CHITIN METABOLIZING ENZYMES IN INSECTS

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INTRODUCTION

Chitin is an essential component of the insect cuticle and is present in the gut, peritrophic membrane and trachea. The concentration of chitin, the orientation of chitin fibres within the exoskeleton and the degree of cross-linking determine the mechanical properties of the cuticle to a considerable extent. Since the exoskeleton is shed and rebuilt in a cyclic manner during moulting cycles, a hormonal regulation of chitin metabolism in arthropods is expected. Chitin is degraded mainly during pre-moult, chitin synthesis takes place preferentially during post-moult. An overlap of both processes occurs during pre-moult, when a new thin cuticle is synthesized before eclosion. The different properties of larval, pupal and adult cuticle require the adaptation of chitin metabolism according to the developmental stage. The involvement of chitin degradation in nutrition leads to tissue specific differences between midgut/ hepatopancreas and epidermis (Spindler-Barth et al., 1990).

Our own studies mainly focused on the epithelial cell line from *Chironomus tentans*. Morphology, enzyme pattern and reaction to moulting hormones (Spindler-Barth et al., 1992; Spindler-Barth and Spindler, 1998) of this cell line correspond essentially to epidermal / imaginal tissue. Cell lines offer the advantage that, in contrast to animal tissue, there is no contamination by fungi, bacteria and protozoa, all containing chitinolytic enzymes. The cells degrade ecdysteroids only very slowly, which allows long term incubations under defined hormonally controlled conditions (Spindler and Spindler-Barth, 1991).

Keywords: Chitinase, chitin synthase, Chironomus tentans, inhibitors

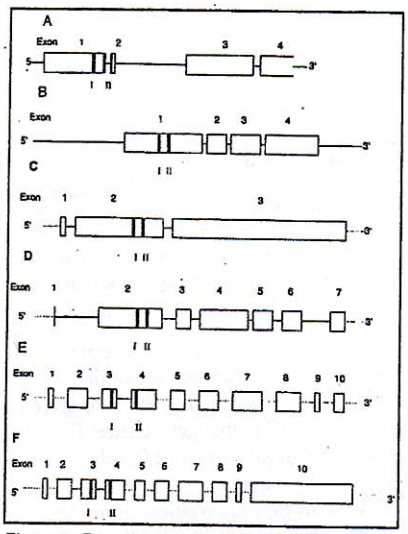

Figure 1. Genomic organisation of chitinase genes from A: Chironomus tentans (3 400 bp), B: Aedes aegypti (accession number AF026491; 3 182 bp), C: Drosophila melanogaster (flybase CG9357; 4 680 bp), D: Drosophila melanogaster (fly base CG9307; 3 000 bp), E: Manduca sexta (accession number L49234; 15632 bp), F: Bombyx mori (accession number AB048355; 15 909 bp). The vertical black bars represent the highly conserved regions I and II.

CHITINASE

The essential role of chitin degrading enzymes during insect life is described previously (Spindler, 1983; Kramer et al., 1985; Kramer and Koga, 1986).

A) Genomic organisation

Only one chitinase gene was determined in *Chironomus* with Southern blots. This was confirmed by *in situ* hybridisation (Feix et al., 2000b), which revealed only one locus for chitinase in *Chironomus tentans*. Only one chitinase gene was identified in *Manduca sexta* (Choi et al., 1997) and *Bombyx mori* (Abdel-Banat and Kogg, 2001) also. In contrast, in *Aedes aegypti* there are four genes coding for chitinase (de la Vega et al., 1998). In the *Drosophila melanogaster* database, we have found seven putative chitinase genes. These genes are distributed all over the *Drosophila* genome and differ remarkably in their genomic organisation (Figure 1). All of them contain the highly conserved regions I and II located in exon two. Biochemically, chitinase is characterised from *Drosophila* species (Spindler, 1976) and cells (Boden et al., 1985), but no correlation with a specific chitinase gene exists so far. It is unknown, whether all seven genes are expressed and code for functional chitinases.

The genomic organisation of chitinases from diptera and lepidoptera varies considerably in length and number of exons and introns (Figure 1). Dipteran genes are much shorter (3000 to 4700 bp) than the lepidopteran ones (15000 bp). Lepidopteran chitinase genes possess more exons (*Bombyx* 11, *Manduca* 10) than dipteran ones (*Aedes* 4, *Chironomus* 4), with the exception of *Drosophila*, which has both variants. Only in the chitinase gene of *C. tentans* the catalytic centre (conserved region II) is split by an intron.

The chitinase gene of *C. tentans* (Figure 2) consists of four exons between 13 and 620bp in length (Table 1). All intron/exon boundaries within the chitinase gene from *C. tentans* (Table 1) are in agreement with the gt/ag rule (Breathnach et al., 1978). Exon one encodes the 5′ untranslated region, the signal peptide, the conserved region I and part of the conserved region II, which codes for the catalytic centre. Within the very short exon two, there is the remaining part of the conserved region II.

Table 1. Exon-intron boundaries of the chitinase gene from *Chironomus tentans* with sizes of introns and exons. Exon sequences are shown in capital letters, introns are in lowercase.

Exon No.	Exon size (bp)	sequence at exon-intron junctions		intron size (bp)	codon interrupted
		5′-splice donor	3′-splice donor		
1	>600	TGGGAgtaaa.....	.gtttagGTATC	68	Glu-158
2	13	ACTCAGgtgag.....	.tacagAGAGG	637	-
3	620	GGTCTGgtaag.....	.tttaagGTCGG	64	Trp-369
4	>319				

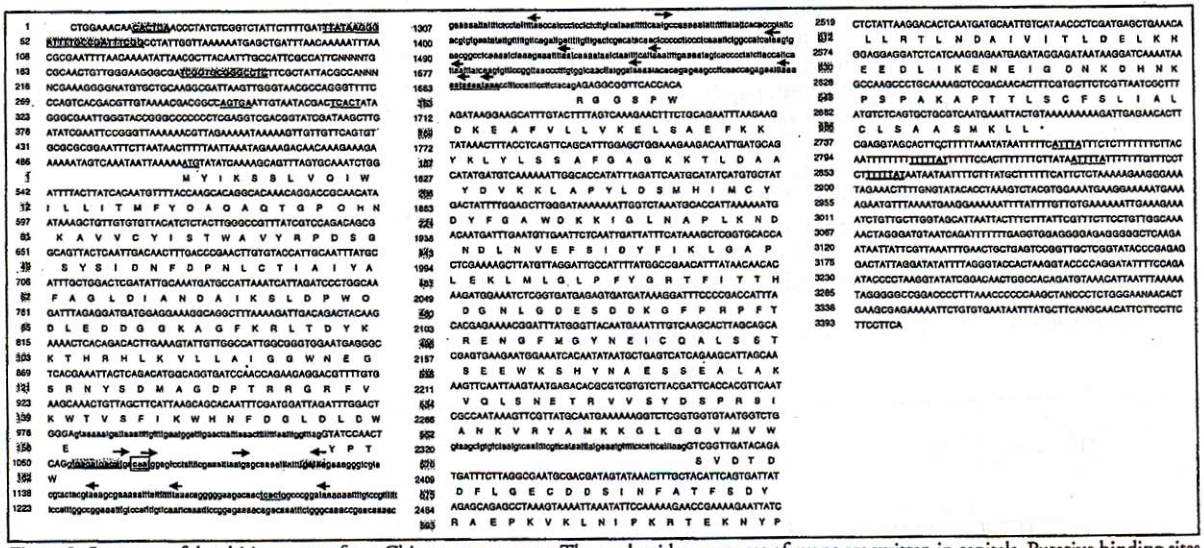

Figure 2: Sequence of the chitinase gene from Chironomus tentans. The nucleotide sequences of exons are written in capitals. Putative binding sites for the transcription factors zeste, gcm and GATA are in grey boxes, the caat-box is boxed, cap site consensus sequences, polyadenylation signals, putative initiation (←) and termination (→) signals within intron two. Genomic DNA from the epithelial cell line of Chironomus tentans was prepared by standard methods (Ausubel et al.,1995). A library of genomic DNA from the epithelial cell line of C. tentans cloned in λ DASH II (Stratagene, La Jolla) was screened with a 869 bp fragment of the chitinase cDNA [α ^{32}P]-dCTP labelled by nick translation (Boehringer, Mannheim). Plaque lifts were performed according to the prescription of the manufacturer. In addition, genomic DNA was analysed by PCR using various primers, which are available upon request.

Intron two of the *Chironomus tentans* chitinase gene may be derived from a transposable element. Several putative open reading frames (ORFs) are present. The longest one codes for a peptide of 40 amino acids. We found considerable sequence similarity (89 – 100%) to the central region of the *Chironomus thummi* foldback transposable element (Hankeln and Schmidt, 1990), to an intron sequence of the *C. tentans* sp 240/420 gene (X70773) and to a noncoding region of the *C. thummi* 7B globin gene cluster (U07703) (Figure 3). At the 5′-end of intron two, there are zeste binding sites (Benson and Pirrotta, 1988), known to be involved in gene regulation, a GATA motif (Kadalayil et al., 1997), a caat-box, and an arthropod capsite consensus sequence TCACT (Cherbas and Cherbas, 1993), which can act as a facultative promoter element. In the 3′ region of intron two the polyadenylation signal AATAAA is present (Proudfoot, 1991). Thus, all elements for a functional gene are found in intron two. Nevertheless, it is unlikely that intron two (637 nucleotides) is still a functional transposable element, since it is neither flanked by terminally inverted repeats nor does it contain an ORF coding for a transposase. Mikitani et al. (2000) also described a transposable element within an intron of the *Bombyx mori* chitinase gene. In both cases the biological significance of this intron is still unknown.

A search for transcription factor binding sites (Transfac; Heinemeyer et al., 1998) by sequence alignment of the non transcribed 5′-end revealed at least 3 gcm-like motifs, which indicate DNA binding and may have transcriptional regulatory activity (Akiyama et al., 1996). A binding site for the zeste protein, which is involved in gene regulation (Benson et al., 1988) is also present in the *C. tentans* chitinase gene. A TATA box was not found in the *C. tentans* sequence, but instead it contains the pentamer TCACT. This element can serve as a facultative promoter element and is normally located -10bp and +10bp from the transcription start point

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A)  a: 324  atattcacaccgtattcacgtgtgaatatattgttttttgttcagattga 372
    b: 3989 atattcacaccgtattcacgtgtgaatttatttttttgttcagattga 4037

B)  a: 324  atattcacaccgtattcacgtgtgaatatattgttt 359
    b: 4136 atattcacaccgtattcacgtgtgaatatattgttt 4101

C)  c: 326 attcacaccgtattcacgtgtgaatatattgtttttgttcagat 369
    b: 638 attcacaccgtttcacgtgtgaatcattggttttgttcagat 595

D)  a: 324  atattcacaccgtattcacgtgtgaatatattgttttgttcagattga 372
    d: 5492 atattaacaccgttttcacgtgtgaatccattggttttgttcagattga 5540
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Figure 3. Sequence alignments of intron two from the *Chironomus tentans* chitinase gene with introns of other *Chironomus* genes. Sequence identities: A and C = 93%, B = 100%, D = 89%. Grey areas represent deviations between the two genes. Introns were compared with the blast program (Altschul et al.,1990).
a = *Chironomus tentans* Sp240/420 gene
b = *Chironomus tentans* chitinase gene, intron 2
c = *Chironomus thummi* histone H1, H2A, H2B, H3, H4 genes with TFB1
d = *Chironomus thummi* 7B globin gene cluster

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  1 mtifygagaq tgpghNKAVV CYISTWAVYR Pdsgsysidn fdPNLCTIAI YAFAGL---D

                157-GKVVG SYFVEWGVYG R---NFTVDK IPAQNLTHLL YGFIPICGGN

  IANDAIKSLD ---------- ---------- ---------- -pwqdleddG GKAGFKRLTD

199 GINDSLKEIE GSFQALQRSC QGREDFKISI HDPFAALQKA QKGVTAWDDP YKGNFGQLMA

  YKKTHRHLKV LLAIGGWNEG SRnysdmAGD PTRRGRFVKQ TVSFIKQHN- FDGLDLDWEY

259 LKQAHPDLKI LPSIGGWTLS DPFF--FMGD KVKRDRFVGS VKEFLQTWKF FDGVDIDWEF

  PTQrg----- GSPQDKEAFV LLVKELSAEF KKYK------ LYLSSAFGAG KKTIDAAYD

317 PGGKGANPNL GSPQDGETYV LLMKELRAML DQLSTETGRK YELTSAISAG KDKIDK-VA

  VKKLAPYLDS MHIMCYDYFG AWD-KKIGLN APLKN---- DNDLNVEFST DYFTKLGAPL

375 YNVAQNSMDH IFLMSYDFYG AFDLKNLGHQ TALNAPAWKP DTAYTTVNGV NALLAQGVKP

  EKLMLGLPFY GRTFI--ttq dgnlgdesdd kgfpgpFTRE NGFMGYNEIC qalsstseew

435 GKIVVGTAMY GRGWTGVNGY QNNIPFTGTA TG-PVKGTWE NGIVDYRQI- -AGQFMSGEW

  ksqyhaesse alakvqlsne TRVVSYDSPR SIANKVRYAM KKGLGGVMVW SVDTDdfl

492 --QYTYDATA EAPYVFKPST GDLITFDDAR SVQAKGKYVL DKQLGGLFSW EIDADN--

  gecddsinfa tfdyraepkv klnipkrtek nYPLLRTLND AIVITLDelk qeedlikene

546 ---------- ---------- ---------- -GDILNSMNA SLGNSAG*

  iqdnkdqnkp spakapttls cfsliaicls aasmkll*
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Figure 4: Sequence alignment of the *Serratia marcescens* and *Chironomus tentans* (gray background) chitinase used for modelling. Parts shown in lower case letters were not modelled.

(Cherbas and Cherbas, 1993). Fifteen nucleotides upstream, there is another TCACT element located on the minus strand, indicating a putative transcription start point of the chitinase gene.

In the 3'-untranslated region AU-rich elements, known to regulate mRNA stability, and adenylate/uridylate-rich elements (ARE) comprising AUUUUA, AUUUA motifs, which represent binding sites for proteins that mediate mRNA degradation (Chen and Shyu, 1995) are present. Two cytoplasmic adenylation control elements UUUUUAU (Jackson, 1993), but no typical polyadenylation signal (AATAAA) were found (Proudfoot, 1991).

B) Protein structure and function

In contrast to chitinases from other taxa, no X-ray analysis of the 3 D structure of an insect chitinase is available so far. A 3D model of *Chironomus tentans* (Ct) chitinase is based on the experimentally determined structure of the *Serratia marcescens* enzyme (Perrakis et al., 1994). Sander and Schneider (1991) have proposed that any two sequences displaying more than 27% identity over at least 80 residues, share a common structure. The level of similarity in the present case (34% identity) is higher than this threshold, implying a common structure. A simple, full-length alignment between these two sequences is insufficient in the present context, for two reasons: first, many insertions-deletions are required; and second, alternative sub-alignments are possible for some parts, both impairing the accuracy of this approach considerably. The possibility for alternative sub-alignments remained, even in a multiple alignment, which incorporated two closely related viral homologues (about 60% identical) to chitinase from *Serratia marcescens*, found in *Autographa californica* NPV (SwissProt: CHIT_NPVAC) and *Orgyia pseudotsugata* NPV (SwissProt: CHIT_NPVOP). Direct diagonal plot techniques identified a few "islands" of similarity, all together about 120 residues. Using these segments as "anchors", the alignment could be inferred for a few more residues in the less similar areas in between, but the missing parts are still unacceptably long. Some of the gaps were corrected manually to conform to the secondary structure of the *Serratia marcescens* chitinase. Figure 4 displays the final alignment in detail.

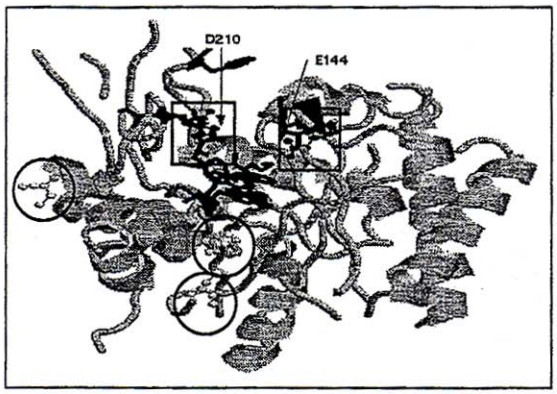

Figure 5. Model structure of the Chironomus tentans chitinase. The proposed amino acids involved in hydrolysis of chitin (E144 and D210) are shown in boxes. They are surrounded by conserved amino acids that apparently form the chitin recognition site (black). Cysteines appear in circles.

The final model structure was predicted computationally using the WHAT IF Homology based molecular modelling and drug design program (Vriend, 1990). 113 identities of about 360 residues of common length (approx. 34%) afforded a multiple alignment to disambiguate the correspondence between the two sequences. The first 15 amino acid residues of *Chironomus tentans* chitinase were not modelled. The last 16 residues of the sequence, probably corresponding to the 8th helix of the $(\alpha\beta)_8$-barrel, were aligned slightly better at the proposed position rather than without, leaving a long intervening loop. The overall topology of the structure of *Chironomus tentans* chitinase is essentially a TIM-barrel with well defined helices and ß-sheets. The connecting loops have not been included, since they can not be properly modelled.

Figure 5 presents the model structure of a significant part of the *Chironomus tentans* chitinase molecule as described above. The proposed amino acids that are involved in the hydrolysis of chitin have been identified (E144 and D210) in the modelled structure. The catalytic amino acids are positioned just inside a cleft formed at the site of the TIM-barrel and are surrounded by conserved amino acids that apparently form the chitin recognition site.

Table 2: Comparison of the putative chitin binding subsites of *Chironomus tentans* and the experimentally defined chitin binding subsites of *Serratia marcescens* chitinase.

Subsite	*Serratia marcescens*	*Chironomus tentans*
-6	solvent	-
-5	Y170	Y29
-4	R172	P31
-3	T276, E473,W167	N104, E285, W26
-2	E540, E473, W539, W275	S356, E285, W355, W103
-1	D391, M388, W539, W275, Y390	D210, M207, W355, W103, Y209
+1	R446 D391opposite to the acidic catalytic residue E315 catalytic residue W275	R259 D210 opposite to the acidiccatalytic residue E144 catalytic residue W103
+2	K369, D391, F396	K186, D210, W215

The following table (Table 2) compares the putative chitin binding subsites of *Chironomus tentans* and the experimentally defined chitin binding subsites of *Serratia marcescens* chitinase. 17 of 21 amino acids involved in chitin binding are identical in *Chironomus* and *Serratia* and demonstrate that not only the catalytic site but also the chitin binding domain is highly conserved. For *Manduca sexta* chitinase it was shown that the chitin binding domain is in fact functional (Arakane et al., 2003).

A Glu/Pro rich region (Pest sequence), which is considered as a putative regulatory site for proteolytic attack was found in the C-terminal end of the protein. Putative phosphorylation and N-glycosylation sites were also present (Feix et al., 2000b). The secreted *Chironomus* enzyme is in fact glycosylated, as demonstrated by lectin binding. Both wheat germ agglutinin and concanavalin A coupled to sepharose retained enzymatic activity, which was eluted from the matrix by the corresponding sugars N-acetylglucosamine and α-methylmannoside (Figure 6).

In all arthropod chitinases tested so far (Spindler and Spindler-Barth, 1999), blockage of free SH-groups with *N*-ethylmaleinimide was without effect on enzymatic activity but enzymatic activity was abolished after addition of mercaptoethanol (Figure 7) indicating

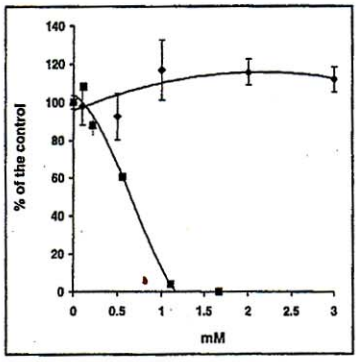

Figure 6. The influence N-ethylmaleinimide and mercaptoethanol on chitinase activity from the epithelial cell line of *Chironomus tentans*. Data were corrected for the quench, especially by mercaptoethanol.

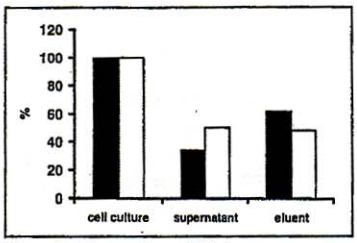

Figure 7. Absorption of *Chironomus tentans* chitinase to lectin-sepharoses. The enzyme was bound to WGA-, resp. Con A-sepharose and eluted by 1 M N-acetylglucosamine and 1 M a-mannoside.

that S-S-bridges are essential for enzyme activity. According to our model Cys21 and Cys46 have the potential to form a disulfide bridge. The cysteines in positions 366 and 445 might be also be good candidates, since they are highly conserved (Feix et al., 2000a) not only in insects, but also in all arthropods and nematodes.

C) Hormonal regulation

In various insect species a positive correlation between moulting hormone titre and chitinase activity has been demonstrated, but the molecular mechanisms of hormone action are only poorly investigated.

Chironomus tentans cells secrete chitin degrading enzymes into the culture medium. Chitinase and N-acetylglucosaminidase activities increase moderately in the presence of 20-OH-ecdysone (0.1 µM or higher), although the effect on N-acetylglucosaminidase is less pronounced (1.6 fold) (Figure 8a). Only about 6% of the chitinase activity remains within the cells and the intracellular level of enzyme activity is not changed by 20-OH-ecdysone. In contrast, the major fraction of N-acetyl-ß-D-glucosaminidase activity is found within the cell (Figure 8b, c). There is a pronounced steroid specificity, which corresponds to the affinity of the hormone to the ecdysteroid receptor (Spindler-Barth, 1993; Quack et al., 1995): vertebrate steroid hormones are without any effect, the biologically less active "prohormone" ecdysone has only a weak influence, compared to 20-OH-ecdysone and the nonsteroidal moulting hormone agonist RH 5992.

In *Manduca sexta* (Kramer et al., 1993) and *Ephestia cautella* (Spindler-Barth et al., 1986) juvenile hormone reduces the effect of moulting hormones. In the *Chironomus tentans* cell line juvenile hormone III and the juvenoid fenoxycarb (data not shown) in increasing concentrations up to 5 µM have no effect, although juvenile hormone (JH) modifies cell proliferation in the *Chironomus* cells (Wyss, 1982; Kissenbeck, personal communication). Chitinase has to partially degrade the old cuticle independent of whether a larval-larval, a larval-pupal or a pupal-adult moult occurs. In all cases the titre of moulting hormones rises before moulting but the titres of juvenile hormone vary according to the developmental stage. Since the interactions between JH and ecdysteroids are dependent on species, developmental stage and tissue, contradictory results are not unexpected.

A slight increase in chitinase transcript concentration is seen after addition of 1 µM 20-OH-ecdysone to the *Chironomus tentans* cell line after three days (Figure 9). It is unknown so far, whether or not the hormone changes transcript levels directly. A more indirect effect seems likely, since the length of the poly A tail of the chitinase mRNA decreases with time (Figure 10), which might influence transcript stability.

CHITIN SYNTHASES

In contrast to chitin synthases in yeasts the corresponding enzymes in insects are only poorly investigated, despite their physiological importance. This is mainly due to loss of enzyme activity after extraction of the membrane bound enzyme with even very mild detergents (Ludwig et al. 1991).

A) Chitin synthase gene

Using the conserved sequence of the catalytic site QRRRW, fragments coding for parts of two chitin synthase enzymes were cloned from *Drosophila melanogaster* (Gagou et al., 2002) and chitin synthase specific cDNA clones were isolated from several insect pests like *Lucilia cuprina* (Tellam et al., 2000), *Aedes Aegyptii* (Ibrahim et al., 2000) and *Manduca sexta* (Zhu, et al., 2002). The sequence identity to yeast enzymes is only modest, but the overall organisation of the chitin synthase genes seems similar in both taxa. Although the molecular weight varies considerably between insects (180.7 KDa, in *Lucilia*, 99.5KDa in *Aedes*), three domains can be distinguished as in yeasts and fungi: An N-terminal region A, a catalytic domain B and a C-terminal region C. A number of putative transmembrane sites (15-18 in *Lucilia*) were identified. Alternative splice sites may indicate the presence of isoenzymes in some species; two different chitin synthase genes were identified by sequence alignments in the genome of *Drosophila* and by Southern blots in *Manduca* (Zimoch and Merzendorfer, 2002). Immunostaining showed either localization in the midgut of *Manduca* (Zimoch and Merzendorfer, 2002) or epithelial cells of *Manduca* (Zhu et al., 2002).

B) Protein structure and function

From crude extracts of the epithelial cell line of *Chironomus tentans* two enzyme activities with different localization within the same cells were separated by ultracentrifugation in a sucrose gradient (Figure 11). As expected, one isoenzyme colocalizes with the apical cell membrane, according to marker enzymes. A second chitin synthase activity is present in the microsomal fraction. Different

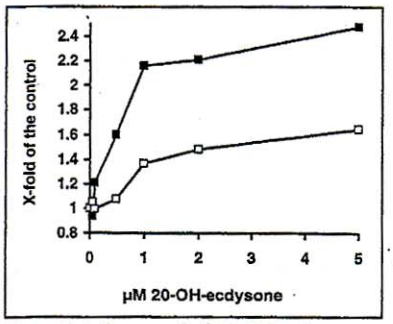

Figure 8. Influence of of 20-OH-ecdysone on chitinase (■) and N-acetyl-ß-D glucosaminidase (□) from *Chironomus tentans* as a total (a), in the cells (b) and in the medium (c). Cells were incubated with the hormone for 7 days (means ± SD, n = 4-6). Enzymatic activity was determined with a microfluorimetric assay, based on the enzymatic degradation of 4 methylumbelliferyl-derivatives of either N-acetyl-ß-D-glucosaminide (MUF-GlcNAc; Sigma, Deisenhofen) or N,N',N"-triacetyl-chitotrioside (MUF-chitotriose; Calbiochem, Soden) as described by Spindler (1997). Protein was determined according to Bradford (1976) using bovine serum albumin as standard.

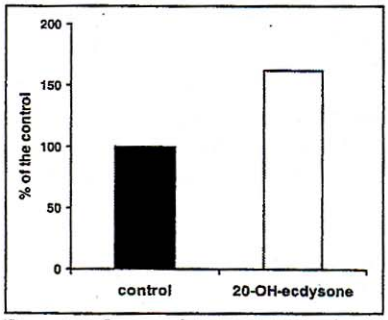

Figure 9. Influence of 1 µM 20-OH-ecdysone (incubation time: 3 days) on *Chironomus tentans* chitinase mRNA (means ± SD, n = 3).

sensitivities against low concentrations of polyoxin D (Figure 12) indicate that not only the localization, but also functional properties vary between both isoenzymes.

In yeasts chitin synthase is associated with chitosomes (Kamada et al., 1991). The molecular weight of insect chitin is much higher and chitin molecules may consist of several thousand monomers. It seems unlikely that these high molecular weight fibres are handled within the cell. The selective inhibition of chitin synthesis in arthropods by benzoylphenylureas and tunicamycin indicates that the molecular mechanisms varies between yeasts and insects, although the catalytic site of chitin synthase is rather conserved.

In vertebrates tunicamycin inhibits an early step in glycoprotein synthesis: addition of N-acetylglucosamine to dolicholphosphate, which takes place in the endoplasmatic reticulum. Dolichophosphate coupled N-acetylglucosamine oligomers were found in *Artemia* (Horst, 1983; Horst and Walker, 1993) and a vesicular transport of N-acetylglucosamine containing material from the endoplasmatic reticulum to the cell membranes, where high molecular weight chitin is synthesized, was demonstrated by autoradiography. Although not proven definitely this two step model of chitin synthesis is in agreement with several experimental data obtained with insect tissues:

1. Localization of chitin synthase isoenzymes in microsomal fraction and apical cell membrane of *Chironomus* cells.
2. Inhibition of chitin synthesis in insects by tunicamycin.
3. Stimulation of chitin synthesis by addition of dolicholphosphate in the presence of ATP (Table 3).
4. Inhibition of vesicular transport of precursor molecules by benzoylphenylureas (Londershausen et al., 1997) and intracellular accumulation of precursor molecules for chitin synthesis. Although the molecular mechanism of inhibition is not yet known and additional target sites for benzoylphenylureas are described, a direct action on chitin synthesis seems unlikely, since the integrity of the target cells is necessary for inhibition.
5. UDP-N-acetylglucosamine incorporation by benzoylphenylureas is inhibited only partially in *Chironomus* cells (Spindler-Barth et al., 1989a), even at high inhibitor concentrations, which may indicate a benzolyphenylurea-sensitive and insensitive mode of N-acetylglucosamine incorporation.

C) Hormonal regulation

An ecdysone responsive element is present in the promoter region of the chitin synthase gene of *Lucilia* (Tellham et al., 2000). This was expected since numerous reports exist on hormonal regulation of chitin synthase activity in various tissues. Both stimulation and inhibition by 20-OH- ecdysone are described (Spindler-Barth, 1993) and may vary according to animal species, developmental stage, tissue and moulting cycle. Before moult, when the ecdysteroid titre is increasing or high, only relatively small amounts of chitin are synthesized to deliver a protective layer, when the old cuticle is degraded partially. Shortly before moult the ecdysteroid titre declines rapidly. During moult the new exoskeleton is rather weak to facilitate eclosion. After moult, when the hormone titre is

Table 3: Influence of dolichol phosphate and ATP on ^{3}H-N-acetylglucosamine incorporation into chitin in the epithelial cell line from *Chironomus tentans*.

Treatment	Incorporation (in% of the control)	n =
control	100.0 ± 11.8	10
DMSO (1%)	97.3 ± 7.3	8
30 µM dolichol phosphate, DMSO (1%)	102.8 ± 8.2	5
30 µM ATP	105.6 ± 17.4	3
30 µM dolichol phosphate, DMSO (1%), 30 µM ATP	153.5 ± 15.1	3

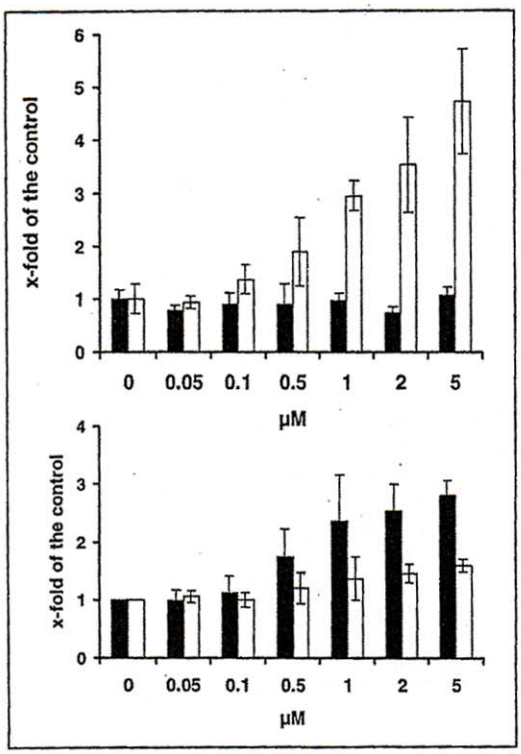

Figure 10. Influence of 1 µM 20-OH-ecdysone on the length of the poly A tail from Chironomus tentans chitinase mRNA (means

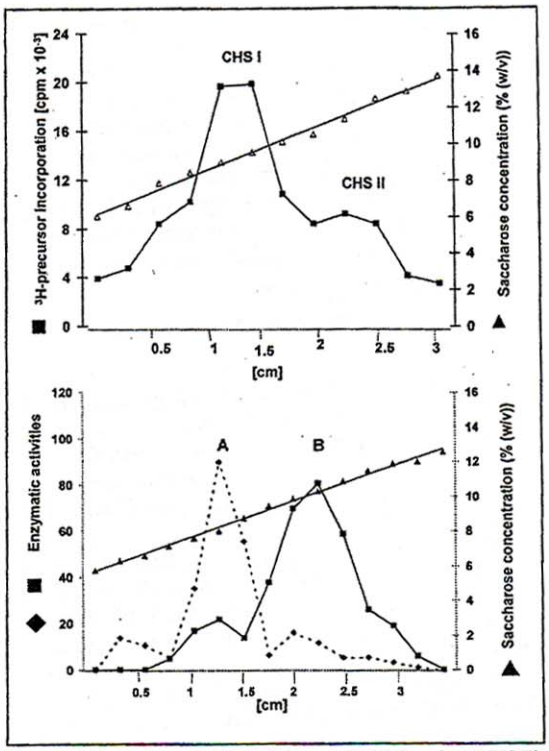

Figure 11. Separation of chitin synthases I (CHS I) and II (CHS II) from cell extracts of the *Chironomus tentans* cell line on a sucrose gradient. Upper panel: Incorporation of ^{3}H-precursor into chitin (measured according to Ludwig et al., 1991). Lower panel: Activities of the two lead enzymes glucose-6-phosphatase (A, microsomal fraction) and 5'-nucleotidase (B, apical membrane) are shown.

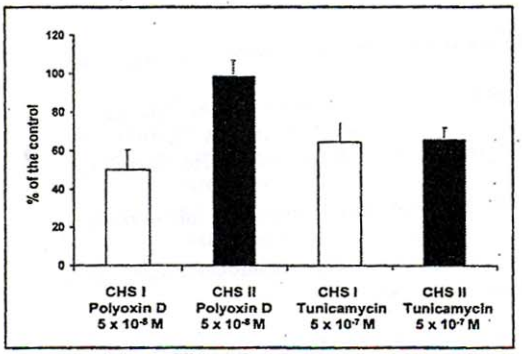

Figure 12. Inhibition of the two chitin synthases CHS I and CHS II from *Chironomus tentans* by polyoxin D and tunicamycin.

low, most of the chitin is synthesized and the new exoskeleton is subsequently sclerotizised. Consequently high ecdysteroid titres inhibit chitin synthesis and decreasing hormone concentrations stimulate chitin synthesis in imaginal discs of *Drosophila* (Apple and Fristrom, 1990). In *Chironomus tentans* cells chitin synthesis is also inhibited by concentrations above 10^{-7} M (Spindler-Barth et al., 1989b). A significant increase in chitin synthase specific transcript concentration is reported after the ecdysteroid pulse ceased in *Drosophila* (Gagou et al., 2002) and during cuticle deposition in *Manduca* (Zhu et al., 2002).

FUTURE PERSPECTIVES

The three-dimensional structure of insect chitinases and chitin synthases are still unknown and only computer models based on sequence alignments are available. Elucidation of the 3D structure of the catalytical domains might help to elucidate, why some inhibitors have pronounced species specific effects on chitinases even within the same protein family (Spindler and Spindler-Barth, 1999). It should also facilitate a more effective search for inhibitors, which could be used as selective and environmentally friendly insecticides (Cohen, 2001; Kramer and Muthukrishnan, 1997; Palli and Retnakaran, 1999; Spindler and Spindler-Barth, 1999; Xie et al., 2001; Lucero et al., 2002; Behr et al., 2003).

Recently, cDNAs from various insect species were cloned, but the biochemical properties of chitin synthase, the pathway of chitin synthesis, which certainly deviates form chitin formation in yeasts, and the highly ordered deposition of chitin fibrils are still rather unknown.

Characterization of the promoter regions and putative regulatory elements will reveal, whether functional hormone responsive elements interacting with the ecdysteroid receptor are present. A consensus sequence for an ecdysone responsive element alone is no proof for hormonal regulation, since the surrounding and the cellular context determine whether a hormone responsive element is active or not (Spindler-Barth and Spindler, 2000). In addition to the hormonal regulation at the transcriptional level there are several indications that ecdysteroids act also post-transcriptionally.

The mechanical properties of the insect cuticle are determined to a considerable extent by the concentration of chitin and the arrangement of chitin fibres. The resistance of the cuticle against pressure varies considerably among insects (Boevé and Schaffner, 2003) as well as the ability to repair wounded cuticle. Our investigations on cuticle repair demonstrate that besides sclerotization, chitin synthesis is involved in cuticle repair. Since chitin synthesis is controlled by ecdysteroids, wound repair might be a suited model to study local differences in hormone response within the same tissue.

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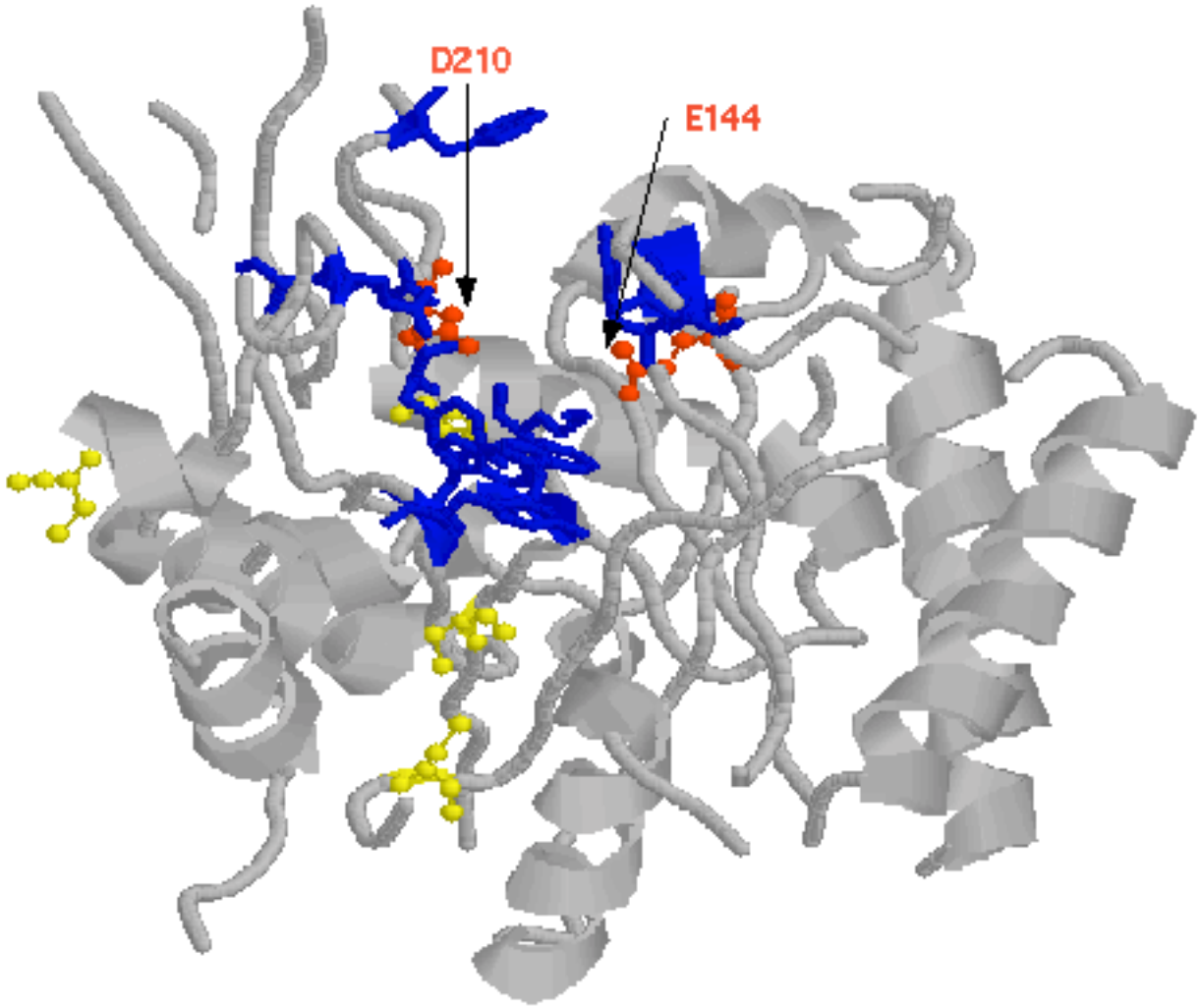
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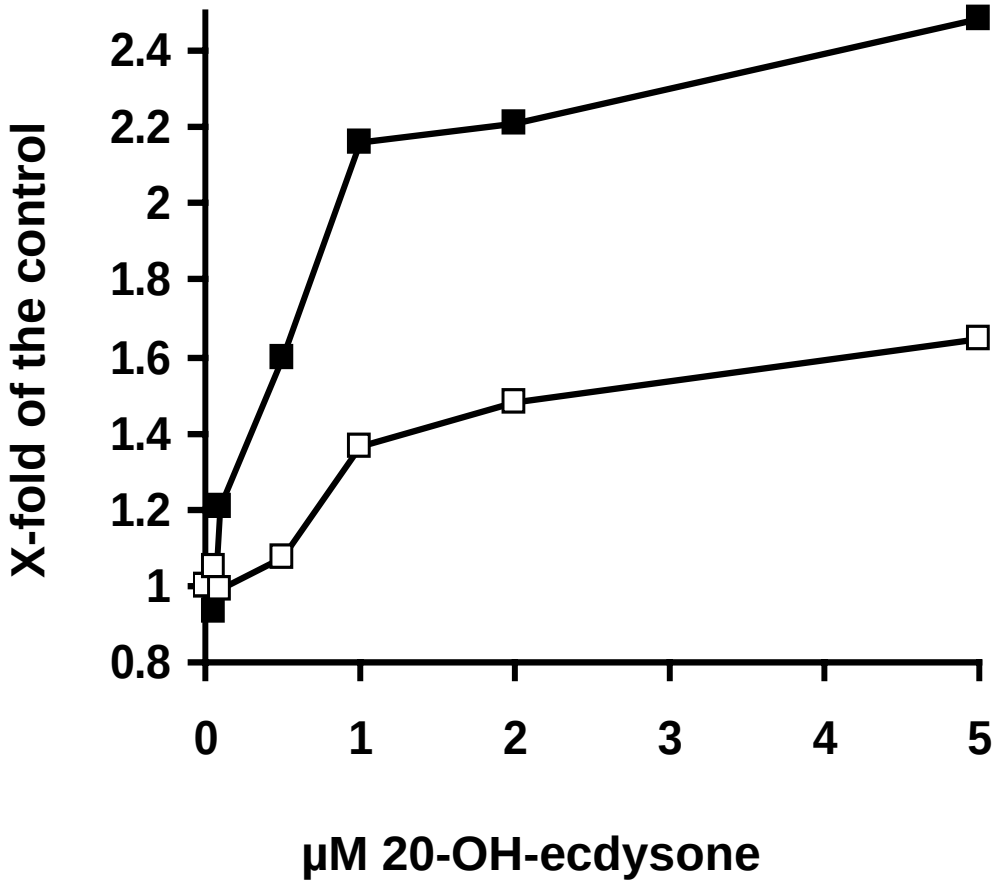
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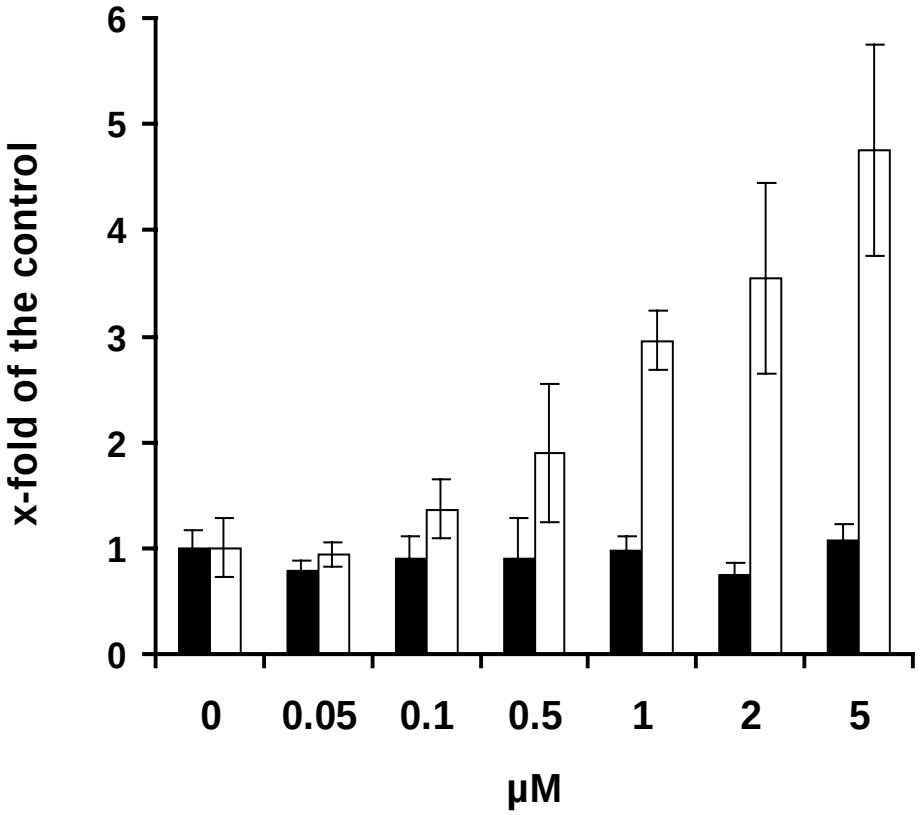
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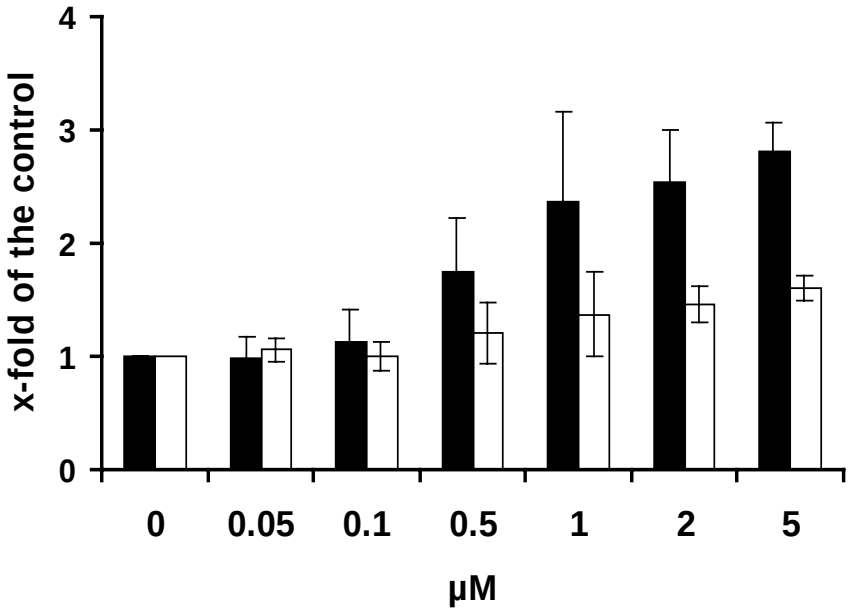
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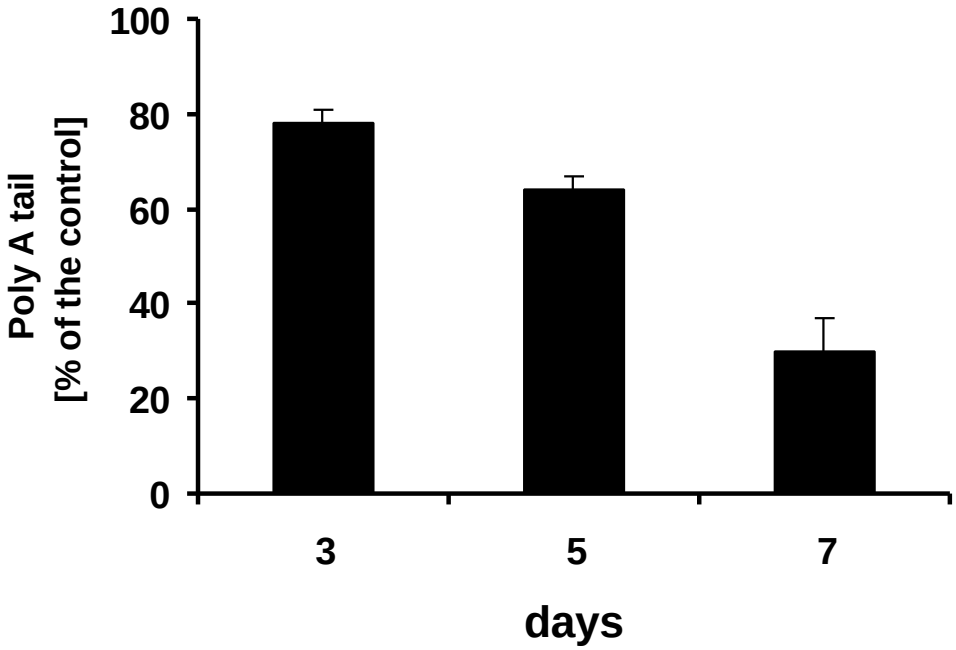
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