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RESEARCH ARTICLE

An HLA-B27 Homodimer Specific Antibody Recognizes a Discontinuous Mixed-Disulfide Epitope as Identified by Affinity-Mass Spectrometry

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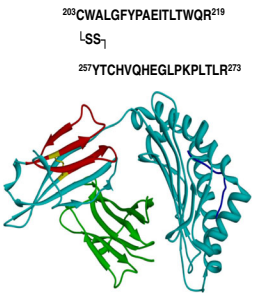

Abstract. HLA-B27 homodimer formation is believed to be a hallmark of HLA-B27 associated spondyloarthritides. Recently, we have generated a homodimer-specific monoclonal antibody (HD6) and have demonstrated that HLA-B27 homodimer complexes are present on monocytes of healthy *HLA-B27* gene carriers at low levels, with significantly increased levels at active disease. The capability of the HD6 antibody to discriminate between correctly formed HLA-B27 heterotrimers and pathology-associated homodimers is striking and cannot be explained by the primary structure of HLA-B27. We hypothesized that HD6 accesses a unique epitope and used affinity-mass spectrometry for its identification. The HD6 antibody was immobilized on an activated sepharose affinity column, and HLA-B27 homodimer characterized for affinity. The epitope was identified by proteolytic epitope excision and MALDI mass spectrometry, and shown to comprise a discontinuous Cys-203- 257-Cys mixed-disulfide peptide structure that is not accessible in HLA-B27 heterotrimers due to protection by noncovalently linked β2-microglobulin. The epitope peptides were synthesized by solid phase peptide synthesis, and the two monomeric peptide components, HLA-B27(203-219) and HLA-B27(257-273), as well as the homo- and hetero-dimeric disulfide linked combinations prepared. The affinity binding constants K_D towards the antibodies were determined using a surface acoustic wave (SAW) biosensor, and showed the highest affinity with a K_D of approximately 40 nM to the HD6 antibody for the (203-219)-SS-(257-273) mixed disulfide epitope.

Keywords: Affinity mass spectrometry, Proteolytic epitope excision, HLA B27, *Ankylosing Spondylitis*, Mixed-disulfide linked epitope, Affinity quantification

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Introduction

The human leukocyte antigen (HLA) covers the genes coding for the major histocompatibility class I complex. HLA- B27, an allele present in only some individuals, is a major susceptibility factor for the development of *Ankylosing spondylitis* (AS) and other spondyloarthritides, a group of arthritis diseases primarily affecting the spine [1–3]. The canonical HLA-B27 heterotrimeric structure comprises a HLA heavy chain noncovalently associated with a monomorphic β2-microglobulin (β2m) light chain, and a short peptide derived from self-proteins, viruses, or bacteria. The HLA-B27 heterotrimer complexes form in the endoplasmic reticulum and egress to the cell surface where they are recognized by CD8+ cytotoxic T cells through their T cell receptors [4]. However, unusual biochemical properties of HLA-B27 include its ability to misfold and form cell-surface β2m-free heavy

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chain homodimers ($B27_2$) [5, 6] that are thought to influence inflammatory responses [7, 8]. It remains hitherto undetermined how the interaction of $B27_2$ leads to disease. However, it has been suggested that ligation of $B27_2$ with immunoregulatory receptors may upset this balance in favor of a pro-inflammatory response [8–10]. Cell-surface $B27_2$ has been detected in B27-transfected cell lines, peripheral blood mononuclear cells (PBMCs) of AS patients, and leukocytes from B27-transgenic rats [8, 11–13].

Unlike most other class I molecules, HLA-B27 possesses an unpaired cysteine residue (Cys67) within the B pocket. This residue has been implicated in the formation of $B27_2$ homodimers [14]. Since these $B27_2$ homodimers do not contain β2m and adopt different conformations to the HLA-B27 heterotrimers, their binding to immunoregulatory receptors does not depend on peptide presentation but on yet unidentified aberrant $B27_2$ interactions. The use of blocking antibodies to reduce or inhibit the interaction of $B27_2$ to ligands is of particular interest due to its potential role in the pathogenesis of HLA-B27-associated diseases.

We have previously reported on a monoclonal antibody (HD6) that recognizes B27 β2m-free-heavy chain forms and $B27_2$ homodimers [13, 15]. The antibody has been generated by screening a human phage display library against an in-house obtained recombinant HLA-$B27_2$. The HD6 antibody has been used as a diagnostic tool to establish that the *HLA-B27* allele in patients correlates with low-level expression of $B27_2$ on monocytes and B-lymphocytes [13]. Furthermore, HD6 has shown that $B27_2$ expression significantly increases in blood and synovial fluid derived monocytes in *HLA-B27*+ patients with clinically active spondylitis [13].

HD6 recognizes an epitope that is not accessible or present when HLA-B27 forms correctly as a heterotrimer and is, therefore, a unique hallmark of the free HLA-B27 heavy or the $B27_2$ homodimer, retrospectively. Since its identification might help to better understand the process of HLA-B27 misfolding, we employed an affinity mass spectrometric approach according to the proteolytic-mass spectrometry method developed and established in our laboratory [16]. The HLA-$B27_2$ was bound to the antibody column, the immobilized immune complex digested with trypsin, and after washing off unbound material, the eluted epitope peptides were identified by MALDI mass spectrometry. The epitope proved to be a discontinuous structure comprising two linear peptide sequences linked by a mixed disulphide, HLA-B27(203-219)-SS-(257-273). The two linear epitope peptides were synthesized by solid phase peptide synthesis (SPPS) and incubated under oxidative conditions, which in the presence of the antibody provided the heterodimeric epitope as the prevailing species eluted from the affinity column. Using a surface-acoustic wave (SAW) biosensor and affinity-MS for affinity confirmation, the affinity constants K_D were determined for all epitope-containing peptides (linear sequences, homo- and heterodimer), and the highest affinity of ca. 40 nM was established for the heterodimer peptide (203-219)-SS- (257-273).

Experimental

Proteolytic Epitope Excision and Affinity Characterization

An affinity column was prepared by loading activated sepharose on a 1 mL micro-column. The HD6 monoclonal antibody was loaded onto the column in sodium phosphate buffer (PBS), pH 7.15, and immobilized by incubation for 2 h at 37 °C. Following incubation, the free active sites were blocked with ethanolamine and the column washed and stored at 4 °C. For epitope excision, the HLA-$B27_2$ protein was loaded on the column in PBS and incubated for 2 h at 37 °C. After excess peptide was washed out and saved for gel electrophoretic analysis, trypsin was added and incubated for additional 2 h. The supernatant was collected and the column washed with PBS and water. The last mL of the washing fraction was collected as a control, and the bound epitope peptides eluted by 4 × 15 min incubation with 0.1% trifluoroacetic acid. Fractions were lyophilized and purified using the ZipTip procedure [17]. For affinity testing of the synthetic epitope peptides, the samples were loaded onto the affinity columns in PBS and incubated for 2 h at 37 °C. In the same manner, the supernatant, the last mL of the washing fraction, and the elution fraction were collected and prepared for MALDI mass spectrometry.

Gel Electrophoresis

For in gel proteolytic digestion and mass spectrometry, the excess HLA-$B27_2$ protein from the affinity column experiments was subjected to SDS- PAGE separation. The fraction (10 μL) was mixed with 10 μL running buffer, 4% SDS, 25% glycerol, and bromophenol blue staining reagent. After loading on a 15% gel, electrophoresis was developed for 15 min at 60 V, and subsequently for 1–2 h at 100 V. Visualization of the bands was performed by Coomassie Brilliant Blue staining.

In-Gel Tryptic Digestion and Peptide Extraction

The protein spot was cut from the gel and washed with MilliQ water. The gel piece was shaken for 30 min at 25 °C with 60% acetonitrile to dehydrate, and dried in a SpeedVac centrifuge (30 min). Coomassie Brilliant Blue spots were destained by addition of 50 mM NH_4HCO_3 (15 min), dehydration with 60% acetonitrile (15 min), and SpeedVac centrifugation (30 min). The gel pieces were then swollen in digestion buffer (12.5 ng trypsin/μL 50 mM NH_4HCO_3) at 4 °C for 45 min, and incubated for 12 h at 37 °C. After removal of supernatant, peptide extraction was performed at 25 °C with a solution of 60% acetonitrile, 0.1% TFA in MilliQ water (three steps of 1 h each). The tryptic digest mixture was analyzed by MALDI-TOF MS, and data were searched against the NCBInr protein database using the Mascot MS Ion search engine.

Synthesis and Purification of Epitope Peptides

The HLA-B27 (203-219) and HLA-B27 (257-273) epitope peptides were synthesized by solid phase peptide synthesis

(SPPS) on a semiautomatic peptide synthesizer (ResPepSL; Intavis Bioanalytical Instruments, Köln, Germany) according to the Fmoc strategy. The synthesis was carried out on a Rink Amide resin (GL Biochem Ltd., Shanghai, China), with a loading capacity of 0.51 mmol/g. The disulfide linked epitope heterodimer was obtained from an equimolar mixture of the two monomeric peptides at 1 mg/mL in a solution of DMSO and 25 mM ammonium acetate 1:1 (v:v), by incubation for 12 h at room temperature under agitation. Following incubation, the disulfide formation reaction mixture was diluted with 0.1% TFA in MilliQ water to reduce the DMSO content from 50% to 10%. The HLA-B27 peptides and the reaction mixture were purified by reversed phase high performance liquid chromatography (RP-HPLC) on a semi-preparative Vydac C12 column, using the mobile phases, 80% acetonitrile, 0.1% TFA in MilliQ water (eluent B) and 0.1% TFA in milliQ water (eluent A). A linear gradient of 0–60% eluent B was used in 60 min at a flow rate of 3 mL/min.

Mass Spectrometry

Lyophilized digestion samples, purified synthetic epitope peptides, and lyophilized affinity fractions were analyzed by MALDI-TOF-MS on a Waters-Micromass TofSpec 2E mass spectrometer (Waters Ltd., Manchester, UK) in linear mode. Aliquots of 0.8 µL of peptide sample were mixed on a metal target with 0.8 µL saturate α-cyano4-hydroxy cinnamic acid (CHCA) in acetonitrile:0.1% TFA in water 2:1 (v:v).

SAW Biosensor Determinations

Affinity K_D determinations were performed with a K5S Sens surface acoustic wave (SAW) biosensor (SAW Instruments, Bonn, Germany). Epitope peptides were immobilized on gold chips as previously described [17, 18] and different concentrations of the HD6 antibody injected. K_D determinations were performed by using OriginPro 7.5 software with the FitMaster plug-in, providing a pseudo first order kinetic constant (k_{obs}). Plotting k_{obs} against the concentration provided association (k_{on}) and dissociation constants (k_{off}), from which $K_D = k_{off}/k_{on}$ was determined.

Sequence Determinations

Sequence determination of the isolated epitope peptide and the synthetic peptides (203-214) and (257-273) was performed with an Applied Biosystems (Darmstadt, Hessen, Germany), model 494 Procise Sequencer attached to a model 140C Microgradient System, a 785A Programmable Absorbance Detector and a 610A Data Analysis System. For sequencing of both blotted and lyophilized samples, the corresponding standard pulsed liquid methods were employed. Prior to sequencing, the peptide samples were wetted with 100% methanol and applied to the sequencing cartridge.

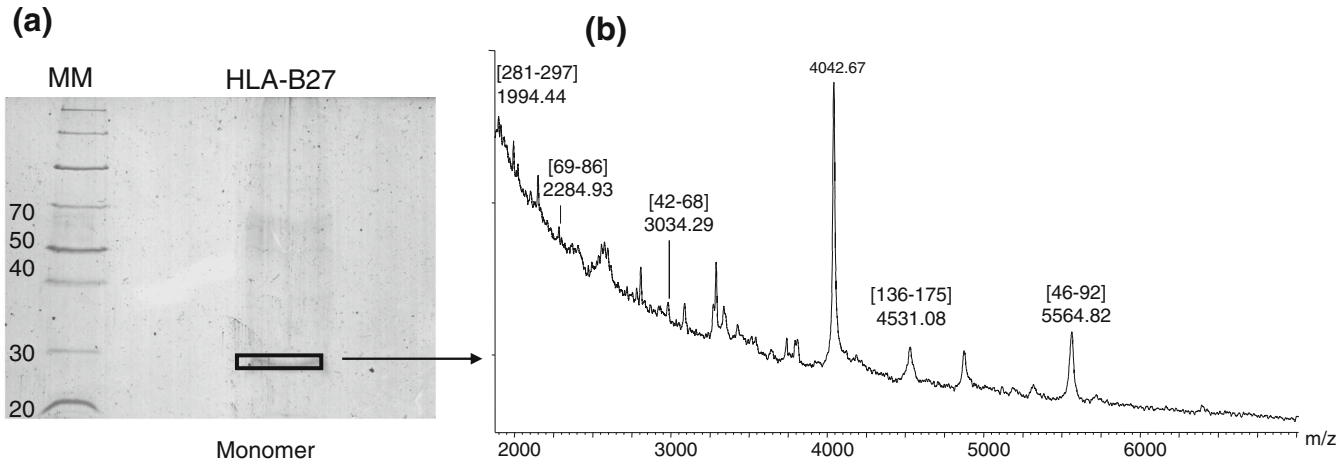
Results and Discussion

Primary Structure Characterization of the HLA-B27Antigen

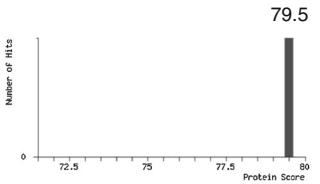
In order to identify the epitope of HLA-B27, the primary structure of the antigen was initially characterized in detail by proteolytic digestion and MALDI-MS. A sample of the free heavy chain homodimer, $B27_2$ was loaded on a sepharose affinity microcolumn containing the HD6 antibody. The eluted excess protein was collected and subjected to SDS-PAGE separation. Upon Coomassie Blue staining, a major band at approximately 30 kDa and a minor band around 60 kDa were observed, corresponding to the free heavy chain monomer and homodimer, respectively (Figure 1a). The monomer band was cut out from the gel and subjected to tryptic in-gel digestion, as described in the "Experimental" section. MALDI-TOF MS was employed to characterize the proteolytic mixture (Figure 1b) and the list of peptide masses obtained was searched against the NCIBnr database with the help of the Mascot database search engine. The MALDI-MS data provided the unequivocal identification of HLA class B27 protein (Figure 1c), thus confirming the molecular identity and homogeneity of the sample.

Affinity-Mass Spectrometric Identification of the HD6 Epitope

The HD6 monoclonal antibody was immobilized on a sepharose column and its affinity to the HLA-B27 ascertained by tryptic digestion and MALDI mass spectrometry. For epitope identification, an epitope-excision MS experiment was performed [17–20]. HLA-$B27_2$ was immobilized on the antibody column and digested with trypsin, and the affinity- eluted fractions subjected to MALDI-MS (Figure 2). After washing out the supernatant, the remaining affinity-bound epitope peptide(s) were eluted from the column with 0.1% TFA, and subjected to MALDI-MS analysis. The epitope proved to be discontinuous, comprising two linear partial peptides HLA-B27(203-219) and HLA-B27(257-273). As shown in Figure 2b, the affinity-elution fraction contained a single peptide mass at *m/z* 4042, consistent with the mixed disulfide, HLA-B27(203-219)-SS-(257-273) (Figure 2a). From the MALDI-MS analysis and upon dissociation, the HLA-B27 epitope was ascertained to comprise a mixed disulfide-bridged peptide, HLA-B27(203-219)-SS-(257-273) (see Figure 2b and location of the discontinuous epitope in the crystal structure, Figure 6). In addition, the epitope was confirmed by Edman sequencing of (i) the isolated epitope peptide, which upon DTT reduction and *N*-iodoacetamide alkylation provided the N-terminal partial sequence pairs, H-C/Y – W/T – A/C – L/H – G/V –, whereas (ii) the corresponding single sequences were obtained from the synthetic component peptides, (203-219) and (257- 273) (s. Figure 2).



(c)



Start-End	Observed $(M+1H)^{1+}$	Calculated $(M+1H)^{1+}$	Delta M	Peptide
42 – 68	3034.29	3032.33	1.96	R. GEPRFITVGYVDDTLFVRFDSDAASPR .E
46 – 92	5564.82	5565.11	-0.29	R. FITVGYVDDTLFVRFDSDAASPREEPRAPWIEQEGPEYW DRETQICK .A
69 – 86	2284.93	2288.43	-3.50	R. EEPRAPWIEQEGPEYWDR .E
136 – 175	4531.08	4531.85	-0.77	R. GYHQDAYDG KDYIALNEDL SSWTAADTAA QITQRKWEAAR .V
281 – 297	1994.44	1993.34	1.10	R. YTCHVQHEGLPKPLTLR .W

Figure 1. Primary structure characterization of HLA-B27 by in-gel tryptic digestion and MALDI-MS of the band cut from SDS-PAGE (**a**), followed by MALDI-MS (**b**), and MASCOT data-base search identification (**c**)

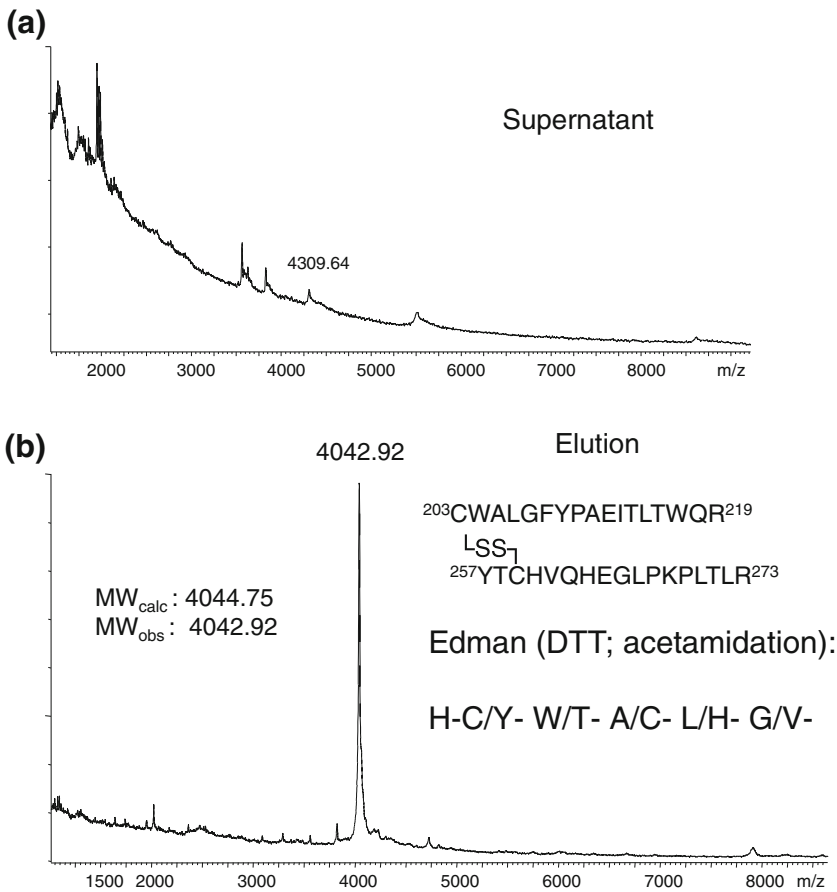


Figure 2. Affinity-MS identification of the HLA-B27 epitope to HD6 by tryptic epitope excision and MALDI-MS: (**a**), non-binding supernatant; (**b**), MALDI-MS of affinity-elution fraction

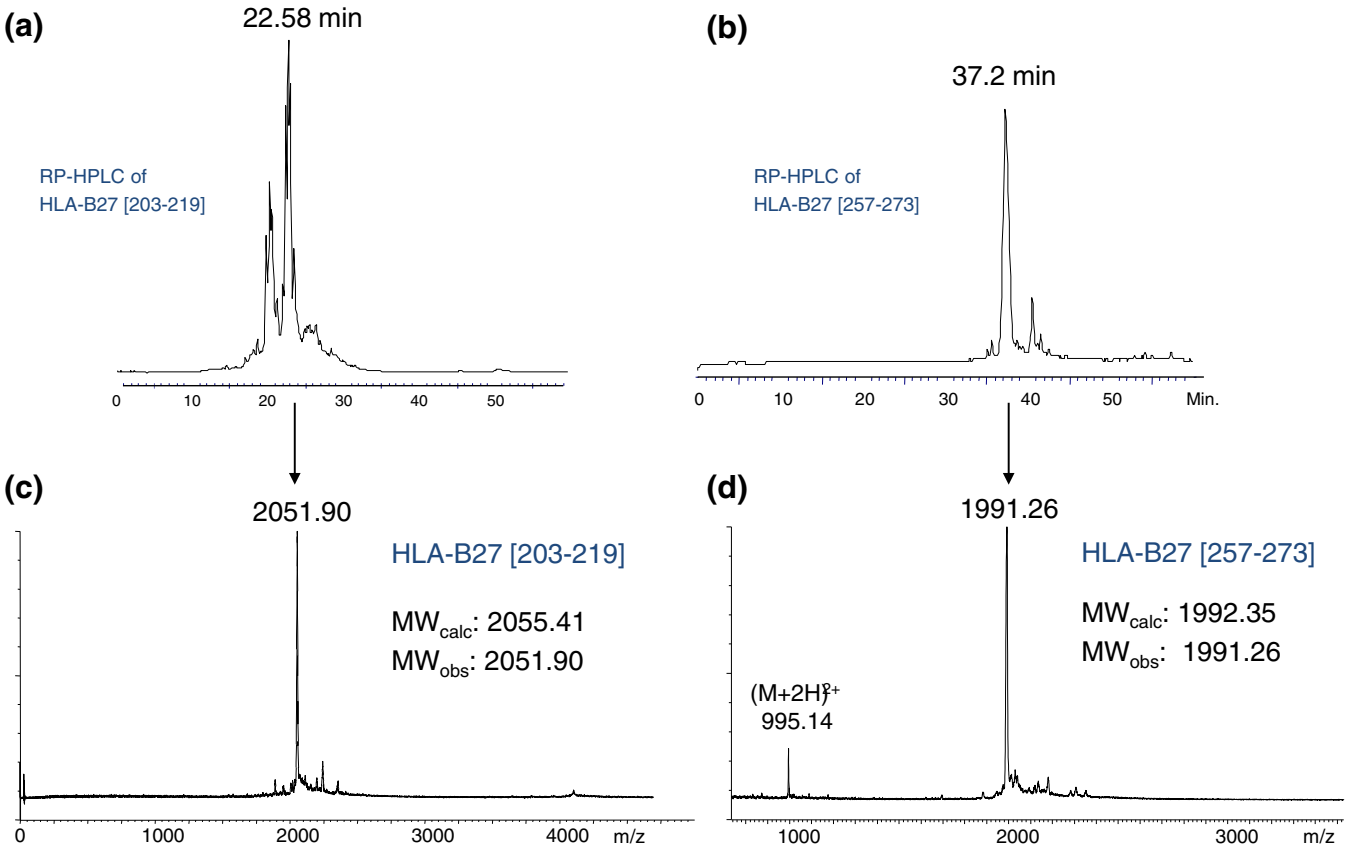


Figure 3. HPLC isolation and MALDI-MS of the synthetic epitope peptides, HLA-B27(203-219) and HLA-B27(257-273)

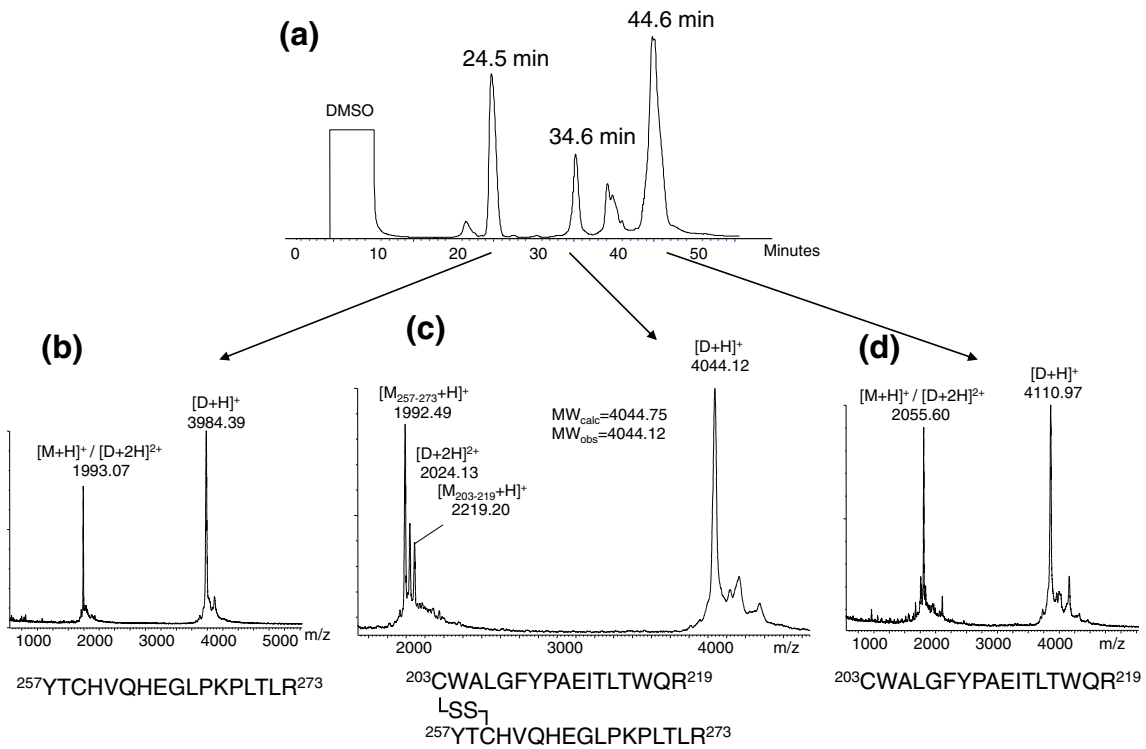


Figure 4. HPLC isolation and MALDI-MS (**a**) of the disulfide- dimers upon incubation of epitope peptides under oxidative conditions (see Experimental section); (**b**) HLA(257-273) homodimer; (**c**) HLA(203-219)-SS-HLA(257-273) heterodimer; (**d**) HLA(203-219) homodimer

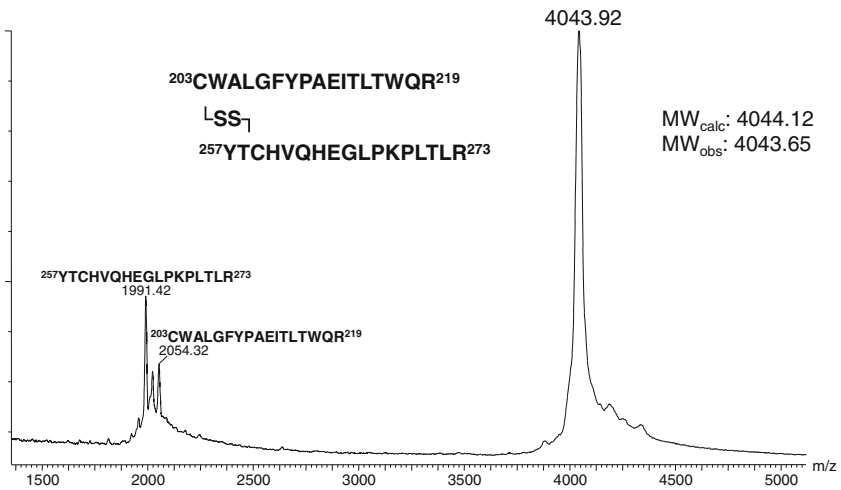


Figure 5. Affinity-MS of the elution fraction of the mixed-disulfide heterodimer, HLA-B27(203-219)-SS-HLA-B27(257-273) from the HD6 antibody column

Affinity-Mass Spectrometric Characterization of Epitope Peptides

The component peptides comprising the epitope, HLA-B27(203-219) and HLA-B27(257-273) were synthesized by SPPS at the 50 μM scale according to the Fmoc strategy. The crude products were purified by RP-HPLC. The linear peptide, HLA-B27(203-219) had a retention time of 22.6 min (Figure 3a), whereas the HLA-B27(257-273) peptide showed a significantly higher retention time of 37.2 min (Figure 3b). The pure peptides, HLA-B27(203-219) and HLA-B27(257-273) were subjected to MALDI-MS analysis (Figure 3c, d), which confirmed the expected molecular masses.

In order to ascertain the affinity and specificity of the two peptide sequences (203-219) and (257-273) comprising the epitope, the peptides were tested both separately and in a mixture against the HD6 antibody affinity column. When tested separately, both peptides showed affinity for the antibody and both formed homodimeric disulfides upon incubation at oxidative conditions (Supplementary Figure S1; supporting information). The homodimers (203-219)-SS-(203-219) and (257-273)-SS-(257-273) also showed significant affinity for the antibody. Upon testing the competitive affinities of a mixture of both linear peptides, however, both peptides showed a pronounced tendency to form a heterodimer in the presence of the antibody (Supplementary Figure S1). From the mixture of all the possible homo- and hetero-dimers, the mixed-disulfide linked heterodimer (203-219)-SS-(257-273) was observed as the predominant peptide eluted from the HD6 antibody column (Supplementary Figure S1c).

Table 1. Affinity Binding Constants of HLA-B27 Epitope Peptides Determined by SAW Biosensor

HLA-B27 peptide	K_D HD6 antibody × 10^{-9} M (R2)
[203–219]-SS-[257–273] heterodimer	41.3 (0.98)
[203–219] homodimer	339.2(0.93)
[257–273] homodimer	>500
[203–219] monomer	–
[257–273] monomer	100 (0.85)

Synthesis and Characterization of the Mixed Disulfide-Linked Epitope Peptide

Due to high yield and the unusual affinity properties of the "heterodimeric" mixed disulfide-linked epitope observed in the affinity experiments, an approach was pursued to prepare the mixed disulfide linked epitope by in vitro incubation of the synthetic linear peptides at oxidative conditions (Supplementary Figure S2).

The linear epitope peptides, HLA(203-219) and HLA(257-273) were incubated as a 1:1 mixture in DMSO/ammonium acetate at 25 °C for 24 h, and the reaction products separated by HPLC. Both the disulfide-linked homodimers $(203\text{-}219)_2$ and

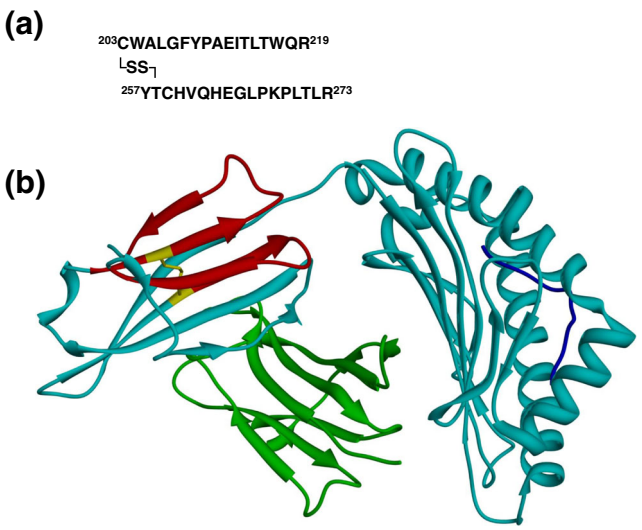


Figure 6. Structure model of the HLA-B27 heterotrimer complex. (**a**, red), with the disulfide bridge (orange) marked on the crystal structure; (**b**), heavy chain – HLA-B27 (cyan), light chain – ß2-microglobulin (green), and antigenic peptide (HCMV, pp65, RPHERNGFTVL (blue)

$(257\text{-}273)_2$ were successfully separated by RP-HPLC, with the HLA-B27(257-273) homodimer eluting earlier and the HLA-B27(203-219) homodimer with a longer retention time (Figure 4). The HLA-B27(203-219)-SS-(257-273) heterodimer eluted with an intermediate retention time, with a good separation (Figure 4a). In contrast, homo-dimeric peptides were only partially separated from the reduced linear peptides, as shown by MALDI-MS analysis (see Figure 4b, c, d).

Determination of Affinity Binding Constants

The disulfide linked HLA-B27(203-219)-SS-(257-273) heterodimeric epitope was subjected to qualitative and quantitative affinity characterization against the HD6 antibody. As proven by the mass spectrum of the affinity elution fraction (Figure 5) the heterodimeric epitope showed high affinity. For quantitative affinity determinations all epitope peptides were immobilized on gold-chips, and binding constants determined using the SAW biosensor at a series of concentrations between 5 and 150 nM. By comparison of the homo- and heterodimeric peptides, the highest affinity with a K_D of approximately 40 nM was obtained for the heterodimeric epitope, HLA-B27(203-219)-SS-(257-273) (Table 1).

A structure model based on the crystal structure with the location of the heterodimeric epitope is shown Figure 6. From the location of the epitope, it is well conceivable that the complete heterodimeric mixed-disulfide becomes accessible to the antibody upon the conformational rearrangement associated with the dissociation of the β2-microglobulin (shown in green in the structure) from the heterotrimer complex, as a basis for the epitope specificity of HD6.

Conclusions

The HLA-B27 epitope recognized by the HD6 antibody was shown in this study to comprise a specific, discontinuous mixed disulfide-linked peptide, HLA-B27(203-219)-SS-(257-273) formed by two linear peptides comprising different distinct parts within the antigen structure. These results thus illustrate the feasibility of the "native-like" proteolytic excision mass spectrometry methodology to identify discontinuous epitopes that are not possible to identify, or present a particular challenge to conventional methods. Although it was considered that one of the peptides might be devoid of affinity and affinity is derived from the shielding effect of the other peptide, we could show that both linear peptides retain significant affinity to the antibody. Moreover, we demonstrate here that the disulfide-linked heterodimeric epitope is preferentially formed from the two monomeric peptides in the presence of the antibody, excluding the formation of homodimeric forms. The presence of the monomers in the affinity elution fractions supports the conclusion that both peptides are preferentially present on the paratope, the part of the antibody recognizing the epitope, with or without a disulfide bridge as a linkage. K_D determination using an SAW biosensor ascertained that this hetero-dimeric epitope has the highest binding affinity among other possible epitope dimers. In summary, the hetero-dimeric mixed-sulfide epitope is identified in this study as a highly specific potential biomarker, presenting lead structure for the development of high specificity and affinity binders of HLA-B27.

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