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Different Proteasome Subtypes in a Single Tissue Exhibit Different Enzymatic Properties

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It is concluded from many experiments that mammalian tissues and cells must contain a heterogeneous population of 20 S proteasome complexes. We describe the purification and separation by chromatographic procedures of constitutive 20 S proteasomes, 20 S immuno-proteasomes and intermediate-type 20 S proteasomes from a given tissue. Our data demonstrate that each of these three groups comprises more than one subtype and that the relative ratios of the subtypes differ between different rat tissues. Thus, six subtypes could be identified in rat muscle tissue. Subtypes I and II are constitutive proteasomes, while subtypes V and VI comprise immuno-proteasomes. Subtypes III and IV belong to a group of intermediate-type proteasomes. The subtypes differ with regard to their enzymatic characteristics. Subtypes I-III exhibit high chymotrypsin-like activity and high peptidylglutamylpeptide hydrolysing activity, while these activities are depressed in subtypes IV-VI. In contrast, trypsin-like activity of subtypes IV-VI is enhanced in comparison to subtypes I-III. Importantly, the subtypes also differ in their preferential cleavage site usage when tested by digestion of a synthetic 25mer polypeptide substrate.

Therefore, the characteristics of proteasomes purified from tissues or cells represent the average of the different subtype activities which in turn may have different functions *in vivo*.

Keywords: 20 S proteasomes; multiple subtypes; separation and purification; enzymatic properties; rat tissues

Introduction

The major cellular non-lysosomal ATP/ubiquitin-dependent proteolytic pathway is catalysed by a protein complex, that consists of about 60 protein subunits. Of these, 28 build up the central 20 S core particle, the multicatalytic proteinase (MCP) (Dahlmann *et al.*,1988) also designated multicatalytic proteinase complex (MPC) (Orlowski & Wilk, 1988), 20 S proteasome (Arrigo *et al.*, 1988), or prosome (Schmid *et al.*, 1984). The other proteins are constituents of a proteasome regulator PA700 that docks on to one or both of the endplates of the barrel-shaped enzyme complex to form the 26 S proteasome. The 20 S proteasome is a dimer of four stacked seven-membered rings. Three of the β-subunits, which form the two inner rings carry the proteolytically active sites, while the two outer rings consist of α-subunits, which bind regulatory complexes, e.g. proteasome inhibitor PI31, proteasome activator PA28, and proteasome regulator PA700 (19 S regulator complex). These regulatory complexes consist of one, two or 17 different subunits, respectively. They not only regulate the proteolytic activities of proteasomes but are also involved in recognition, binding, and defolding of substrates, functions which can be performed by the 20 S proteasome itself only to a limited extent (Coux *et al.*, 1996).

Despite high structural conservation of proteasomes during evolution from archaebacteria to man (Baumeister *et al.*, 1998), considerable variation exists with regard to their subunit

Abbreviations used: MCP, multicatalytic proteinase; Suc, succinyl; MCA, 7-amino-4-methylcoumarylamide; Bz, benzoyl; Z, benzoyloxycarbonyl; PAGE, polyacrylamide gel electrophoresis; PGPH, peptidylglutamylpeptide hydrolyzing; MCMV IE, murine cytomegalovirus immediate early.

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composition, activity and cellular distribution. Probably the most prominent variability in proteasome subunit composition and expression has been reported for *Drosophila melanogaster* (Haass & Kloetzel, 1989). Here, the subunit pattern changes throughout development, suggesting that this may reflect a functional adaptation to developmental requirements (Falkenburg & Kloetzel, 1989). In addition, a number of subunit isoform genes have been described which differ in sequence and which are differentially expressed during ontogenesis of the fly (Yuan *et al*., 1996). As a consequence, proteasomes exhibit different subunit compositions at different stages of development or in different tissues (Hutson *et al*., 1997; Chae & Meada, 1998).

For the mammalian system, Hong *et al*. (1994) have classified the α and β-subunits of 20 S proteasomes into three categories: constitutive, tissue-specific, and development specific, indicating that the subunit pattern of 20 S proteasomes differ depending on their origin in the organism. This phenomenon has been further scrutinized by Orlowski and co-workers (Cardozo *et al*., 1995; Eleuteri *et al*., 1997) who compared the features of 20 S proteasomes from different mammalian tissues. A major reason for the differences in properties and activities of proteasomes is the extent of replacement of the constitutive, active site harbouring β-subunits, delta, Z, and MB1, by the γ-interferon inducible active site containing subunits, LMP2, MECl1 and LMP7, respectively. This replacement results in the formation of so called ''immuno-proteasomes'', which efficiently generate peptides that are presented by MHC class I complexes to cytotoxic T-lymphocytes (Schmidtke *et al*., 1998; Sijts *et al*., 2000). Since these exchanges of β-subunits affect the proteasome activities towards fluorogenic peptide substrates (Gaczynska *et al*., 1996; Eleuteri *et al*., 1997), differences in these activities could also be observed between proteasomes from various tissues (Aki *et al*., 1994; Hong *et al*., 1994; Cardozo *et al*., 1995; Eleuteri *et al*., 1997; Cardozo & Kohanski, 1998).

However, this heterogeneity in proteasome subtypes not only exists between different tissues, but also within a single cell (Palmer *et al*., 1996). Differences within a single cell are due to various degrees of different subunit composition, such as constitutive *versus* inducible subunits, subunit isoforms and varying degrees of secondary modifications. Several α-subunits are products of differential splicing events (Yuan *et al*., 1996) or are phosphorylated, a process which seems to affect the proteolytic activities of proteasomes (Mason *et al*., 1996). In addition, subpopulations of proteasomes are located within different cellular compartments (Benedikt *et al*., 1995; Hori *et al*., 1999). Interferon-γ-inducible subunits seem to be enriched in the microsomal fraction, whereas proteasomes from the nucleus contain only small amounts of these subunits (Palmer *et al*., 1996). Also, function and ontogenesis-induced variations of proteasomes and experimentally induced changes in subunit composition of proteasomes, as a result of treatment of cells with phorbol esters and retinoic acid dihydro-oxycholecalciferol, have been described (Henry *et al*., 1996; Baz *et al*., 1997). Thus, in this investigation we have chromatographically separated 20 S proteasome subtypes and analysed the properties of various proteasome subtypes from a given tissue.

Our data provide first evidence that in addition to constitutive and immuno-proteasomes, proteasomes of intermediate-type exist, and each of these subpopulations comprises more than one proteasome subtype. Since each subtype exhibits slightly different enzymatic activities, the proteolytic activities and characteristics determined for proteasomes purified from a given tissue or cell represent the average of the sum of the different activities of the proteasome subtypes.

Results

Separation and identification of 20 S proteasome subtypes

To identify and separate 20 S proteasome subtypes, proteasomes purified from rat skeletal muscle tissue were subjected to a Mini Q anion exchange column and eluted by a linear gradient of NaCl. As shown in Figure 1(a), six proteasome peaks, three major and three minor, designated subtype I-VI, eluted within a very narrow range of the salt gradient (Table 1) and could be identified as proteasomes based on their hydrolytic activities towards several fluorogenic peptide substrates (Figure 1(b)) as well as due to their characteristic 2D-PAGE protein pattern (not shown).

Table 1. NaCl concentrations specific for elution of rat muscle proteasome subtypes from Mini Q

	Proteasome subtype					
	I	II	III	IV	V	VI
NaCl (mM)	322.8	328.2	334.2	338.6	343.9	347.7
S.D.	1.5	1.3	1.9	2	0.8	1.1
p-value for comparison with the next peak	0.00001	0.00002	0.0013	0.00009	0.00006	

Data are means from seven different muscle proteasome preparations.

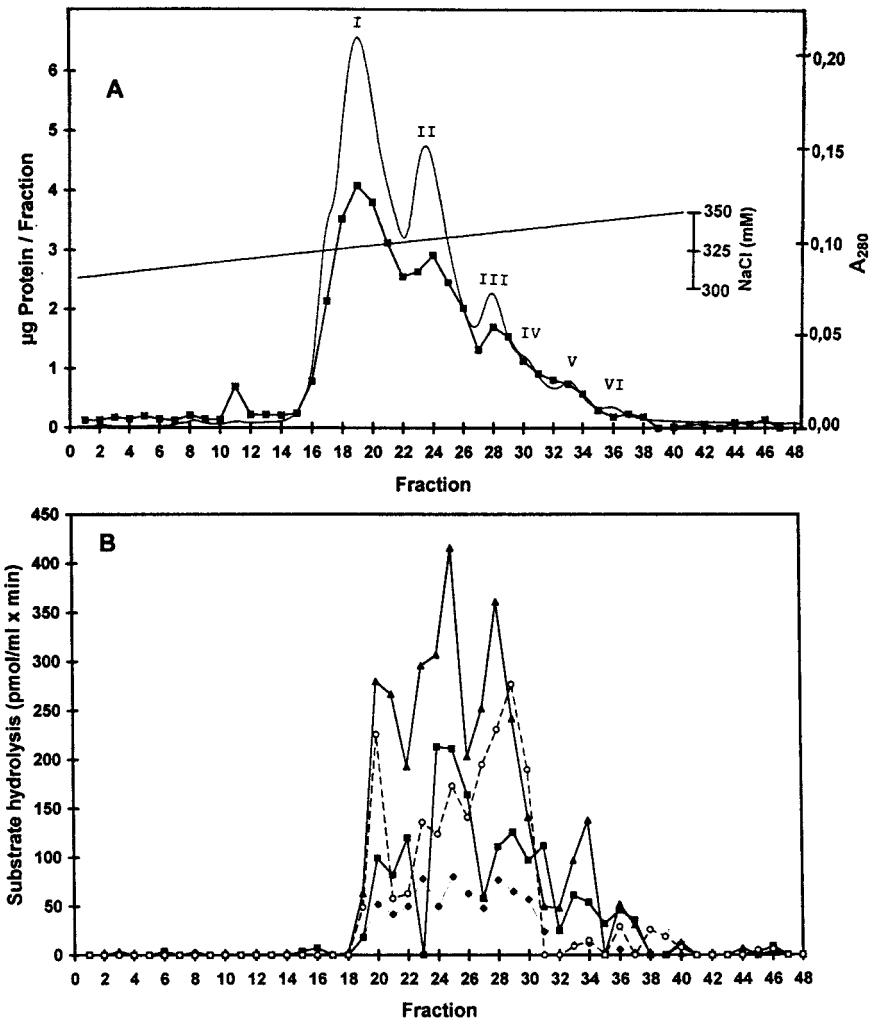

Figure 1. Chromatographic separation of proteasome subtypes from skeletal muscle. About 100 µg (a) and 50 µg (b), respectively, of highly purified 20 S proteasomes from rat skeletal muscle were subjected to chromatography on Mini Q column equilibrated in TEAD buffer and eluted with a linear increasing gradient of 0 mM-500 mM NaCl in TEAD buffer. Proteins eluting between 300 and 360 mM NaCl were collected in fractions of 20 µl. (a) Diagonal line shows the NaCl concentration (—) A_{280}. Protein as determined by Bradford assay (Friedenauer & Berlet, 1989) is given in µg/fraction (■—■). Proteasome subtypes are indicated consecutively as I, II, III, IV , V, VI. (b) Aliquots of each fraction were assayed for proteasome activity with the following substrates: (◆—◆) Suc-LLVY-MCA; (■—■) Bz-VGR-MCA; (▲—▲) Z-GGL-MCA; (○—○) Z-LLE-MCA.

To demonstrate that the differences in chromatographic behaviour of the proteasome complexes are due to their intrinsic physicochemical properties, aliquots of the most prominent peaks, namely I, II, III, and V, were rechromatographed on the same column under identical conditions. Again each peak eluted at its characteristic salt concentration and ''contaminating'' proteasome subtypes from neighbouring peaks were separated (Figure 2). Furthermore, when re-chromatographed proteasomes from peak I to III were pooled, and again subjected to MiniQ chromatography, the three proteasome subtypes eluted successively, indicating that the various proteasome subtypes are clearly different entities which can be separated due to their different surface charges.

Immuno-proteasomes are separated from constitutive and intermediate-type proteasomes

An obvious explanation for the existence of proteasome subtypes is the variable extent of exchange of constitutive subunits by γ-interferon inducible subunits. To investigate whether the separation of proteasome subtypes is due to their different content in immuno-subunits, proteasomes fractionated by anion-exchange chromatography as shown in Figure 1, were analysed by SDS-PAGE and immuno-blotting with subunit specific antibodies.

As shown in Figure 3 the constitutive subunits MB1 (β5), Z (β2), and delta (β1) are present in proteasome subtypes I and II, whereas the γ-interferon-inducible homologues LMP7 (β5i)

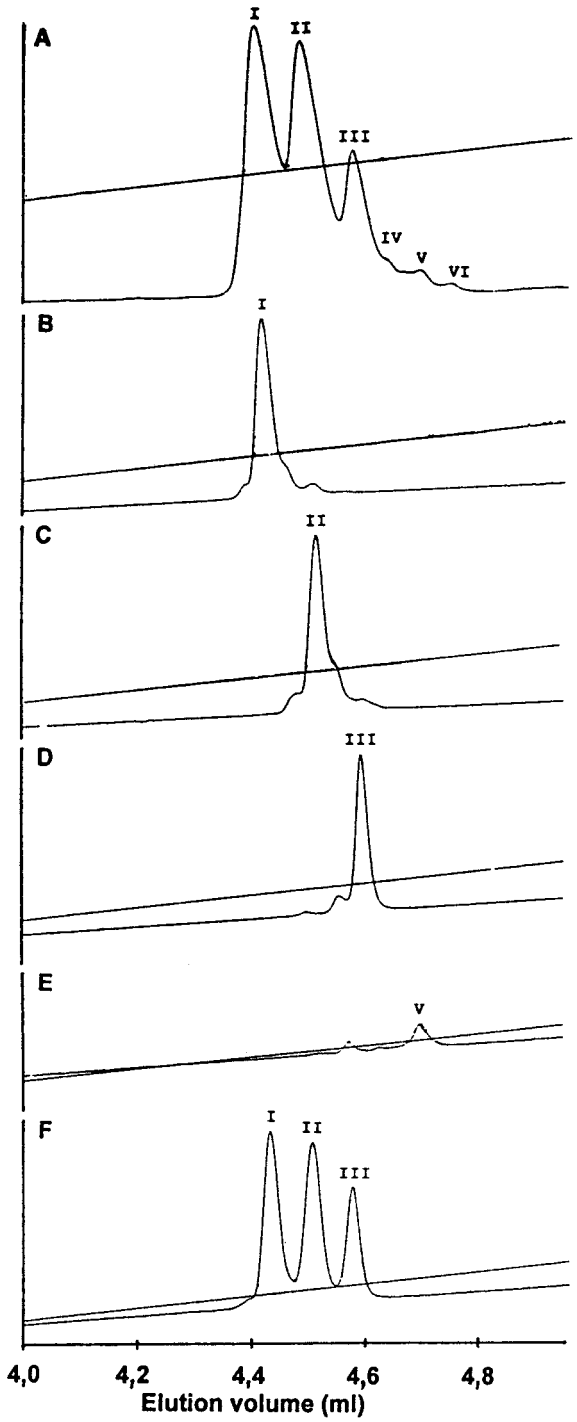

Figure 2. Re-chromatography of muscle proteasome subtypes on Mini Q. (a) Rat muscle 20 S proteasomes were fractionated by chromatography on Mini Q into six subtypes (I-VI) as described in legend to Figure 1. (b)-(e) Material from subtypes I, II, III, and V, respectively, was subjected to a second chromatography under the same conditions. (f) Peak fraction of subtypes I, II, and III were each pooled and subjected to re-chromatography on Mini Q. Diagonal line shows the NaCl concentration (300 mM-360 mM) of the gradient.

and LMP2 (β1i) are present in subtypes V and VI. This indicates that the former contain constitutive proteasomes and the latter immuno-proteasomes. Since immunoreaction with anti-LMP7 increases in proteasomes eluted in fractions 27 to 30, and concomitantly their reaction decreases with anti-Z and anti-delta, subtypes III and IV are proteasomes of subunit composition intermediate between constitutive and immuno-proteasomes. Thus, muscle tissue contains predominantly constitutive-type proteasomes (subtypes I and II), considerable amounts of intermediate-type proteasomes (subtypes III and IV), but only a small amount of pure immuno-type proteasomes (subtypes V and VI).

In contrast to muscle tissue spleen contains predominantly immuno-proteasomes (Eleuteri *et al.*, 1997; Cardozo & Kohanski, 1998). We have therefore prepared proteasomes from rat spleen tissue and additionally from liver and kidney and compared their subtype pattern by anion exchange chromatography (Figure 4). A subunit analysis by SDS-PAGE and immunoblotting of spleen proteasome subtypes is shown in Figure 5. No immunoreaction could be obtained with anti-MB1 and only low levels of delta were visible in the fractions of subtype I and II from spleen. In contrast, a strong reaction was obtained with anti-LMP7 and anti-LMP2 in subtypes I-IV and II-IV, respectively. Since subunit Z was not detectable at all, we probed the blot with an antibody raised against mouse subunit MECl1 (β2i). Its pattern of reaction clearly indicates the presence of MECl1 in spleen proteasome subtypes I-IV. Thus, the predominant subtypes III and IV in spleen tissue are proteasomes of the immuno-type, whereas proteasomes containing exclusively constitutive subunits are obviously not present in this tissue. On the other hand, and similar to subtypes III and IV from skeletal muscle, subtype I and II contain both delta and LMP2 and thus are proteasomes of intermediate composition.

These results clearly show that both two main subpopulations of proteasomes, constitutive and immuno-type, exist in multiple different subtypes. Furthermore, they show that different intermediate forms exist between immuno and constitutive proteasomes. A comparison of all four intermediate-type proteasomes found in muscle and spleen (Figures 3 and 5) reveals that they differ in composition with respect to their active-site β-subunits (β1-β2-β5): muscle subtype III contains delta-Z-LMP7; muscle subtype IV contains delta/LMP2-Z-LMP7; spleen subtype I contains delta-MECl1-LMP7; spleen subtype II contains delta/LMP2-MECl1-LMP7.

Considering that all subunits contribute to the overall surface charge of the proteasome complexes, it is not surprising that subtypes from different tissues indicated by us with the same number elute at different salt concentrations (Figure 4). The reason is their non-identical subunit composition, e.g. subtype I from muscle contains

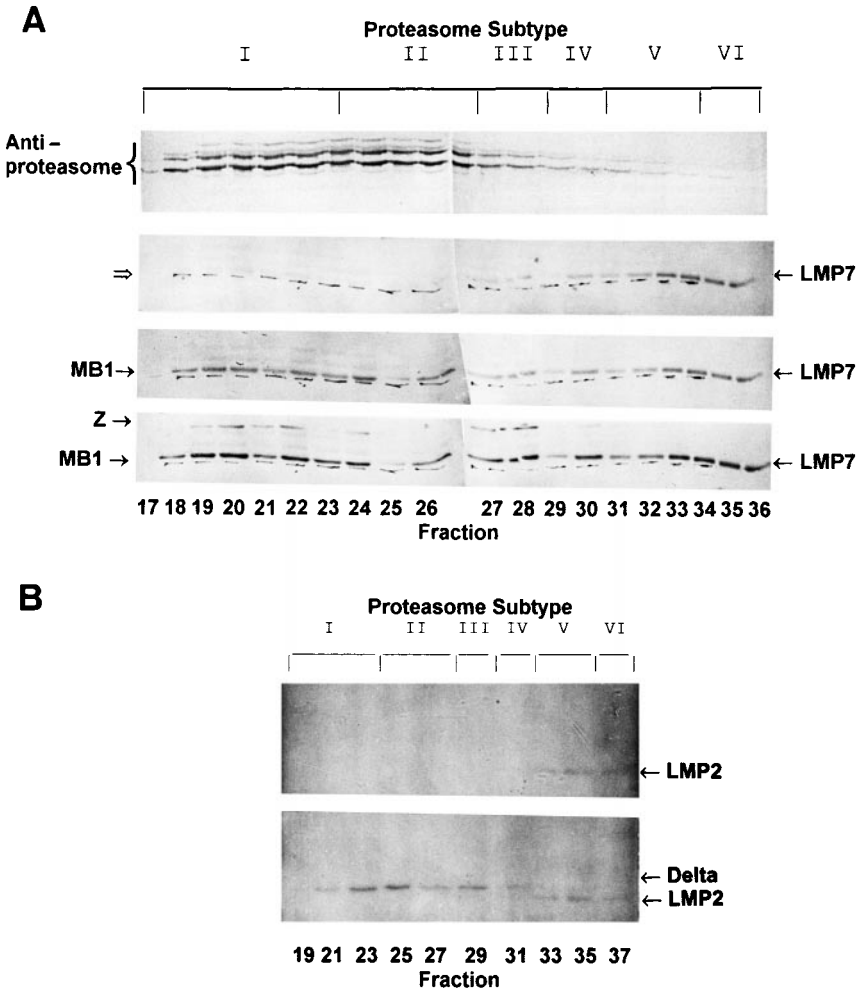

Figure 3. Immunochemical detection of subunit LMP7,LMP2, MB1, delta, and Z in proteasome subtypes from rat skeletal muscle. Material of each (a) or of every second fraction (b) between number 17-36 obtained by Mini Q chromatography of rat muscle proteasome (see Figure 1) was subjected to SDS-PAGE and Western blotted. (a) Upper row shows reaction of an antibody raised against whole 20 S proteasome from rat skeletal muscle, indicating the distribution of proteasomes within the spectrum of subtypes I-VI. The next three rows show a second blot incubated sequentially with three different antibodies: 1, with an antibody raised against LMP7; reaction is only seen with fraction 27-36; 2, with an antibody raised against MB1; reaction is only detected in fractions 17-27; 3, with an antibody to subunit Z; this subunit is only detected in fractions 17-28. The open arrow (second row left hand) indicates pencil marks that were set after Ponceau staining of the blots to locate proteasome bands before immunoreactions. (b) A blot first incubated with an antibody raised against LMP2, giving a reaction only with fractions 33-37. Afterwards the same blot was incubated with antibody specific for subunit delta, clearly present only in fractions 19-31.

only constitutive β subunits, whereas subtype I from spleen is of intermediate-type composition. We have confirmed this different chromatographic behaviour by co-chromatography of proteasome subtypes from different tissues (not shown). However, the fact that subtypes I from liver and kidney elute even earlier from the column than the purely constitutive subtype I from muscle emphasizes that the relative content of immuno-subunits is not the only determining factor for the existence of proteasome subtypes.

Proteasome subtypes differ in their proteolytic properties

Conflicting results have been reported on the activities of immuno-proteasomes when compared with constitutive proteasomes (for a review, see Tanaka & Kasahara, 1998). The analysis of the peptide hydrolysing activities of the six different proteasome subtypes identified in rat muscle tissue using fluorogenic peptide substrates demonstrates that the activities measured are not simply a function of proteasome concentration (Figure 6).

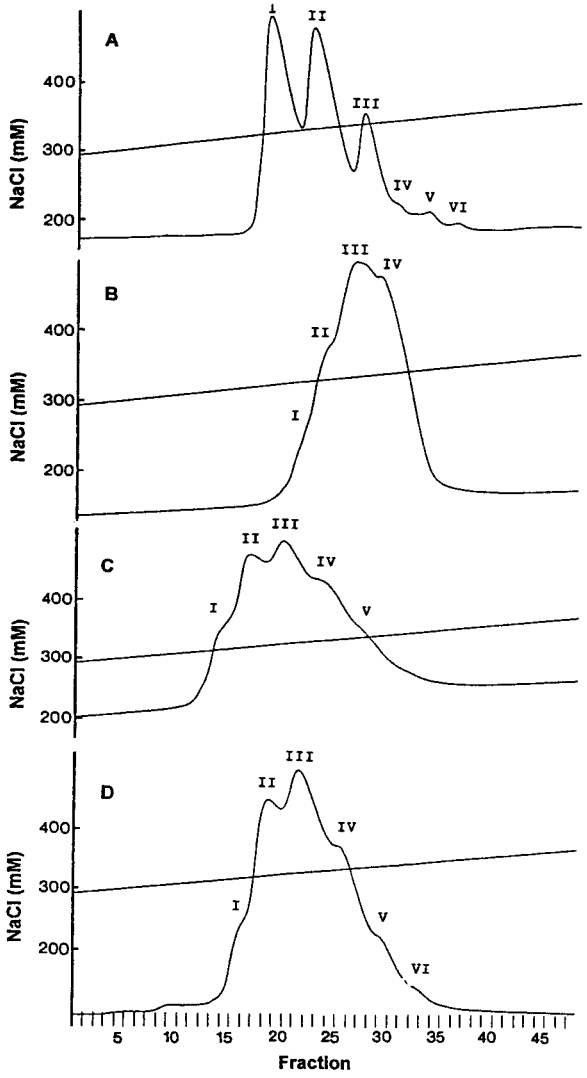

Figure 4. Pattern of proteasome subtypes in different rat tissues. (a)-(d) 20 S proteasomes were purified from rat skeletal muscle (a), spleen (b), liver (c), and kidney (d), respectively, and about 50 μg each were then subjected to chromatography on Mini Q as detailed in legend to Figure 1. The diagonal lines indicate the actual concentration of NaCl in the fractions. Proteasome subtypes of the different tissues are indicated by Roman numerals I-VI.

Clear-cut differences exist between pure constitutive and pure immunosubtypes. The former (subtypes I and II) exhibit high chymotrypsin-like (measured with Suc-LLVY-MCA) and PGPH activity (measured with Z-LLE-MCA), while both activities are significantly suppressed in pure immuno-proteasomes (subtypes V and VI). The converse is found for the trypsin-like activity (measured with Bz-VGR-MCA) which is enhanced in subtypes VI, and especially in V, when compared to subtypes I-II. Thus, in proteasome subtypes containing immuno-subunits the PGPH and the chymotrypsin-like activity are depressed, whereas trypsin-like activity is enhanced. These data support previously obtained results using transfected cell lines or interferon-γ-treated cell lines (Boes *et al*., 1994; Ustrell *et al*., 1995).

Interestingly, the velocity of fluorogenic peptide hydrolysis by those proteasomes belonging to the intermediate subtypes is generally also intermediate between that measured for pure constitutive and pure immuno-type proteasomes. Subtype III shows closer similarity to constitutive, and subtype IV to immuno-type proteasomes. A clear exception to this rule is the PGPH activity of subtype III which is the highest measured within the six muscle proteasome subtypes.

The differences in activities found for the various subtypes are most likely due to their different content in immuno- and constitutive β-subunits. However, additional structural features must be important for expression of the different activities, since our experimental data reveal that activities are also different between individual subtypes belonging to one of each of the major groups of proteasomes. Thus, the two constitutive subtypes I and II clearly have different V_{max} for hydrolysis of Suc-LLVY-MCA. Similar differences are apparent for the PGPH activity of the two immuno-proteasome subtypes V and VI.

Proteasome subtypes differ with respect to polypeptide substrate turnover rates and cleavage specificity

The limited length is an obvious disadvantage of fluorogenic peptide substrates for exact characterisation of the enzymatic properties of 20 S proteasomes. Therefore, to compare the proteolytic activities of muscle proteasome subtypes towards non-fluorogenic substrates we have also used the pp89 25mer polypeptide substrate whose sequence is derived from the MCMV IE protein pp89 (Figure 7). This synthetic polypeptide substrate was incubated with isolated proteasome subtypes from muscle and the extent of degradation was analysed by HPLC and mass spectrometry. These experiments show (Figure 7(a)) that after 16 hours of incubation proteasome subtypes IV and V degraded about 50 % of the substrate. Under the same conditions only 5-12 % of substrate was degraded by subtypes I and II, which exclusively contain constitutive β-subunits. Subtype III and VI are of intermediate activity. So, despite their high level of chymotrypsin-like and PGPH activity the constitutive proteasome subtypes I and II exhibit significantly lower activity towards the 25mer polypeptide substrate than intermediate proteasome subtypes IV and immuno-proteasome V.

To detect qualitative differences in the cleavage pattern of proteasomes, subtypes II, III, and IV from rat spleen were incubated with the pp89 25mer peptide substrate for different periods of time and analysed by HPLC coupled with mass spectrometry. Analogous to the results obtained with subtypes from muscle tissue, the specific

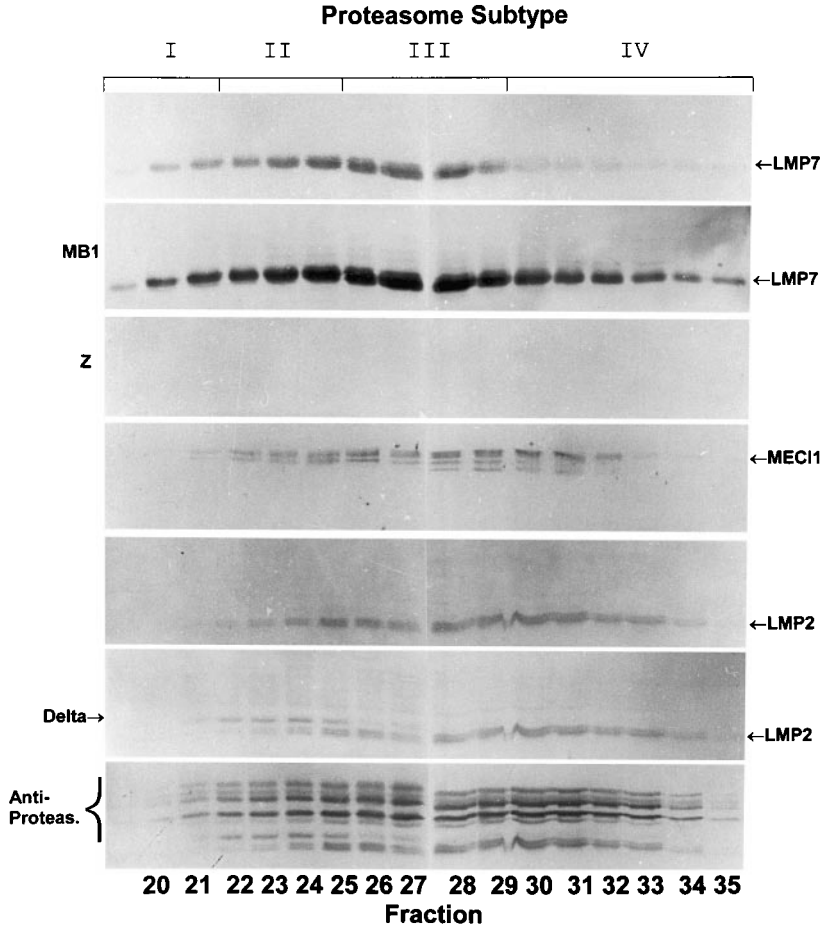

Figure 5. Immunochemical detection of subunit LMP7, MB1, Z, MECl1, LMP2, and delta in proteasome subtypes from rat spleen. Material from fractions 20-35 obtained by Mini Q chromatography of rat spleen proteasomes (see Figure 4) was subjected to SDS-PAGE and Western blotted. Upper two panels show the same blot first incubated with anti-LMP7 that is detected in all fractions. Subsequent incubation with anti-MB1 results in an intensification of the same signal, only. The next two panels show the same blot first incubated with anti-Z, which shows no reaction at all, and subsequently incubated with anti-MECl1, which reacts with proteasomes in fraction 22-35. The lower three panels show a blot first incubated with anti-LMP2, reacting with proteasomes in fraction 22-35, then incubated with anti-delta, that reacts with proteasomes in fractions 20-25, and finally with an antibody raised against whole rat muscle proteasomes to illustrate the distribution of proteasomes throughout the chromatographic eluate.

proteolytic activities of immuno-proteasome from spleen (subtype III and IV) towards this polypeptide are higher than that of subtype II, which contains intermediate type proteasomes (Figure 7(b)). Additionally, we compared the preferential cleavage site usage by these subtypes. After three hours of incubation when only small amounts of substrate are degraded and the generation of products is still in its linear phase, we have measured the generation of the peptide product residues 8-25 indicative for cleavage after Y7 and of peptide product residues 16-25 indicative for cleavage after L15. Product 16-25 is generated with 1.5-fold efficiency compared to product 8-25 by subtype II. Independently of the observation that both products are generated more efficiently by subtypes III and IV, the ratio of these two digestion products is reversed (Figure 7(c)). Thus, subtype II preferentially cleaves behind residue L15 while subtypes III and IV prefer cleavage behind Y7, indicating that subtle differences exist between the different proteasome subtypes with regard to their cleavage-site usage.

Discussion

The detailed biochemical analysis of proteasomes from mammalian tissues, in particular with regard to their hydrolysing activities, has so far been obscured by the heterogeneity of proteasome populations. In consequence, analysis of proteasomes isolated from a given tissue in most cases reflects the properties of the predominant subtype (either with regard to amount or activity) and neglects the others.

A major reason for the existence of different proteasome subtypes is the competition of ten different types of β-subunits for integration into the seven-membered β-ring. Seven of these ten β-subunits are constitutively expressed, but three, delta (β1), Z (β2), and MB1 (β5), can be replaced by the subunits LMP2 (β1i), MECl1 (β2i), and LMP7 (β5i), respectively, expressed under the control of interferon-γ, a process leading to the formation of immuno-proteasomes. Consequently, two main groups of proteasomes must exist, which, due to small differences in their surface charge, can be separated by chromatographic means.

At present it is not known whether with regard to the immuno-subunits the two halves of the proteasome-cylinder are built up by two identical sets of β-subunits. If unequal replacement in both halves is possible, 36 different arrangements of subunit composition are conceivable. If an identical subunit arrangement in both halves is exclusively

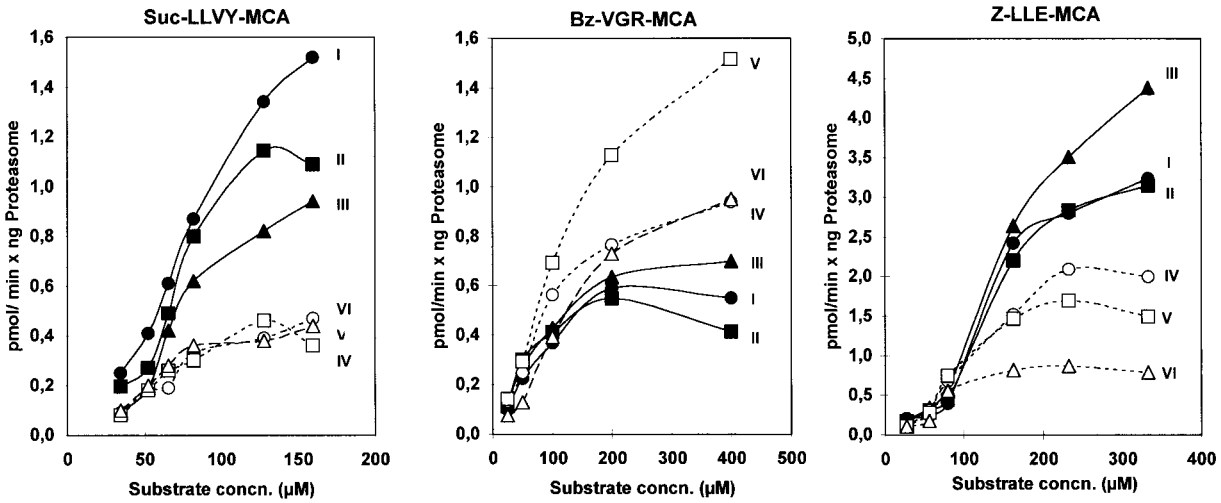

Figure 6. Velocity plots of fluorogenic peptide substrates with proteasome subtypes from rat skeletal muscle. Specific hydrolytic activities of muscle proteasome subtypes I-VI were measured with various concentrations of substrates Suc-LLVY-MCA, Bz-VGR-MCA, and Z-LLE-MCA, respectively. The data are mean values of two to four independent experiments.

allowed, this number is reduced to eight. Although theoretically all configurations may occur, investigations on the biogenesis of immuno-proteasomes have revealed that cooperative incorporation of the immuno-subunits is favoured (Groettrup *et al.*, 1997; Griffin *et al.*, 1998). Thus, MECl1 and LMP2 are mutually required for incorporation into proteasomes but independent of LMP7. On the other hand, incorporation of LMP7 is favoured in the presence of MECl1 and LMP2. Together, this might lead to the preferential formation of immuno-proteasomes, containing all three interferon-γ-inducible subunits. In addition, formation of proteasomes containing LMP7 as a single immuno-subunit or LMP2 plus MECl1 also appears to be possible. Hence, these data suggest the favoured biogenesis of three major forms of immuno-proteasomes. Our experiments show that the minor subtypes V and VI of muscle proteasomes contain only ''complete'' immuno-proteasomes, while subtypes III and IV are proteasomes with only one or two of the three immunosubunits and thus are intermediate-type proteasomes. On the other hand, subtypes I and II, the major constituents of muscle proteasomes, are pure constitutive proteasomes. As expected, this latter type of proteasome is, if at all, only scarcely present in spleen.

All these findings raise several questions: what are the structural differences between the different constitutive subtypes and on the other hand between the different complete immuno-subtypes? Post-translational modification such as phosphorylation could effect the small difference in surface charge between subtypes I and II as well as between V and VI, respectively. What is the exact subunit composition of intermediate-type proteasomes? Does their existence only result from the fact that during their biogenesis immunosubunits are not available in equal amounts, or do they have specialized functions that they fullfil only in certain types of cells or within a sequence of events, e.g. during immune response?

The latter suggestion is supported by our finding that the different subtypes actually differ in their enzymatic characteristics. Thus, a clear difference exists, in that the chymotrypsin-like activity of subtype I is higher than that of subtype II, while there exist only minor differences with regard to the trypsin-like and PGPH-activities between these two subtypes of constitutive proteasome complexes. The differences in proteolytic activities are also evident in their distinct rates of turnover of the pp89 25mer peptide. This becomes particularly obvious when the activities of the different subtypes towards various substrates are compared. For instance, muscle subtype I shows the highest chymotrypsin-like activity among the six subtypes but turns over the pp89 25mer peptide at the lowest rate. On the other hand, subtype V with low chymotrypsin-like and PGPH activity, but exhibiting the highest trypsin-like activity among these subtypes, reveals the highest turnover rate of the pp89 25mer peptide. Subtype IV exhibits a similiar peptide turnover rate, nevertheless, it has a lower trypsin-like activity than subtype V. When considering that the peptide bonds K20-R21 and R21-V22 in the 25mer peptide, that are potential trypsin-like cleavage sites are not split, these results do not present a straightforward interpretation.

The fact that different proteasome subtypes not only differ in their hydrolytic activities but also in their initial cleavage site usage, and thus in the quality of products generated, is demonstrated by our kinetic analysis of pp89 25mer digests. The

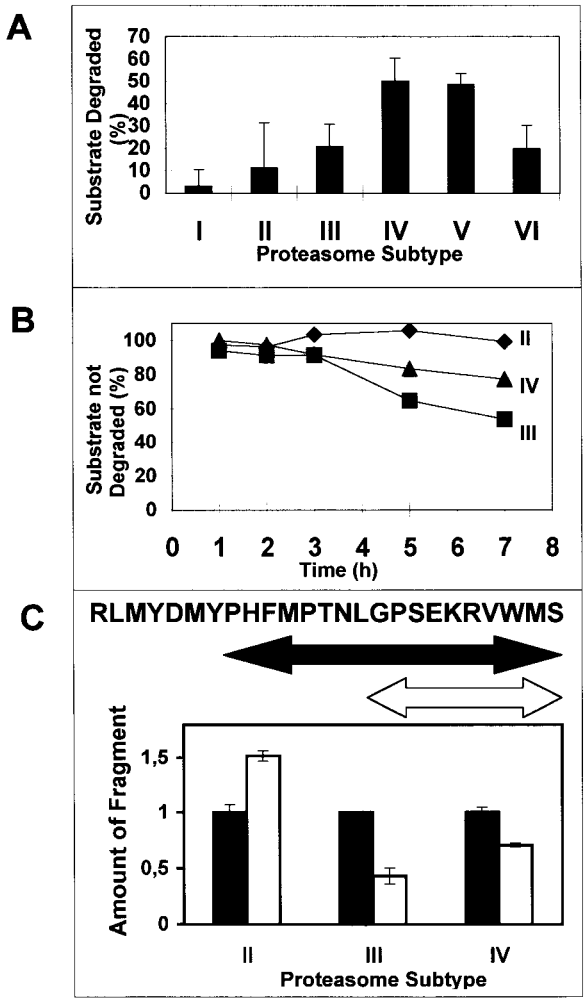

Figure 7. Degradation of murine cytomegalovirus IE protein pp98 (25mer polypeptide) by proteasome subtypes. (a) 10 µg of 25mer polypeptide RLMYDMYPHFMPTNLGPSEKRVWMS of MCMV IE protein pp89 was incubated with 1 µg proteasome subtype I-VI purified from rat skeletal muscle and incubated 16 hours at 37 °C. Degradation of the polypeptide substrate was measured by HPLC analysis and results given as percent degraded substrate peptide. Data are means ± S.D. of three to five independent experiments. (b) The relative amount of non-degraded substrate at the indicated time points is shown after incubation of 1 µg pp89 derived synthetic 25mer polypeptide with 0.5 µg of the 20 S proteasome subtypes II, III, and IV from rat spleen. (c) Using the relative amounts of the peptide product 8-PHFMPTNLGPSEKRVWMS-25 (closed columns) indicative for cleavage after Y7 and of the product 6-GPSEKRVWMS-25 (open columns) indicative for cleavage after L15 the ratio of cleavage site usage by the subtypes II, III and IV after three hours is shown. For this purpose the intensity measured for the 8-25mer product generated by each subtype was arbitrarily set to 1. Data are means ±S.D. of two analyses.

analysis of the degradation process of pp89 25mer peptide by two immuno-proteasome subtypes from spleen, subtypes III and IV, and the intermediate-type subtype II, reveals that there is an inverse relationship in the extent of preferential cleavage of peptide bond L15-G16 *versus* peptide bond Y7-P8.

These data, in combination with the observation that subtype patterns differ with the various tissues investigated, indicate that the quality of proteasome activity may differ depending on the physiological state of a tissue or given cell type. It is not likely that all types of cells within a given tissue contain the same set of proteasome subtypes. As proteasomes from human erythrocytes can also be resolved into three or four different subtypes (data not shown), a given type of cell contains several proteasome subtypes and future investigations concerning the control of proteasome functions we will have to consider the existence of proteasome subtypes with potential specialized functions.

Materials and Methods

Materials

Chromatographic resins for purification of 20 S proteasomes including MiniQ PC 3.2/3 were obtained from Amersham Pharmacia Biotech. Fluorogenic peptide substrates Suc-LLVY-MCA and Z-LLE-MCA were from Calbiochem, Bz-VGR-MCA from Bachem. The 25mer polypeptide (RLMYDMYPHFMPTNLGPSEKRVWMS) deriving from the murine cytomegalovirus IE pp89 protein was synthesised according to standard procedures. Subunit-specific antibodies K93251 (anti-MB1 (β5)), mcp168 (anti-Z (β2)), mcp421 (anti-delta (β1)) were obtained from Dr K. Hendil (Copenhagen, Denmark); K63 (anti-LMP7 (β5i)), K864 (anti LMP2 (β1i)), MC14 (anti-MECl1 (β2i)) were raised in rabbits. Anti-mouse IgG and anti-rabbit IgG alkaline phosphatase-conjugated secondary antibodies were obtained from CALTAG and Roche Diagnostics, respectively.

Animals

For all experiments, male Wistar rats of about 180 g body weight were used. Rats were killed by an overdose of diethyl ether, and hindleg skeletal muscles, liver, spleen, and kidneys were excised. The tissues were used directly for preparation of proteasomes or were immediately frozen in liquid nitrogen and stored at −80 °C until use.

Determination of proteolytic activities

Fluorogenic peptide hydrolysis by proteasomes was measured by incubation of 10 µl enzyme solution with 20 µl of 100 µM substrate solution and 10 µl of substrate buffer, which was 50 mM Caps, 0.5 mM dithiothreitol, 1 mM NaN_3 (pH 10.5) for Bz-VGR-MCA, and 20 mM Tris HCl, 1 mM EDTA, 1 mM NaN_3, 1 mM DTT, (pH 7.5) (TEAD buffer), for Suc-LLVY-MCA, Z-LLE-MCA as well as for Z-GGL-MCA. After 20-60 minutes of incubation at 37 °C the enzymatic reaction was stopped by addition of 200 µl of 100 mM sodium chloroacetate dissolved in 30 mM sodium acetate, 70 mM acetic acid (pH 4.3). Fluorescence was measured at 355 nm exitation and 460 nm emission.

Purification of 20 S proteasomes

All purification steps were performed at 4-6 °C. Proteasomes were monitored by their proteolytic activity towards Suc-LLVY-MCA. 20 S proteasomes were isolated either by the method described (Dahlmann *et al.*, 1985) or as by-product of the procedure for isolation of 26 S proteasomes (Dahlmann *et al.*, 1995). During the latter procedure, proteasomes are eluted from TSK-Fractogel DEAE 650S resin by a linear gradient of 75 mM-400 mM KCl. 20 S proteasomes elute at a KCl concentration of 160 mM just ahead of the 26 S proteasome peak. Fractions containing 20 S proteasomes were pooled and the enzymes concentrated by ultracentrifugation for 16 hours at 100,000 *g*. 20 S proteasomes were then further purified by gel filtration on Superose 6 (2 cm × 50 cm) in TEAD buffer (flow rate 25 ml/hour; fraction size 1 ml) and chromatography on Mono Q in TEAD buffer (Dahlmann *et al.*, 1985). Thereafter, $(NH_4)_2SO_4$ (final concentration 1.2 M) was added to the 20 S proteasome containing solution. This was then subjected to phenyl-Superose and proteins bound to the resin were eluted from the column by a linear decreasing gradient of $(NH_4)_2SO_4$. 20 S proteasomes eluted from the column were extensively dialysed against TEAD buffer. All proteasome preparations were of apparent homogeneity as judged by SDS-PAGE.

Separation of proteasome subtypes

Purified 20 S proteasomes were further separated by chromatography on Mini Q in conjunction with the SMART system (Amersham Pharmacia Biotech). The column was equilibrated in TEAD buffer. Proteasomes (50 μg) bound to the resin were eluted at a flow rate of 0.2 ml/min by a 6 ml linear increasing gradient of 0 M-0.5 M NaCl in TEAD buffer. Fractions of 0.02 ml were collected. For investigation of the enzymatic properties of proteasome subtypes the fractions comprising a subtype were pooled and re-chromatographed on Mini Q for complete separation from other subtypes. Thereafter, the enzyme solution was dialysed against TEAD buffer.

Electrophoretic techniques

SDS-polyacrylamide gels of 12.5 % (w/v) polyacrylamide concentration were run according to the method of Laemmli (1970). The first dimension of two-dimensional gel electrophoresis was performed under non-equilibrium pH-gradient electrophoresis (NEPHGE) conditions using pH gradients between 3 and 10 (O'Farrell *et al.*, 1977; Wehren *et al.*, 1996).

Digestion and analysis of fragments of pp89 25mer polypeptide

Samples of 10 μg of pp89 25mer polypeptide and 0.5 or 1 μg of purified 20 S proteasome subtypes were incubated in 100 or 400 μl, respectively, of 20 mM Hepes/NaOH, 2 mM $MgCl_2$, 1 mM dithiothreitol, (pH 7.8) at 37 °C for various periods of time as indicated in the Figure legends. The reaction was stopped by freezing the mixtures, and the fragments were separated by HPLC. The extent of digestion was determined by measuring the peak height of undigested 25mer polypeptide and the fragments were identified by mass spectrometry (Ossendorp *et al.*, 1996; Theobald *et al.*, 1998).

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