20S proteasome from LMP7 knock out mice reveals altered proteolytic activities and cleavage site preferences

R. Stohwasser[a], U. Kuckelkorn[a], R. Kraft[b], S. Kostka[b], P.-M. Kloetzel[a,*]

[a] *Institut für Biochemie der Charité, Humboldt Universität, Berlin, Hessische Str. 3–4, D-10115 Berlin, Germany*
[b] *Max Delbrück Centrum für Molekulare Medizin, Robert-Rössle Str. 10, D-13122 Berlin-Buch, Germany*

Received 22 August 1995; revised version received 17 November 1995

Abstract 20S proteasomes of tissues from LMP7 knock out mice which show reduced MHC class I restricted antigen presentation were analyzed with regard to their subunit composition, peptide hydrolyzing activity and their ability to cleave a synthetic 25-mer polypeptide. LMP7 deficiency results in an enhanced incorporation of subunit MB1 and in a 2–3.8-fold increase in V_{max} for the Suc-LLVY-MCA hydrolyzing activity. Since LMP7 deficiency also affects the cleavage site preference of 20S proteasomes the reduced MHC class I antigen presentation of LMP7 knock out mice is most likely due to an impairment in peptide generation.

Key words: 20S Proteasome; LMP7 knock out mouse; Subunit MB1 peptidase activity

1. Introduction

The 20S proteasome is the proteolytic core of several intracellular high molecular mass multisubunit proteinase complexes. ATP-dependent assembly of the 20S proteasome and a regulatory 19S complex results in the formation of the 26S proteasome, which is involved in ubiquitin-dependent protein degradation [1]. Association of 20S proteasome with the 11S regulator complex constitutes another proteinase complex which stimulates the peptide hydrolyzing activity and the proteinolytic activity in vitro [2,3]. With regard to function the 20S proteasome seems to occupy a central role in intracellular protein metabolism, being involved in processes such as cell cycle [4], apoptosis [5], transcriptional regulation [6], and cellular immune response [7]. Its role in the generation of peptides which bind to MHC class I molecules was recently demonstrated by the use of inhibitors of proteasome function [8].

The 20S proteasome complex is a dynamic cellular structure, which conditionally can alter its subunit composition and function. Three of the 14 constitutively expressed subunits can be supplemented by the homologous subunits, MECL1, LMP2 and LMP7, which are incorporated into the 20S proteasome complex in response to interferon-γ (IFN-γ). The incorporation of LMP2 results in the substitution of subunit δ, MECL1 replaces subunit Z and LMP7 substitutes subunit MB1 [9–11].

The IFN-γ inducible LMP2 and LMP7 subunits which are encoded within the MHC class II region in close neighbourhood to the peptide transporter genes TAP1 and TAP2 are non-essential with regard to the viability of cells and their presence is not a prerequisite for antigen presentation [12,13]. Nevertheless, they are assumed to play an important role in modifying the antigen processing capacity of a cell [14,15]. The mechanism by which single subunits may alter the peptide set presented to $CD8^+$ T lymphocytes is still controversial, but from cytokine induced and transfectant cell lines there exists experimental evidence that LMP incorporation changes proteasome subunit composition and the proteasomal peptide and polypeptide cleavage properties [15–17]. Furthermore, recent results from LMP2 and LMP7 knock out mice [18,19] show that LMPs can affect MHC class I expression at the cell surface. Thus MHC class I surface expression of H-2K, H-2D and I-A molecules and presentation of an endogenous self-antigen was significantly reduced in LMP7 deficient mice compared with that of wildtype littermate controls [19]. In contrast, breeding, health status and the numbers of T and B lymphocytes in the peripheral lymphoid organs of mice was not influenced by the LMP7 deficiency [19]. To explain this observation the authors discussed the possibility that the LMP7 subunit might be a docking molecule that physically links the proteasome and the TAP transporters to increase the efficiency of peptide translocation into the endoplasmic reticulum or alternatively, that LMP7 alters the cleavage properties of the 20S proteasome complex and in consequence influences the generation of antigenic peptides [19].

In the present communication we therefore isolated 20S proteasomes from LMP7 knock out mice and analysed their subunit composition and proteolytic activities in response to the LMP7 deficiency.

2. Materials and methods

2.1. Purification of 20S proteasomes from mouse tissue

Proteasomes from wild-type mice and from mice lacking LMP7 (C57BL/6 or 129/O1a; H-2^b [19]) were purified in parallel and were stored under identical conditions at each step of the purification procedure.

Tissues of 10 mice were dispersed (T25 Ultra-Turrax /S25 N-10 G; Janke & Kunkel, Staufen, Germany) in 10 (thymus, spleen) to 30 ml (liver) lysis buffer A (80 mM KAc, 5 mM $MgAc_2$, 10 mM HEPES, pH 7.2) and were further processed using a Dounce homogenizer. The purification procedure was continued by DEAE-Sephacel chromatography, sucrose gradient centrifugation and Mono-Q chromatography [3]. Protein quantification, analysis of 20S proteasome subunit composition by non-equilibrium two-dimensional electrophoresis and measurement of peptidase activities were carried out as previously described [15].

2.2. Assay of 20S polypeptide cleavage properties

To analyze the polypeptide cleavage properties we used a synthetic

*Corresponding author. Fax: (49) (30) 2846-8217.

Abbreviations: HPLC, high performance liquid chromatography; MCA, 7-amido-4-methylcoumarin; MHC, major histocompatibility complex; NEPHGE, non-equilibrium pH gradient electrophoresis; PAGE, polyacrylamide gel electrophoresis; Suc, succinyl; TAP, transporter associated with antigen presentation.

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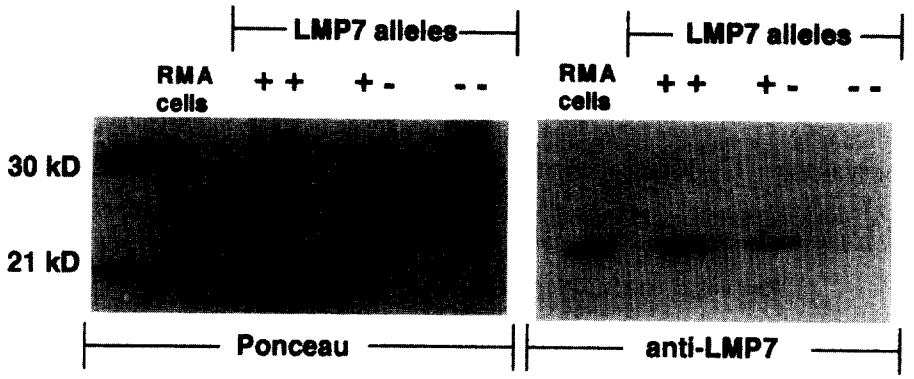

Fig. 1. Analysis of 20S proteasomes from LMP7 knock out mice by immunoblot analysis. Left panel: Ponceau-stained SDS-PAGE pattern of sucrose gradient purified liver 20S proteasomes from LMP7-deficient (−−) knock out mice, from heterozygous mice (+−) or from wild-type littermate controls (++). Only the proteasome subunit region of the blot is shown. Right panel: immunoblot probed with polyclonal rabbit anti-LMP7 antiserum [21]. 5 μg of purified RMA cell derived mouse 20S proteasome were loaded as control.

25-mer polypeptide RLMYDMYPHFMPTNLGPSEKRVWMS derived from the sequence of murine cytomegalovirus pp89 IE protein [20]. The 25-mer peptide covers the antigenic nonamer region (underlined). 20 μg peptide were dissolved in 300 μl of buffer (20 mM HEPES/NaOH, pH 7.8, 2 mM $MgAc_2$, 1 mM dithiothreitol) and digested with 1 μg purified proteasome for 48 h at 37°C. Kinetic studies revealed, that proteolysis was completed after 24 to 48 h [3] (data not shown).

75 μl of the peptide mixtures were separated by reversed HPLC using a mRPC C2/C18; SC2, 1/10 column, Pharmacia LKB, and Pharmacia LKB equipment (SMART system). Eluents: Solution A = 0.1% TFA in water (v/v); Solution B = 0.1% TFA in acetonitrile; flow rate = 100 μl/min. Gradient used: 0–10 min, 10% B; 10–70 min, linear increase to 50% B; 70–90 min, 90% B, 90–95 min held at 90% B; 95–105 min, decrease to 10% B; 15 min equilibrated at 10% B before the start of a new run.

3. Results and discussion

3.1. *Proteasomes from LMP7^{--} mice reveal altered subunit composition*

To study the influence of LMP7 deficiency on the biochemical properties of the 20S proteasome the enzyme complex was isolated from tissues of mice which carry or lack the LMP7 gene. Previously, the absence of the LMP7 gene was confirmed only by Southern blot analysis [19]. Therefore to ascertain the erased expression of the LMP7 protein and to exclude the occurrence of additional non-MHC-linked LMP7 or LMP7-like genes we checked LMP7 subunit expression by immunoblotting with an LMP7 specific antibody. Partially purified proteasome from liver of wild-type (LMP7^{++}) mice and mice heterozygous (LMP7^{-+}) or homozygous (LMP7^{--}) with respect to the LMP7 deletion was subjected to SDS-PAGE and immunoblot analysis. As shown in Fig. 1 LMP7 can be detected in proteasomes of LMP7^{++} mice, but is absent in LMP7^{--} mice. In heterozygous LMP7^{+-} mice the LMP7 subunit is also detectable. Since we did not obtain any other immune-signal these data demonstrate that there exists no other LMP7 gene or LMP7 homologous gene which encodes a protein which can be detected by the LMP7 antibody. As calculated from the amount of purified RMA cell proteasome loaded (5 μg) and the LMP7 signal obtained by immunoblotting only approximately 30% of the mouse liver proteasomes contain the LMP7 subunit. This is in agreement with previous data showing that different tissues and cell lines can differ in their relative amount of LMP containing 20S proteasomes [21].

Presuming that altered MHC class I surface expression in LMP7-deficient mice might be due to alterations of subunit composition and in consequence proteasome function we purified proteasomes of thymus, spleen and liver and analyzed proteasomal subunit composition by 2D gel electrophoresis. 20S proteasome purified from thymus (Fig. 2A) reveals a 20S proteasome pattern of 16 subunits in a molecular range of 21–31 kDa. Additional protein spots are most likely due to modifications of proteasomal subunits. Individual subunits were identified by immunoblot (LMP7, LMP2) and protein sequencing (MB1, LMP2 and LMP7). Comparison of subunit pattern of LMP7^{++} and LMP7^{--} proteasome reveals that the disappearance of the LMP7 protein is accompanied by an increased incorporation of the homologous subunit MB1 into the 20S proteasome (Fig. 2A–C). The absence of LMP7 has no obvious influence on the amount of LMP2 or any other proteasome subunit as has been suggested before [14,22].

3.2. *Absence of LMP7 increases the peptidase activities both in tissue and transfectant 20S proteasome*

To determine the influence of LMP7 deficiency on the peptidase activities of 20S proteasomes in tissues of LMP7 knock out mice we monitored the enzymatic activities using the fluorogenic peptide substrates Suc-LLVY-MCA (chymotrypsin-like; CHT-L), Z-LLE-βNA (peptidyl-glutamyl-peptide-hydrolyzing activity; PGPH) and Bz-VGR-MCA (trypsin-like) (Fig. 3). Analysis of the kinetic parameters reveals that LMP7 deficiency results in an approximately 74% increase of the CHT-L activity of 20S proteasome from thymus. In contrast, the lack of LMP7 had almost no significant effect on the PGPH activity and the trypsin-like hydrolytic activity (Table 1; Fig. 3A–C). As judged by the chymotrypsin-like activity the lack of LMP7 had no significant influence on $S_{0.5}$ or on the positive cooperative between proteasome subunits as determined by the Hill coefficient (Table 1). These results are in good agreement with data obtained with proteasomes from the transfectant human T2/LMP2^{+}/LMP7^{+} ('wild-type situation') and the T2/LMP2 cell line which expresses LMP2 but lacks LMP7 (Table 2 [15]). In 20S proteasomes from thymus and spleen of LMP7 knock out mice the increase in CHTL

activity coincides with the enhanced incorporation of subunit MB1 (Fig. 2A–C). In contrast there exists no detectable increase in MB1 incorporation into liver 20S proteasomes (Fig. 2D).

Different tissues have been shown to differ in their amount of LMP mRNAs and LMP protein levels [21]. Hepatocytes contain low amounts of LMP mRNA. Nevertheless LMP7 can be detected by Western blot analysis of sucrose gradient fractions from liver extracts (Fig. 1) which is most likely due to higher levels of LMP7 in liver macrophages.

Purification of 20S proteasomes was performed under conditions known to completely remove the 11S regulator and amino-peptidases which could influence enzyme kinetics [23]. Minor amounts of high molecular weight proteins present in some proteasome preparations were identified as 19S regulator components of the 26S protease (data not shown). However, independent proteasome preparations did not affect peptidase activity profiles in response to LMP7 deletion (Table 2). Furthermore experiments in which the aldehyde inhibitor *N*-benzyloxycarbonyl-Ile-Glu-(*O*-*t*-butyl)-Ala-leucinal (Z-IE-(OtBu)-AL-CHO) was used [24], demonstrated that the proteasomal chymotrypsin-like activity is inhibited in a dose-dependent manner (Fig. 3F). An inhibitor concentration of 0.25 μM reduces the CHTL activity down to 44% and 42%, respectively, whereas PGPH remains unaffected.

From our data it can be deduced that the presence of both LMP2 and LMP7 in 20S proteasomes of different mouse tissues results in a relative down regulation of Suc-LLVY-MCA hydrolyzing activity since proteasomes of tissues with low LMP2 levels such as liver (Fig. 3E) reveal a higher Suc-LLVY-MCA hydrolyzing activity than proteasomes of tissues with high LMP2 content (thymus, spleen; Fig. 3A,D). This stands in contrast to experiments performed with crude extracts of LMP2 knock out mice (i.e. $LMP7^{+}$ mice) in which a relative decrease of Suc-LLVY-MCA hydrolyzing activity was observed [18]. Crude extracts contain a number of other proteinase complexes for which Suc-LLVY-MCA can serve as substrate [3,23]. Therefore enzyme activity measurements of independently obtained tissue extracts without chromatographic purification of the proteasome complex may lead to contradictory results.

In summary, our experiments show that despite the fact that tissues normally represent a collection of different cell types the enzymatic properties of 20S proteasomes from tissues of mutant mice are compatible with data obtained with 20S proteasome of clonal cell lines [15].

3.3. *Polypeptide cleavage properties of $LMP7^{--}$- and $LMP7^{++}$-20S proteasomes are different*

To address the question whether deletion of LMP7 influ-

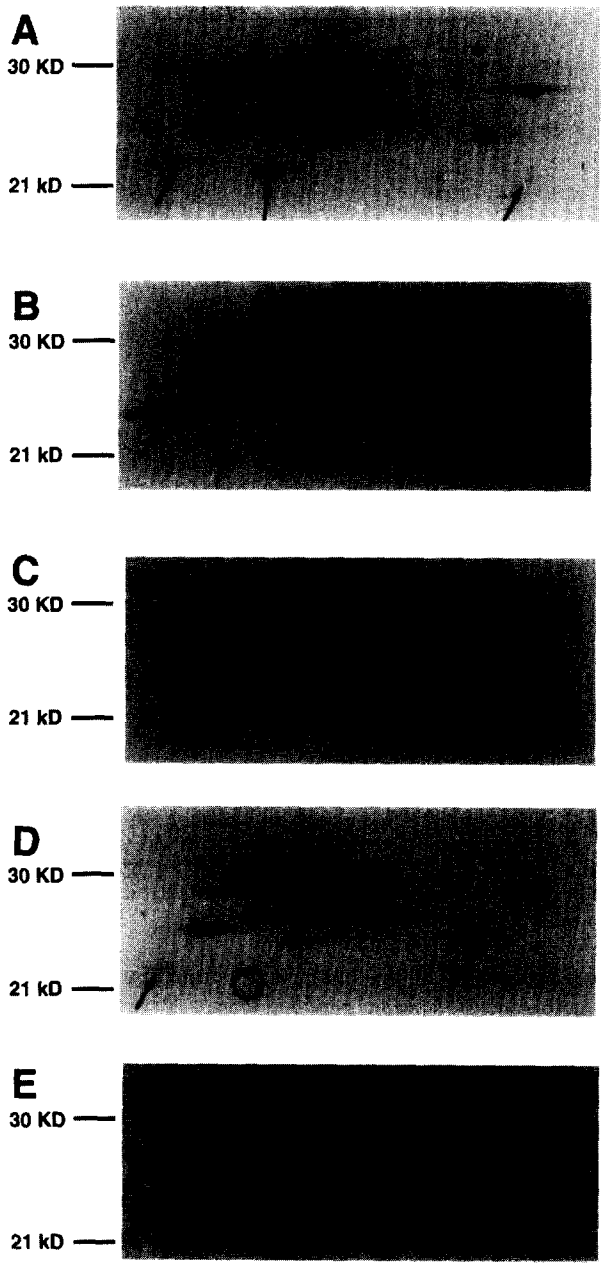

Fig. 2. Two-dimensional gel electrophoresis and silver staining of purified 20S proteasomes (10 μg) isolated from thymus (A,B), spleen (C) and liver (D). (A) Demonstrates the wild-type proteasome pattern of control mice ($LMP7^{++}$). (B–D) shows tissue specific pattern from mice lacking LMP7 ($LMP7^{--}$). (E) Identification of proteasomal subunits: 14 = MB1, 15 = LMP7, 16 = LMP2. Arrows in panel A mark subunits MB1, LMP7 and LMP2. Arrows in panel B, C and D mark subunit MB1, whose amount is increased, and subunit LMP2. Subunit LMP7 is missing (circle).

Table 1
Kinetic parameters of hydrolysis of fluorogenic peptides performed by 20S proteasomes from thymus

	Suc-Leu-Leu-Val-Tyr-MCA		Bz-Val-Gly-Arg-MCA		Z-Leu-Leu-Glu-βNA	
	$LMP7^{++}$	$LMP7^{--}$	$LMP7^{++}$	$LMP7^{--}$	$LMP7^{++}$	$LMP7^{--}$
V_{max}	0.72 ± 0.02	2.72 ± 0.07	3.46 ± 0.46	3.53 ± 0.27	8.78 ± 0.69	8.88 ± 0.24
$S_{0.5}$	28.30 ± 2.20	27.40 ± 1.60	115.9 ± 38.8	197.9 ± 32.1	221.8 ± 27.8	124.6 ± 5.7
H	2.04 ± 0.35	2.49 ± 0.39	1*	1*	1.40 ± 0.10	1.64 ± 0.07

For data calculation, a kinetic Hill model was used: $v = V_{max}/[1 + (S/S_{0.5})^H]$ [27]. Data are mean values ± standard error of the mean (S.E.M.) from three separate determinations ($n = 3$). V_{max} = maximal reaction velocity in nmol/h/μg proteasome; $S_{0.5}$ = Michaelis constant in μM; H = Hill coefficient.
*Value was fixed to $H = 1$ because of the large redundancy between $S_{0.5}$ and H.

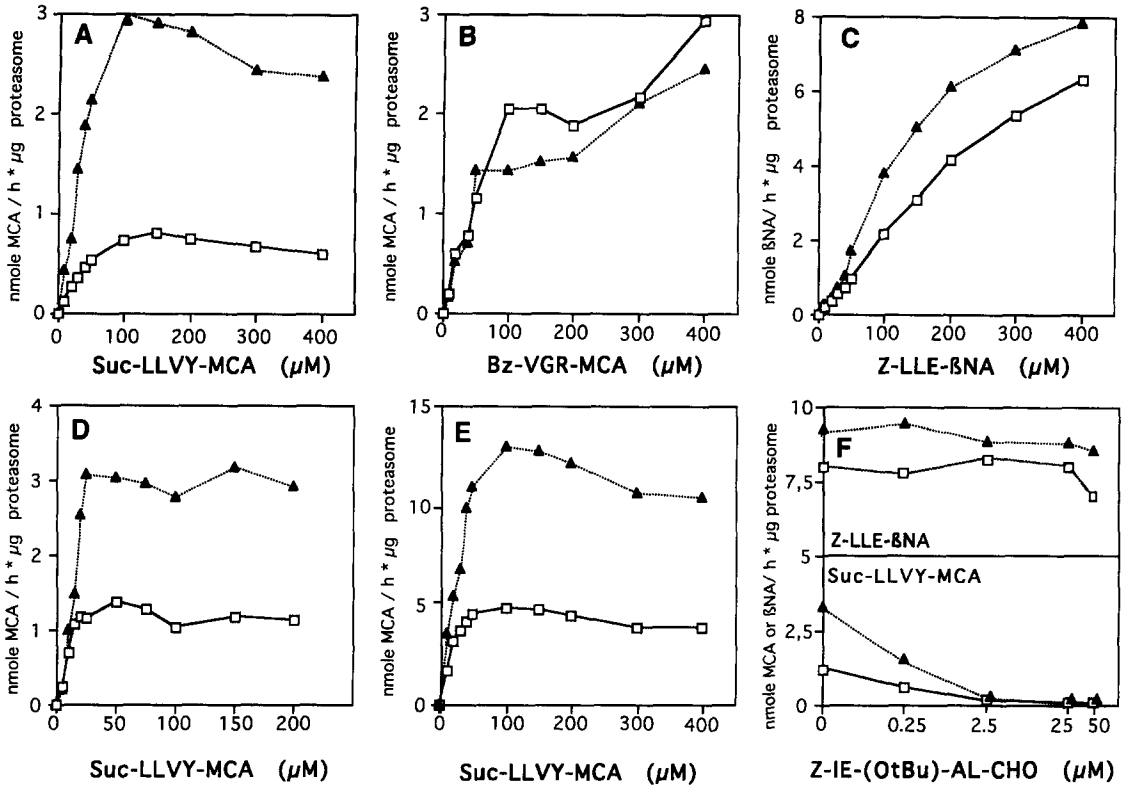

Fig. 3. Effect of LMP7 deficiency on peptidase activities of proteasomes isolated from thymus (A, B, C and F), spleen (D), and liver (E). Fluorogenic peptide substrates were digested at indicated concentrations by application of 100 ng purified proteasome. Filled symbols are indicating LMP7^{--} knock out proteasomes, LMP7^{++} proteasomes isolated from wild-type control mice are symbolized by open symbols. (F) Inhibition of chymotrypsin-like, but not of the PGPH activity by proteasome inhibitor Z-IE-(OtBu)-AL-CHO. Fluorogenic peptide substrates were applied at a concentration of 400 µM.

ences the ability of the proteasomes from tissue of mutant mice to generate specific peptides from a larger peptide substrate we used a synthetic 25-mer peptide derived from the sequence of Murine Cytomegalovirus pp89 IE protein [20] as substrate for proteasomes. The 25-mer peptide covers the antigenic nonameric T-cell epitope (H-2D) of pp89. Peptide digests were subjected to HPLC analysis. As shown in Fig. 4 the cleavage pattern generated by 20S proteasomes from mouse thymus or spleen differs significantly dependent on the presence or absence of the LMP7 subunit. For more detailed analysis the sequence of the cleavage products collected from several peaks was determined by N-terminal peptide sequencing. The analysis of HPLC product files reveals that LMP7 influences the cleavage site preference of the 20S proteasome. Thus the relative amount of the DMYPHFMPTNL 11-mer product (peak 4, aa positions 5–15) generated by dual cleavages and of peptide GPSEKRVWMS (Peak 1, aa positions 16–25) are reduced in LMP7^{--} proteasome digests. On the other hand proteasomes from LMP7 knock out mice generate more efficiently the PHFMPTNL 8-mer peptide (peak 3, aa positions 8–15). The increase in the P_8–L_{15} 8-mer generated by dual cleavages is accompanied by an increase of peptide RLMYDMY (aa positions 1–7). Thus LMP7 deficiency in proteasomes of mouse tissues leads to a change in cleavage site preference.

Recently we were able to show that using purified 20S proteasome in in vitro antigen processing experiments closely reflects the in vivo situation [25]. Thus based on the experiments reported here, the previously observed reduction in MHC class I surface expression in LMP7 knock out mice

Table 2
Comparison of V_{max} of hydrolysis of fluorogenic peptides in tissues and transfectant T2 cell lines

Substrate	V_{max} of peptide hydrolysis in various LMP phenotypes				Ref.
	LMP2$^+$ LMP7$^+$	LMP2$^+$ LMP7$^-$	Percentage of change	Origin of proteasome	
Suc-L-L-V-Y-MCA	0.72 ± 0.02	2.72 ± 0.07	+74	Thymus	This work
Z-L-L-E-βNA	8.78 ± 0.69	8.88 ± 0.24	±0	Thymus	This work
Bz-V-G-R-MCA	3.46 ± 0.46	3.53 ± 0.27	±0	Thymus	This work
Suc-L-L-V-Y-MCA	1.20 ± 0.04	3.00 ± 0.10	+60	Spleen	This work
Suc-L-L-V-Y-MCA	5.09 ± 0.22	9.99 ± 0.42	+49	Liver	This work
Suc-L-L-V-Y-MCA	9.89 ± 1.37	18.9 ± 1.40	+48	Liver	This work
Suc-L-L-V-Y-MCA	1.56	6.72	+77	T2 transfectant	[15]
Z-L-L-E-βNA	0.77	0.59	−23	T2 transfectant	[15]

Calculation of the 'percentage of change' value was performed by setting V_{max} of the LMP7 mutant as 100%. V_{max} in nmol/h/µg proteasome; data are mean values ± S.E. of the mean (S.E.M.) (n = 3). Data from two separate liver 20S proteasome purifications are shown.

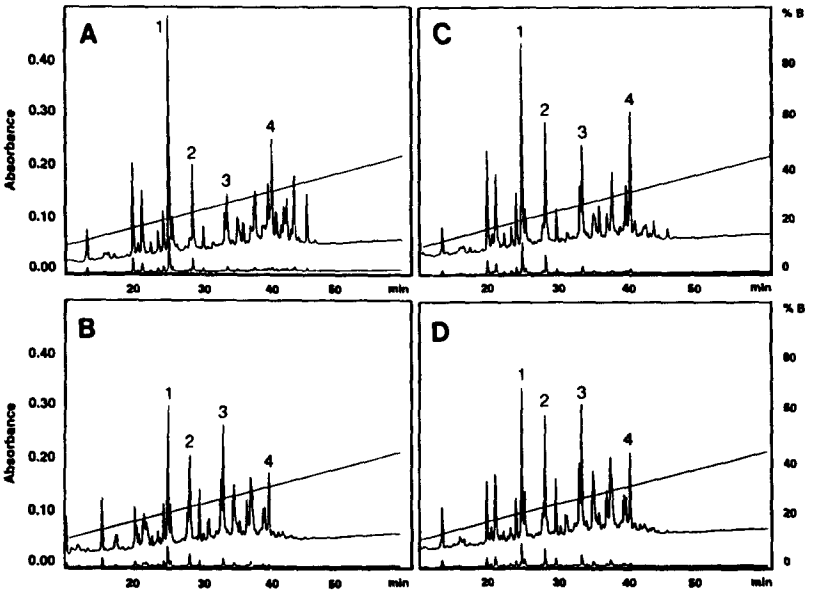

Fig. 4. Analysis of the polypeptide cleavage properties of 20S proteasomes from thymus (A, B) and spleen (C, D) of control mice (upper panels: A, C) and of mice lacking LMP7 (lower panels: B, D). MCMV pp89 protein derived synthetic 25-mer peptide RLMYDMYPHFMPTNLGPSEKRVWMS was digested for 48 h with 1 μg proteasome. Cleavage products were separated by reverse phase HPLC. Numbers are indicating N-terminal sequenced peak fractions: 1 = GPSEKRVWMS (aa positions 16–25); 2 = RLMYDMY (aa positions 1–7); 3 = PHFMPTNL (aa positions 8–15); 4 = DMYPHFMPTNL (aa positions 5–15).

may be explained by partial impairment of peptide generation due to the imbalance of LMP subunits. It has to be pointed out, however, that despite our increased knowledge on proteasomal antigen processing activities, precise predictions concerning potential effects of the LMP subunits on antigen presentation are so far not yet possible and observed positive or negative effects may have been dependent on the type of antigen analysed [26].

Acknowledgements: We thank Dr. H.J. Fehling for providing tissues from LMP7 knock out mice. We acknowledge Dr. H. Holzhütter for evaluation of kinetic measurements, Dr. P. Henklein for the synthesis of peptides, and Inga Becker for technical assistance. This work was supported by the Deutsche Forschungsgemeinschaft (DFG K1 427/5-1).

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