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# PROTEOLYTIC ACTIVITY OF 26S PROTEASOMES ISOLATED FROM MUSCLES OF THE TOBACCO HORNWORM, *MANDUCA SEXTA:* DIFFERENCES BETWEEN SURVIVING MUSCLES AND THOSE UNDERGOING DEVELOPMENTALLY PROGRAMMED CELL DEATH*

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The intersegmental muscles (ISMs) of tobacco hornworm, *Manduca sexta* are a well-characterised model system for examining the biochemical changes that accompany programmed cell death during development. When the ISMs become committed to die, there are dramatic increases in both the ubiquitin-expression, and ubiquitin-dependent proteolysis. Since the 26S proteasome is responsible for ATP/ubiquitin-dependent proteolysis in cells, we examined its enzymatic properties. Specific chymotrypsin-like proteolytic activity of 26S proteasomes isolated from ISM is four times higher than that of surviving flight muscle (FM). However, specific activity does not change between developmental stages within ISM or FM. The difference between proteolytic capacity of the two kinds of muscles is even higher when the ISM become committed to die because 26S proteasome content of ISM increases just before cell death. These observations underline the role of 26S proteasome in programmed cell death.

*Keywords: Manduca sexta* – 26S proteasome – enzyme activity – intersegmental muscle – flight muscle

## INTRODUCTION

Metamorphosis in insects is one of the most dramatic developmental processes involving programmed cell death (PCD), cell proliferation and differentiation. The intersegmental muscles (ISMs) of the tobacco hornworm, *Manduca sexta* are a particularly useful model for examining the biochemical events mediating PCD. The ventral intersegmental muscles of abdominal segments 3–6, which are retained from the larval condition through the 3-week pupal stage, are used during the emergence (eclosion) of the adult moth from the pupal case, but then degenerate and die. The muscles begin to regress in the ca. 48 h period prior to eclosion, a change dependent on the pre-emergence decline in titre of the insect's ecdysteroid moulting hormone, 20-hydroxyecdysone, and then abruptly regress during the ca. 36 h following eclo-

*Dedicated to Professor János Kovács on the occasion of his 70th birthday.

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sion [9, 14]. In contrast thoracic flight muscles (FM) and other organs develop in preparation for adult flight.

Muscle proteins to be eliminated are labelled by ubiquitin and digested by a multicatalytic enzyme complex, the 26S proteasome [10]. This proteolytic machine consists of a 20S proteasome core and two regulatory 19S 'caps', one at either end of that core. Two juxtaposed rings of 7 β-type subunits flanked on both the top and bottom by a ring of 7 α-type subunits form the barrel-shaped 20S core complex. Together, these four rings enclose three inner compartments, two antechambers and one central proteolytic chamber. Each regulatory 19S cap contains a 'base' and a 'lid' complex. The 'base' complex, which directly contacts an α type ring of the 20S core, is currently thought to comprise all six ATPases (S4, S6, S6', S7, S8, S10b), and the two largest subunits (S1, S2), as well as S5a, the ubiquitin binding subunit. The 'lid' complex consists of eight subunits and is necessary for degradation of ubiquitinated target proteins. Several functions have been proposed for the ATPases. The hydrolysis of ATP is supposed to promote the assembly of 26S proteasomes. The ATPases could have a role in the gating of the translocation channel on the 20S particle. Substrate proteins are possibly bound and unfolded by the ATPases. The ATPases might assist in the translocation of unfolded substrate proteins into the central proteolytic chamber of the 20S proteasome [18]. Consistent with these proposals the 'base' complex of the 19S cap has recently been shown to have chaperone-like activity [3, 19].

Recent studies in *Manduca sexta* have emphasised the importance of the ubiquitin-dependent cytosolic protein degradation system in the elimination of ISMs, including an enormous increase in polyubiquitin gene expression [15], conjugation of ubiquitin to muscle proteins [7], changes in 20S core subunits [8] and extensive reprogramming of the ATPase regulators of the 26S proteasome [4]. However, there has as yet been no detailed study of the enzymatic properties of proteasomes of different tissues during development. We examined for the first time the enzyme kinetics of the 26S proteasome in ISM and FM in the period leading up to eclosion. The studies show that there are not only more proteasomes present in ISM but they digest ubiquitinated proteins at a higher rate during the elimination of ISM. These observations strengthen the role of 26S proteasome in programmed cell death.

## MATERIALS AND METHODS

### *Insects*

Tobacco hornworms, *Manduca sexta* (L.) (Lepidoptera; Sphingidae) were reared at 25 °C, under a 17 h light –7 h dark photoperiod, on a wheat germ-based artificial diet using standard procedures [1]. Different stages of pharate adult development were recognised by a staging scheme adapted from that of Schwartz and Truman [16] and described fully in [13]. Briefly, stages of development mentioned in this paper are as

follows: pharate adult stage 0: greater than 100 h before eclosion; pharate adult stage 7: about 6 h before eclosion.

### *Separation of 26S proteasomes on glycerol gradients and assay of peptidase activity*

Muscle samples taken from insects were dissected under a simple insect saline solution [6], immediately frozen on a metal surface cooled with liquid nitrogen, and kept at –70 °C until needed. Soluble muscle extract (5 mg protein) prepared from stage 0 and stage 7 ISM and FM were loaded onto a 14 ml 10–40% (v/v) glycerol gradient according to the method of Orino et al. [11]. Samples were centrifuged at 70,000 g in a SW 6×15 rotor for 22 h. Fractions of 0.5 ml were collected and assayed for chymotrypsin-like activity and protein. The chymotrypsin-like activity of the 26S proteasomes were measured using the fluorogenic substrate Suc-LLVY-MCA (Peptide Institute, Inc., Osaka, Japan). Enzyme activity was assayed by incubating glycerol gradient fractions with 0.2 mM substrate in 100 mM Tris-HCl (pH 7.5) for 20 min at 37 °C in a final volume of 105 µl. The reaction was stopped by the addition of 80 mM acetate (pH 4.5), and fluorescence was measured on a Perkin-Elmer LS 5B Luminescence Spectrometer at an excitation of 380 nm and an emission of 460 nm. Protein was determined according to the method of Bradford [2].

### *Enzyme kinetics*

Enzyme kinetical data were collected by incubating a constant amount of purified 26S proteasome (0.5 mg) from one of the glycerol gradient peaks at varying initial fluorogenic substrate concentration ($S_0$, 24.8–248 µM Suc-LLVY-MCA at seven points) in a reaction mix of 500 µl of total volume containing 1 mM ATP, 2 mM $MgCl_2$ in 20 mM Tris-HCl (pH 8.0) for 1 min at 25 °C while the initial rate ($v_0$) was registered on a recording spectrophotometer (Perkin-Elmer LS 5B). The acquired data showed that proteasomes with this substrate follow Michaelis kinetics then the Michaelis constant ($K_m$) and maximum velocity ($V$) were calculated. The whole measurement was performed with every 26S proteasome samples prepared from stage 0 and stage 7 ISM and stage 0 and stage 7 FM.

## RESULTS

26S proteasomes were isolated from ISM and FM both at stage 0 and 7 by glycerol gradient centrifugation. Total muscle protein loaded on the gradient was the same (5 mg) in all cases. Fractions were assayed for protein and chymotrypsin-like activity (Fig. 1). Total chymotrypsin-like activity increased in ISM from stage 0 to stage 7 by a factor of 7.9-times based on area under curve (Fig. 1A, B). However, protein con-

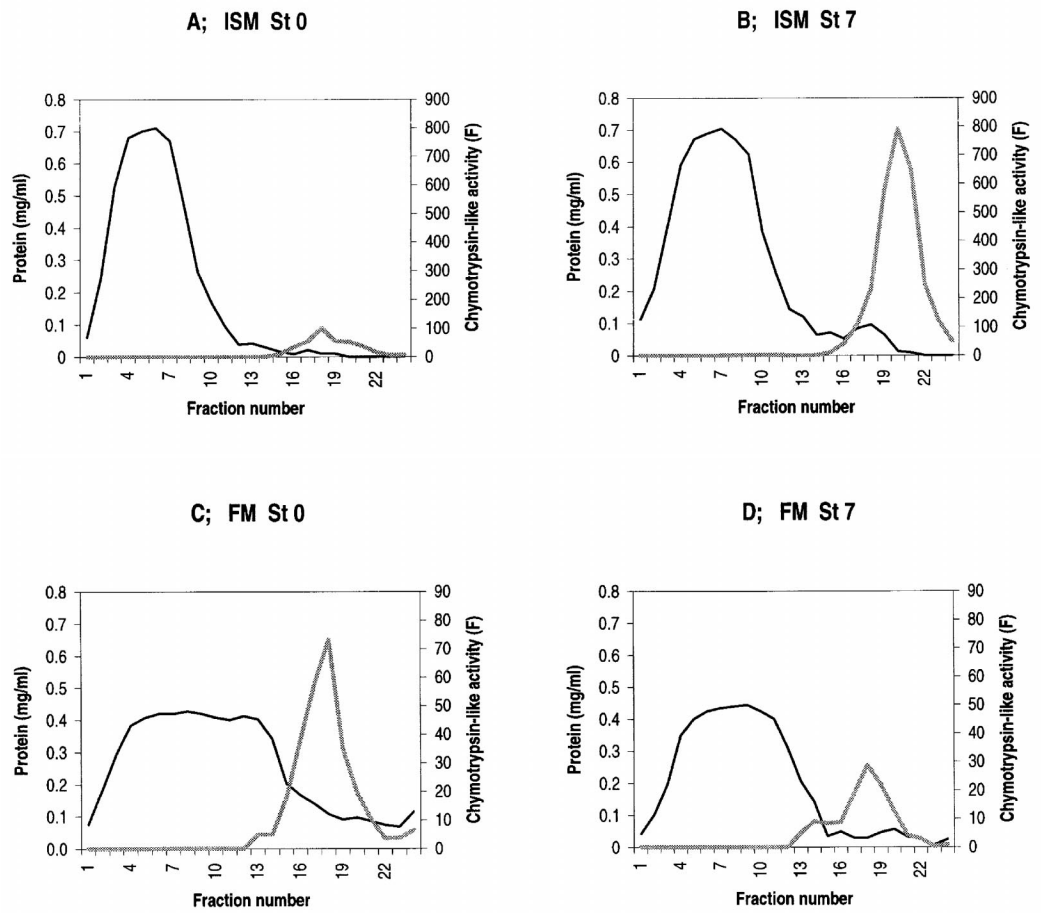


*Fig. 1.* Glycerol gradient preparation of 26S proteasomes from intersegmental muscles (ISM) and flight muscles (FM) of *Manduca sexta* at developmental stage 0 and stage 7. Soluble muscle extracts were fractionated on 10–40% glycerol gradients. Samples of each fraction were assayed for protein (black line) and chymotrypsin-like activities (grey line). Panels A, B, C and D show gradient analysis of proteasomes from ISM at stage 0 and stage 7, and FM at stage 0 and stage 7, respectively. The scale of chymotrypsin-like activities on panels A and B is 10-times higher than on panels C and D. F – fluorescent units

tent was 6.1-times higher in stage 7 fraction of the enzyme activity peak. The overall activity was higher due to the fact that much more enzyme complex was present. Specific activity showed only a slight increase of 1.3-times from stage 0 to 7. Total chymotrypsin-like activity seemed to decrease in FM from stage 0 to 7 by a factor of 2.4-times based on the area under curve (Fig. 1C, D). Nevertheless, this was accompanied by an even bigger drop (3.2×) in protein content of the corresponding fractions. All in all the specific activity of 26S proteasomes in FM increased from stage 0 to stage 7 by 1.3-fold. Comparing total as well as specific activities of 26S proteasomes of the two tissue types the values for ISM were much higher at all stages (Fig. 1).

We decided to collect enzyme kinetical data to determine the difference between enzymatic properties of 26S proteasomes of the two kinds of muscles. Data show that the purified 26S proteasomes of both muscles (ISM, FM) follow Michaelis kinetics

*Table 1*

Maximum velocity ($V$) and Michaelis constant ($K_m$) values of proteasomes isolated from intersegmental muscles (ISM) and flight muscles (FM) of *Manduca sexta* at developmental stages 0 and 7 measured with the fluorogenic substrate Suc-LLVY-MCA

| | $V$ (nM/min/g) | $K_m$ (μM) |
|---|---|---|
| ISM St 0 (n = 5) | 57.2 | 84.6 |
| ISM St 7 (n = 5) | 62.4 | 108.1 |
| FM St 0 (n = 3) | 13.0 | 34.6 |
| FM St 7 (n = 3) | 13.2 | 35.9 |

n – number of independent isolations and activity assays of which the average value was calculated

in both stage 0 and stage 7. There was no statistically significant difference in maximum velocity ($V$) and Michaelis constant ($K_m$) values between stages 0 and 7 in either of the muscles ($V$: F = 0.154, p = 0.701; $K_m$: F = 0.194; p = 0.667), although values of stage 7 ISM were somewhat higher than those of stage 0 ISM (Table 1). However, there was a significant difference in the values between the muscle types ($V$: F = 6.615, p = 0.023; $K_m$: F = 7.670; p = 0.016) (Table 1). Initial rate ($v_0$) against fluorogenic substrate (Suc-LLVY-MCA) concentration ($S_0$) are plotted in Fig. 2 for proteasomes of the two tissue types using the average values of Table 1 (ISM: $V$ = 59.8 nM/min/g, $K_m$ = 96.4 μM; FM: $V$ = 13.1 nM/min/g, $K_m$ = 35.2 μM). 26S proteasomes of dying ISM had 4-times higher maximum velocity ($V$) and 3-times bigger Michaelis constant ($K_m$) (Table 1, Fig. 2) at the constant enzyme concentration (1 mg/l) used.

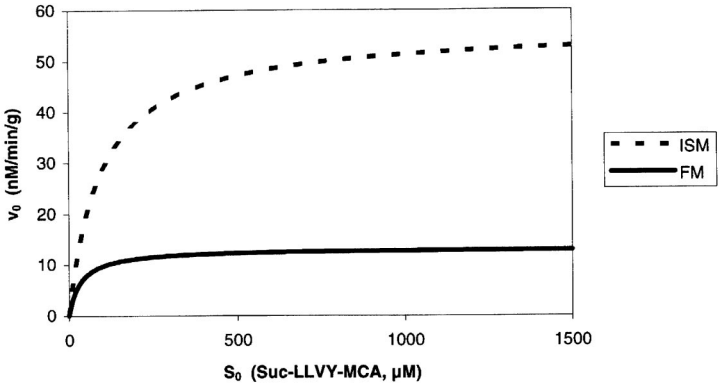


*Fig. 2.* Plot of initial rate ($v_0$) against fluorogenic substrate (Suc-LLVY-MCA) concentration ($S_0$) for proteasomes of intersegmental muscles (ISM, dashed line) and flight muscles (FM, solid line) of *Manduca sexta.* Curves were plotted using the average values of Table 1 (ISM: V = 59.8 nM/min/g, $K_m$ = 96.4 µM; FM: $V$ = 13.1 nM/min/g, $K_m$ = 35.2 µM)

## DISCUSSION

We isolated 26S proteasomes from intersegmental muscles (ISM) and flight muscles (FM) of *Manduca sexta* and measured and compared the chymotrypsin-like proteolytic activities of these proteasomes before and after the commitment of ISM to undergo programmed cell death. The total activity increased from stage 0 to stage 7 in ISM while it was much lower and even decreased in FM (Fig. 1). Taking the protein content into account it turned out that the specific activity did not change from stage 0 to stage 7 in either tissue. However, the specific activity of proteasomes in ISM was four times higher than that of the FM (Table 1, Fig. 2).

The loss of the *Manduca* ISMs involves a massive proteolytic effort in order to remove these giant cells within 36 h at 26 °C. This is presumably why there is a higher level of proteasomes (compared to stage 0 or levels in FM) and these proteasomes are more active (compared to FM) in ISM at stage 7. There is an intensive restructuring of larval tissues in pupae of Holometabola insects which involves protein degradation as well as synthesis. The increased level of proteasomes in FM at stage 0 correlates very well with this process. At stage 7 in the developed FM we suppose that a lower level of proteasomes is sufficient to perform the normal housekeeping functions.

Several studies have examined the role of proteasomes in programmed cell death and the possible changes in its activity and subunit composition during the process (reviewed in [5,12]). Now it is generally accepted that the 26S proteasome plays a major role in the programmed elimination of ISM in *Manduca sexta*. Some authors found changes in the subunit composition of the regulatory part and also the core particle (20S proteasome) during the cell death process [4, 8, 17]. They also observed a

major increase in the proteolytic activity of the proteasome in the same time. We focused our studies on the enzymatic properties of the 26S proteasome. However, according to our measurements although the total chymotrypsin-like activity increased the amount of proteasomes was also higher so the specific activity remained unchanged in ISM from stage 0 to stage 7 (Fig. 1A, B). Increased amount of or newly added cell death specific subunits may increase the activity of 26S proteasomes *in vivo* on ubiquitinated target proteins.

We isolated intact 26S proteasomes under conditions in which they remained intact. Regulatory subunits in 19S particles are believed to recognise, bind, and unfold ubiquitinated proteins and translocate them into the proteolytic core [8]. For large proteins the enzymatically active centre of the complex is accessible only with the help of the regulatory part. However, small peptides can get into the central chamber without the involvement of other proteins [8]. In this work, a tetrapeptide with a fluorogenic moiety (Suc-LLVY-MCA) was used for measuring the chymotrypsin-like activity of the intact 26S proteasome. This substrate is small enough to enter the enzyme's catalytic core bypassing the regulatory part of the proteasome. Thus we measured the proteolytic activity of the 20S core of the proteasome itself rather than that of the whole 26S complex. According to our data this intrinsic proteolytic activity does not change during the programmed cell death of ISM. But since we know from previous work that the regulatory subunits of the ISM proteasomes change at the time that programmed cell death is occurring [4], and that these regulatory subunits are likely to selectively control access of proteins to the enzymatically active core of the organelle, it is probable that the proteolysis of key muscle fibre proteins by 26S proteasomes could still change even while core chymotryptic activity (as measured here) remained constant. The key finding of the present work is that differences in the kinetics of the intrinsic proteolytic (chymotryptic) activity of 26S proteasomes between those muscles that are destined to die (ISM), and those that are destined to survive (FM). Thus there are probably key differences in the core subunit make-up of these organelles between the two types of muscle. The differences could reflect the increased need for intracellular proteolysis in ISM during programmed cell death. Studies described in this paper provide additional evidence that the ubiquitin-proteasome system plays a potentially important role in the events contributing to programmed cell death in the intersegmental muscles of *Manduca sexta*.

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