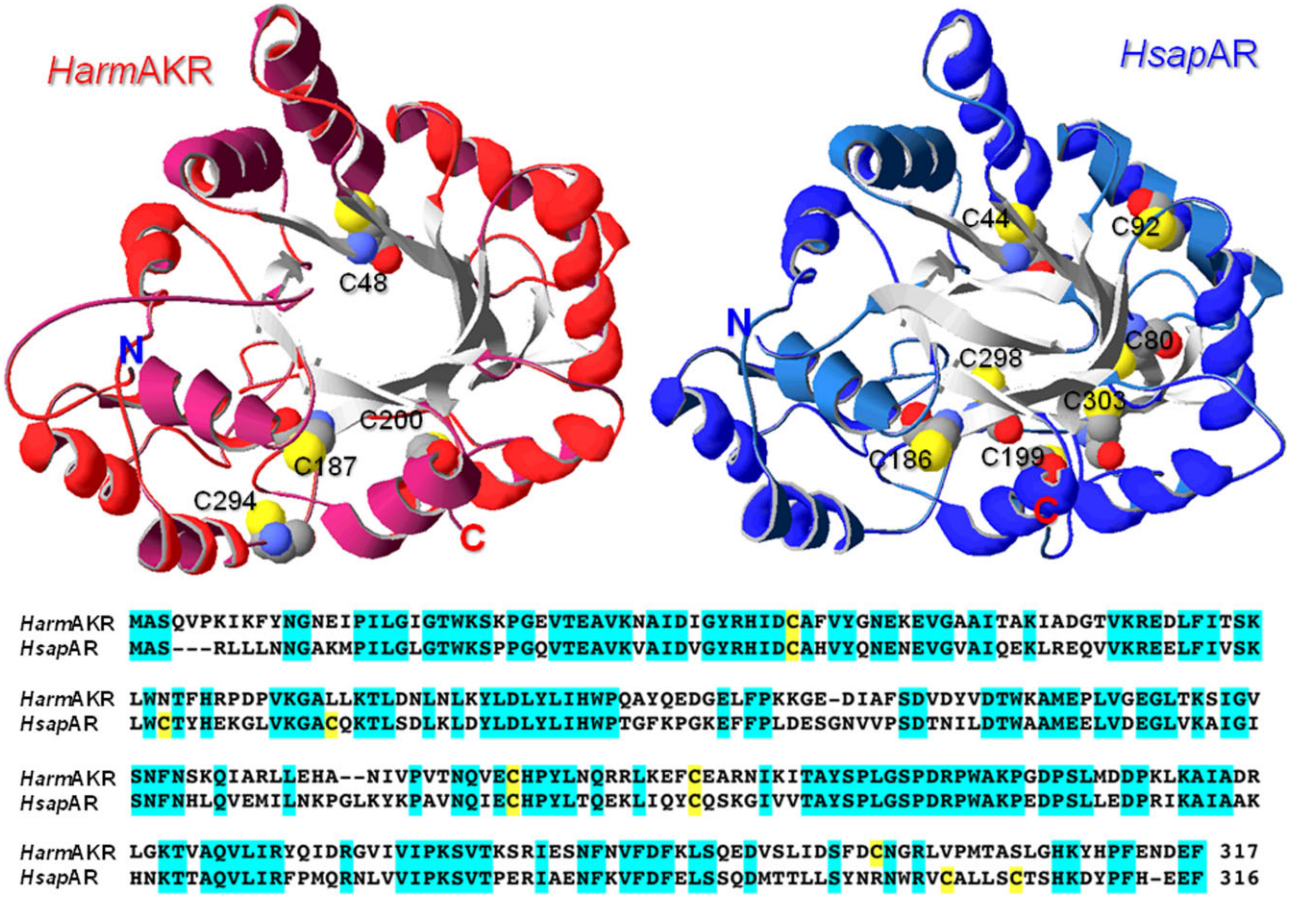

Figure 5. Three-dimensional model of aldo-keto reductase of *Helicoverpa armigera* (*Harm*AKR), built on the structure of human aldose reductase [(AR) PDB acc. 3rx2A]. Although the two proteins only share 54% of their amino acids, they appear remarkably similar in three-dimensional folding. The main difference is in the number of cysteines, seven in the human enzyme (Cys 44, 80, 92, 186, 199, 298, 303), four in *Harm*AKR. All cysteines are in their reduced form despite the distances between some of them (Cys186-Cys199: 10.9 Å; Cys186-Cys298: 12.8 Å) might favour the establishment of disulphide bridges without great energy expenses. *Hsap*AR, *Homo sapiens* aldose reductase.

Summarizing our results, we have described an enzyme that:

1 reduces aldehydes of medium length with broad selectivity;

2 is antennae-rich, being found in the cells at the base of sensilla and in the nerves;

3 is structurally similar to AKRs of mammals, for which a detoxifying role has been convincingly suggested.

Based on these observations, we can certainly leave out a direct and specific action on pheromones, similar to that of 'pheromone-degrading enzymes' reported in several species (Vogt *et al.*, 1985; Rybczynski *et al.*, 1990; Ishida & Leal, 2002; Durand *et al.*, 2012; Leal, 2013); however, the almost exclusive expression in antennae and particularly in the sensillum auxiliary cells strongly suggests a connection between pheromones and this enzyme. We suggest, therefore, that the *Harm*AKR described in the present study is involved in detoxification from breakdown products of pheromones. For a large number of insect species pheromones are 16–20 carbon chains with a functional group in position 1 and one or more double bonds. Degradation of such molecules in α-position relative to the unsaturation produces aldehydes of medium length. Such compounds are toxic for the cell and have to be transformed in safer derivatives, such as the corresponding alcohols.

The ability of the recombinant enzyme to reduce both aldehyde components of the pheromone, revealed by GC/MS experiments (Fig. S6), is not in contrast with the poor activity measured in spectrophotometric assays. In fact, the samples analysed by GC/MS had been incubated for a long time and in the presence of concentrations of the enzyme much higher than in the spectrophotometric assays. We can then conclude that, although pheromone components are not the target substrates for the AKR we have characterized, they can be reduced in extreme conditions.

The enzyme described in the present study, however, is not the only one present in the antennae of *Helicoverpa* species. In fact, we have also detected NADH-dependent reductase activity caused by a different enzyme, whose identity is still unknown and worth investigating.

Experimental procedures

Insects

Helicoverpa armigera and *H. assulta* were collected as larvae from Zhengzhou, Henan, China. The larvae were reared in the laboratory on an artificial diet, the main components of which were wheat germ and tomato paste. Rearing took place at a temperature of 27 ± 1 °C with a photoperiod of 16h light:8h dark. Pupae were sexed and males and females were put into separate cages for eclosion. Adults were fed with 10% honey solution.

Reagents

The enzymes used for molecular biology were from New England Biolabs (Beverly, MA, USA). Oligonucleotides were custom-synthesized at SinoGenoMax, Beijing, China. HNE was from Alexis (San Diego, CA, USA). 2-[(5Z)-5-[(E)-2-methyl-3-phenylprop-2-enylidene]-4-oxo-2-sulfanylidene-1,3-thiazolidin-3-yl] acetic acid (epalrestat) was from Haorui Pharma-Chem Inc. (Edison, NJ, USA). All other chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA) and were of reagent grade.

RNA extraction and cDNA synthesis

Total RNA was extracted from TRI® Reagent (Invitrogen, Carlsbad, CA, USA), following the manufacturer's protocol. The RNA was treated by RQ1 DNase (Promega, Madison, WI, USA) to avoid genomic DNA contamination, followed by purification by phenol, chloroform and isoamyl alcohol (Sangon, Shanghai, China). cDNA was prepared from total RNA by reverse transcription, using 200 units of M-MLV Reverse Transcriptase (Promega) and 0.5 μg of an oligo-dT primer in a 25 μl total volume. The mixture also contained 0.5 mM of each dNTP (TaKaRa, Kyoto, Japan), and 5 μl reaction buffer. The reaction mixture was incubated at 42 °C for 60 min and the product was directly used for PCR amplification or stored at –20 °C.

PCR

Aliquots of 1 μl of crude cDNA were amplified in a Bio-Rad Gene Cycler™ thermocycler, using 2.5 units of Thermus aquaticus DNA polymerase (TaKaRa), 1 mM of each dNTP (TaKaRa), 1 μM of each PCR primer and 2.5 μl reaction buffer.

For the recombinant *Harm*AKR (NCBI acc. no. JF417982), at the 5′ end we used the following specific primer: 5′-CATATGGC TTCTCAAGTGCCTAAA-3′ encoding the first seven amino acids of the protein preceded by an Nde I restriction site. At the 3′ end, the primer: 5′-GAATTCTCAAAACTCATCGTTTTCAAA-3′ contained the sequence encoding the last six amino acids, followed by a stop codon and an *Eco*RI restriction site. After a first denaturation step at 95 °C for 5 min, we performed 35 amplification cycles (1 min at 95 °C, 30 s at 55 °C, 1 min at 72 °C) followed by a final step of 10 min at 72 °C.

Gene expression

In order to approach the expression of *Harm*AKR in different parts of body, a 954-bp length gene fragment was amplified and a 254-bp length of actin was amplified as a reference. The following forward (fw) and reverse (rv) primers were used in the reverse transcription-PCRs: *Harm*AKR, fw5′-ATGGCTTCTCAAGTGCC TAA-3′ and rv5′-TCAAAACTCATCGTTTTCAAAAG-3′; Actin, fw5′-ACCAACTGGGACGACATGGAG-3′ and rv5′-CGTCAGGA TCTTCATGAGGTAGTC-3′. The PCR procedure was as follows: first denaturation step at 95 °C for 5 min, 35 amplification cycles (30 s at 95 °C, 30 s at 55 °C, 1 min at 72 °C) followed by a final step of 10 min at 72 °C.

Cloning and sequencing

The crude PCR products were ligated into a pGEM-T (Promega) vector without further purification, using a 1:5 (plasmid:insert) molar ratio and incubating the mixture overnight at 4 °C. After transformation of *Escherichia coli* Top10 competent cells with the ligation products, positive colonies were selected by PCR using the plasmid's specific primers SP6 and T7, grown in LB/ampicillin medium and custom sequenced at SinoGeno Max, Beijing, China.

Cloning in expression vectors

A pGEM plasmid containing the sequence encoding the protein, flanked by the two restriction sites, was digested with *Nde*I and *Eco*RI restriction enzymes for 2 h at 37 °C and the digestion product was separated on agarose gel. The obtained fragments were purified from gel using a TaKaRa MiniBest Plasmid Purification Kit (TaKaRa) and ligated into the expression vector pET28a, previously linearized with the same enzymes. The resulting plasmid was sequenced and shown to encode the protein.

Preparation of the Harm*AKR protein*

For expression of the recombinant *Harm*AKR, pET-28a vectors containing the sequences encoding the proteins were used to transform BL21 *E. coli* cells that were subsequently cultured in 1 l Luria–Bertani (LB) medium (1% NaCl, 1% tryptone and 0.5% yeast extract). Protein expression was induced by addition of IPTG to a final concentration of 0.4 mM when the culture had reached a value of O.D.600 = 0.8. Cells were grown for further 12 h at 25 °C, then harvested by centrifugation with RCF 2632 g, 4 °C for 10 min (Allegra ™ X-22R, Beckman Coulter, Brea, CA, USA), incubated with lysozyme (final concentration 1 mg/ml) on ice for 30 min and sonicated. After centrifugation with RCF 94833g, 4 °C for 1 h (Avanti ™ J-30 I; Beckman Coulter), the protein was entirely present in the supernatant. Purification was accomplished by affinity chromatography on a HisTrap™ HP column (GE Healthcare, Uppsala, Sweden) along with the protocol provided by the manufacturer. The purified enzyme (5.5 U/mg of protein, measured with 4.7 mM glyceraldehyde as substrate, see below for enzyme assay details) was kept at 4 °C at a protein concentration of 0.13 mg/ml in a saturated solution of ammonium sulphate. No significant changes in activity were observed for at least 4 weeks. The enzyme preparation was dialysed at 4 °C against 10 mM sodium phosphate buffer pH 7.0 before use. One unit of enzyme activity (U) is defined as the amount of the enzyme able to reduce 1 μmole of substrate per min in the standard assay conditions.

Preparation of the antiserum

An antiserum against *Harm*AKR was obtained by injecting a rabbit subcutaneously with 300 μg of the recombinant protein, followed by three additional injections of 200 μg after 7 days each time. The protein was emulsified with an equal volume of Freund's complete adjuvant for the first injection and incomplete adjuvant for further injections. The rabbit was bled one week after the last injection and the serum was used without further purification. The rabbit was housed in a large cage, at constant temperature, and all operations were performed according to ethical guidelines to minimize pain and discomfort to the animal.

Western blot analysis

After electrophoretic separation, performed according to Laemmli (1970) under denaturing conditions (14% SDS-PAGE), duplicate gels were stained with 0.1% Coomassie blue R250 in 10% acetic acid, 20% ethanol or electroblotted on Trans-Blot nitrocellulose membrane (Millipore, Tullagreen, Ireland) according to the procedure described by Kyhse-Andersen (1984). After treatment with 2% powdered skimmed milk/Tris overnight, the membrane was incubated with the crude antiserum against the protein at a dilution of 1:500 (2 h), then with goat anti-(rabbit IgG) horseradish peroxidase conjugate (dilution 1:1000; 1 h). Immunoreacting bands were detected by treatment with 4-chloro-1-naphthol and hydrogen peroxide.

Immunostaining

The antennae, which were dissected from 3- or 4-day old male adults, were immediately fixed by 50 mM Tris-HCl buffer, pH 7.4, containing 4% formaldehyde and 0.5% Triton X-100, for 30 min. The antennae were cut longitudinally along the middle line using a razor blade under a microscope. The sections were incubated in 5% powdered skimmed milk/Tris-HCl buffer overnight. After washing three times/15 min by Tris–HCl buffer, the first antibody was added by dilution 1:200 and incubated for 2 h. Following three times/15 min washes, the sections were treated against goat anti-(rabbit IgG) horseradish peroxidase conjugate (dilution 1:1000; 1h). Immunoreaction was detected by treatment with 4-chloro-1-naphthol and hydrogen peroxide. The pictures were taken using an Olympus S071 microscope.

Detection of AKR activity in antennae

Ten male antennae were homogenized in 400 μl of 50 mM Tris-HCl buffer, pH 7.4. The homogenate was incubated with either 1 mM NADPH or NADH and 250 μM of a 1:1 mixture of the two pheromone components, (*Z*)-9-hexadecenal and (*Z*)-11-hexadecenal, for 1 h at 28 °C, followed by extraction with 400 μl of dichloromethane (Acros Organics, Fair Lawn, NJ, USA). The incubation solution without adding the homogenate was used as the control. The organic phase was concentrated to 20 μl and 2 μl were injected using a splitless technique and analysed with an Agilent Technologies 5975 C MS coupled with an Agilent Technologies 7890 A GC equipped with a polar HP-5 fused silica column (30 m × 0.32 mm ID, 0.25-μm film thickness; J&W Scientific Inc) using the following programme: 50 °C for 5 min; 10 °C/min to 220 °C; 220 °C for 37 min; 5 °C/min to 250 °C; finally held at 250 °C for 5 min. Helium was used as the carrier gas at a constant flow of 1.0 ml/min. The ionization voltage was 70 eV and the emission current was 34.6 mA. The identity of the compounds was confirmed by comparison of their retention times and mass spectra with those obtained injecting authentic samples in the same conditions.

To determine the activity of the recombinant *Harm*AKR, we incubated the solution of the aldehyde (100 μM), NADPH (400 μM) and enzyme (2.8 μM) at 28 °C for 1 h. The GC analytical procedure was as described above except that the GC column used was a DB-WAX column (30 m × 0.25 mm ID, 0.25 μm film thickness, J&W Scientific Inc.). The incubation solution without recombinant AKR was used as the control.

Measurement of enzymatic activity for kinetic characterization

Aldose reductase activity was determined at 37 °C by following the decrease in absorbance at 340 nm linked to the oxidation of the cofactor. The assay mixtures contained, in 0.25 M sodium phosphate buffer pH 6.8, 0.18 mM NADPH, 0.5 mM EDTA, 0.4 M ammonium sulphate, 17–90 nM enzyme and appropriate amounts of different substrates. Reaction rate measurements were performed within 5 min from the addition of the substrate. In particular, in order to measure kinetic parameters of both *Harm*AKR and *bov*AR for different substrates and cofactor, the following ranges of concentration were adopted: from 0.015 to 0.1 mM for linear alkanals; from 0.11 to 0.6 mM for HNE; from 0.2 to 4.7 mM for glyceraldehyde and from 0.04 to 0.18 mM for NADPH. Values relative to the oxidation of NADPH measured in the absence of the substrate were subtracted. K_M and k_{cat} values were determined by linear regression analysis of kinetic data of double reciprocal plots using GraphPad Software.

Purification of bovine lens aldose reductase

The *bov*AR was purified to electrophoretic homogeneity as previously described (Del Corso *et al.*, 1990); the pure enzyme displays a specific activity of 1.2 U/mg of protein, using D,L-glyceraldehyde as substrate. One unit of enzyme activity (U) is defined as the amount of the enzyme able to reduce 1 μmole of substrate per min in the standard assay conditions.

Analysis of sequences

Sequences of AKRs were searched using Blast and HarmAKR as a query against NCBI databases. Both pairwise and multiple alignments were performed with Clustal-W2 using a Blosum matrix and the following parameters: gap open 10, gap extension 0.1. The similarity tree was constructed based on neighbour joining and processed with FigTree v1.4.0 (http://www.expasy.org/spdbv/).

Molecular modelling

The three-dimensional model of *Harm*AKR was generated using the online programme swiss model (Guex & Peitsch, 1997; Schwede *et al.*, 2003; Arnold *et al.*, 2006) and the structure of human AR (PDB acc. 3rx2A, identity 54%) as template. Models were displayed using the SwissPdb Viewer programme 'DeepView' (Guex & Peitsch, 1997) (http://www.expasy.org/spdbv/).

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. Alignment of selected sequences of insect aldo-keto reductases from species whose genomes have been sequenced. Despite high overall similarity (identical amino acids: 58–84%) sequences greatly differ by the number of cysteines (4–7). Residues identical in all the sequences are highlighted in blue, cysteines in yellow. Abbreviations and accession numbers are as in Fig. S2.

Figure S2. Similarity tree of sequences of aldo-keto reductases (AKRs) from different species. Insects in black, vertebrates in red, non-insect arthropods in blue, others in green. Vertebrates and insects AKRs segregate into two distinct groups, but sequence similarity does not correlate with phylogenetic distance within each group. Abbreviations are as follows (NCBI acc. numbers in parentheses): Harm: *Helicoverpa armigera* (AEB26313); Dple: *Danaus plexippus* (EHJ71423); Aaeg: *Aedes aegypti* (XP_001648461); Mrot: *Megachile rotundata* (XM_003708536); Agam: *Anopheles gambiae* (XP_308086); Dpon: *Dendroctonus ponderosae* (BT128266); Bimp: *Bombus impatiens* (XM_003484742); Tcas: *Tribolium castaneum* (XP_969456); Cflo: *Camponotus floridanus* (EFN69299); Amel: *Apis mellifera* (XP_624353); Aech: *Acromyrmex echinatior* (EGI65078); Nvit: *Nasonia vitripennis* (NM_001159939); Gmor: *Glossina morsitans* (ADD20387); Cqui: *Culex quinquefasciatus* (XM_001844786); Apis: *Acyrthosiphon pisum* (NP_001155638); Rfla: *Reticulitermes flavipes* (JX513906); Bmor: *Bombyx mori* (ADQ89807); Dpul: *Daphnia pulex* (EFX86055); Rpul: *Rhipicephalus pulchellus* (JAA57295); Sinv: *Solenopsis invicta* (EFZ11360); Cint: *Ciona intestinalis* (XM_002127698); Hsal: *Harpegnathos saltator* (EFN78750); Iric: *Ixodes ricinus* (JAA69488); Amac: *Amblyomma maculatum* (AEO34008); Phum: *Pediculus humanus* (XM_002427630); Acar: *Anolis carolinensis* (XM_003220809); Oana: *Ornithorhynchus anatinus* (XM_001512073); Bflo: *Branchiostoma floridae* (XM_002598964); Panu: *Papio anubis* (XM_003896622); Cgri: *Cricetulus griseus* (XM_003503181); Ocun: *Oryctolagus cuniculus* (NP_001075756); Cjac: *Callithrix jacchus* (XP_002752015); Fcat: *Felis catus* (XM_003983075); Hgla: *Heterocephalus glaber* (XM_004892622); Cpor: *Cavia porcellus* (XM_003467875); Clup: *Canis lupus* (NM_001252416); Ecab: *Equus caballus* (XP_001500782); Ggal: *Gallus gallus* (NP_989960); Mmus: *Mus musculus* (EDK98457); Drer: *Danio rerio* (AAH78366); Hsap: *Homo sapiens* (3rx2A); Aimel: *Ailuropoda melanoleuca* (XP_002927549).

Figure S3. Bacterial expression and purification of *Helicoverpa amigera* aldo-keto reductase (*Harm*AKR). The protein was abundantly expressed (20 mg/l) after induction with IPTG (Ind), as compared to the pre-induction sample (Pre), and entirely present in the supernatant (SN). Purification was accomplished by affinity chromatography on Ni-containing columns (His-trap). Western blot immunostaining with an antiserum against the His-tag (WB) revealed a single band at the expected molecular weight. Molecular mass markers (M) are, from the top, 66, 45, 29, 20 and 14 kDa.

Figure S4. Expression of *Helicoverpa amigera* aldo-keto reductase (*Harm*AKR) in different parts of the body. (A): antennae, (P): proboscis, (L): legs, (W): wings of males (m) and females (f), (G): pheromone glands. PCR experiments revealed selective expression in the antennae of both sexes, although weak bands were obtained with cDNA from other tissues. Internal standard is actin. Western blot analysis also revealed antennal-biased expression, but all through the body significant cross-reactivity was observed. Molecular weight markers for sodium dodecyl sulphate polyacrylamide gel electrophoresis were as in Fig. S3.

Figure S5. Expression of *Helicoverpa amigera* aldo-keto reductase (*Harm*AKR) during development revealed by Western blot experiments. (1–8): the days of moths after emergence; (E): eggs; (L1–L3): the different instars of larvae; (P1–P14): the days after pupation. The protein was detected in antennae at all ages of adult life, as well as in eggs, larvae and pupae. Molecular weight markers for sodium dodecyl sulphate polyacrylamide gel electrophoresis were as in Fig. S3.

Figure S6. Reduction of (Z)-9-hexadecenal by recombinant aldo-keto reductase (AKR) of *H. armigera*. Top panel. Gas chromatographic profile of incubation solution with recombinant AKR. Similar results were obtained with the other pheromone component (Z)-11-hexadecenal. Lower panel. Gas chromatographic profile of incubation solution without recombinant AKR. In the absence of recombinant protein, no corresponding alcohol was detected after the incubation.