# Antigenic Surveillance of the Influenza Virus by Mass Spectrometry†

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ABSTRACT: The use of a mass spectrometric-based immunoassay to survey the antigenic identity of a type A influenza strain is described. Antigenic surveillance of the influenza virus remains a critical step in the identification of new viral strains and the subsequent use of such strains or synthetic constructs in vaccine preparations. The immunoassay is shown to be capable of detecting a determinant of a single hemagglutinin antigen, responsible for the initial stages of infection, in a mixture of viral proteins with high sensitivity and specificity. The determinant comprises residues 207−225 of the hemagglutinin HA1 chain of the type A strain which occupies a highly accessible region of $\beta$-sheet atop the antigen. Peptide determinants are identified without the need to immobilize antibody or isolate antibody−peptide complexes which are shown to be preserved during mass spectrometric analysis. This immunoassay achieves optimal sensitivity (femtomole level) with minimal sample handling and is amenable to high sample throughput and automation. Determinants are identified by a direct comparison of the matrix-assisted laser desorption ionization mass spectra obtained for an antibody reaction mixture and unreacted control. The sequence and antigenic identity of a component antigen can be rapidly identified by searching protein databases with the mass spectral data in conjunction with tandem mass spectrometric sequencing.

The influenza virus is a formidable disease which continues to afflict almost the entire human population. The hallmark of the virus is its ability to change its antigenic identity frequently, so that immunity established in response to one strain may offer little to no protection against other strains. Although most individuals are able to recover from the effects of the virus in a week to ten days, influenza continues to pose a serious health risk to large segments of the community. The elderly and those whose immune systems are suppressed by illness are particularly susceptible, and influenza remains one of the ten most common causes of death in the United States (*1*). Tens of thousands of deaths are attributed annually to the influenza virus absent of any pandemic threat (*2*, *3*). Widespread international travel among the world's population enables local epidemics to escalate into global pandemics. In addition to the cost to human health, the influenza virus imposes a considerable burden on the world economy. It is estimated that influenza is responsible for the loss of 2.5 million work days each year in the United States alone with subsequent productivity losses estimated in the billions of dollars (*4*).

To prepare for the next influenza pandemic, an event which most consider inevitable (*5*, *6*), public health officials (*7*) have devised strategies and have made recommendations to minimize the mortality and social disruption from the virus. In a report of the panel discussion, several research priorities were recommended (*8*). A major priority area involves the timely recognition of new variants of the influenza virus. Virologic surveillance remains a critical step in the identification of new antigenic variants and the subsequent use of such strains in vaccine preparations.

Four antigens are associated with the influenza virus. These are the surface antigens hemagglutinin and neuraminidase, the internal nucleocapsid protein, and the membrane or matrix protein. Hemagglutinin is the focus of most attention since this antigen is responsible for the attachment of the virus to cell receptors of the respiratory tract (*9*) and for the induction of neutralizing antibodies. HA, the major surface antigen of type A influenza strains, is translated as a single protein $HA_0$. For viral activation, $HA_0$ assembled as trimers must be cleaved into two subunits $HA_1$ and $HA_2$ by cellular proteases, though the polypeptide remains linked through a single disulfide bond. Type A influenza strains are responsible for all of the major pandemics this century, and these strains are typified by their high rates of evolution.

Since the antigenic determinants or epitopes involved in protection against influenza are present on viral HA, it is reasonable to expect that vaccines which contain this viral antigen should have certain advantages over those with superfluous components present in the virus as a whole. Indeed, vaccine toxicity has been shown to be reduced merely by disrupting virons into separate subunits; further improvements were achieved by the preparation of vaccines of highly purified HA and/or NA, free from other viron components (*10*). The identification of the antigenic determinants on viral HA should lead to the use of such determinants and constructs that contain these determinants rather than whole HA for immunization purposes.

Antibodies remain indispensable for screening the antigenic identity of the influenza virus. Monoclonal antibodies, in particular, are important during interpandemic periods

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where slow and irregular antigenic changes in the viral proteins emerge. These would go undetected in assays which use polyclonals; in contrast, the reactivity of monoclones decreases as new variants appear (*11*). We report a rapid, new immunoassay utilizing monoclonal antibodies and mass spectrometry to identify the determinants of a single antigen in a complex biological mixture with high specificity and sensitivity (*12*). The basis for the approach has previously been reported for a model protein (*13*). Here the immunoassay is shown to detect the antibody binding site of a hemagglutinin antigen present in a mixture of viral proteins from an influenza isolate at the femtomole ($10^{-15}$ mol) sample level. Importantly, the assay alleviates the need to immobilize antibody, isolate antibody−peptide complexes, and dissociate the bound epitopic peptides that are all requirements of other methods (*14*, *15*). No radiolabels or chemical indicators are required to monitor the binding process as peptide determinants are identified directly on the basis of a comparison of the matrix-assisted laser desorption ionization (MALDI) (*16*) mass spectra of the antibody reaction mixture versus an unreacted control. This mass spectrometric-based assay can be performed rapidly since both spectra are acquired in less than 1 min, and the approach is amenable to high sample throughput and automation.

## EXPERIMENTAL PROCEDURES

*Proteolysis of Viral Antigens*. The type A influenza virus was cultured in the allantoic cavity of embryonic hen eggs for 10 days. Viral proteins were isolated by repeated ultracentrifugation using a sucrose density (10 to 40%) gradient. Dithiothreitol (0.1 M) in 50 mM ammonium bicarbonate was added to a solution of the viral proteins (53 $\mu$g) in 50 mM ammonium bicarbonate (50 $\mu$L, pH 8.0) to produce a final concentration of 2 mM. The solution was incubated for between 3 and 4 h at 37 °C. A solution of modified trypsin (Promega, Madison, WI) or chymotrypsin (Boehringer Mannheim, Indianapolis, IN) (20 $\mu$g) in 50 mM ammonium bicarbonate (100 $\mu$L, pH 7.8) was added (5.4 $\mu$L, 2% w/w) and the combined solution incubated for 15 h at 37 °C.

*Reaction with Antibody*. An aliquot of the tryptic digest solution (4.1 $\mu$L) was added to a solution of monoclonal anti-influenza IVC102 (Research Diagnostics, Flanders, NJ) at a concentration of approximately 5 $\mu$M in 50 mM Tris-HCl (30 $\mu$L, pH 7.8) containing 150 mM sodium chloride. The reaction solution was incubated at 4 °C for 2 h, and a portion was subjected directly to mass spectrometric analysis.

*Peptide Mixtures*. A mixture of levitide, the synthetic analogue to the binding peptide AIYHTENAYVSVVSSHY-NR, and ACTH 18-39 at 2.5, 7.5, and 5 $\mu$M, respectively, in 50 mM Tris-HCl (30 $\mu$L, pH 8.0) was analyzed by MALDI-MS. The same solution containing 150 mM sodium chloride was treated with 1 mole equiv of monoclonal IVC102 over total peptide (15 $\mu$M) and reacted at 4 °C for 2 h. The second mixture comprised peptides AIYHTENA, Met-Enkephalin-lysine, and angiotensin at concentrations of 15, 2.5, and 10 $\mu$M, respectively.

*Mass Spectrometry.* A portion (1 $\mu$L) of the antibody reaction mixture or unreacted control without antibody was added to a saturated solution (10 $\mu$L) of α-cyano-4-hydroxycinnamic acid in acetonitrile and water (30:70 by volume). Peptide/matrix solutions (500 fmol of peptide/$\mu$L) were applied to the sample plate by heat-assisted deposition by applying 1 $\mu$L of the solution and rapidly evaporating the solvent from the droplet using a variable-temperature heat gun (Master Appliance, Racine, WI) mounted vertically over the plate. MALDI-MS was performed on a Voyager STR reflecting time-of-flight mass spectrometer (PE Biosystems, Framingham, MA) equipped with a 337 nm nitrogen laser operating in the linear, positive ion mode with an accelerating voltage of 20 kV and an extraction delay of 200 ns. A timed ion selector was used to deflect ions with low mass-to-charge ratios (<300) from the detector. Spectra were acquired by averaging data of approximately 100 laser shots to improve data quality and ion statistics. Mass spectra were calibrated externally using the singly protonated ions of glucagon and ions produced from the matrix compound α-cyano-4-hydroxycinnamic acid. Peak intensities where measured using the program utilities Interpol and Integrat running within GRAMS software (Galactic Industries Corp., Salem, NH).

Electrospray ionization (ESI) mass spectra were recorded on a Finnigan (San Jose, CA) LCQ quadrupole ion trap mass spectrometer. Digested antigen (10 pmol) was introduced into the ion source using a Waters Corp. (Milford, MA) 626 binary pump and 600 gradient controller liquid chromatograph (LC) and a VYDAC (Hesperia, CA) 1 mm × 150 mm C18 reverse-phase column. Proteolytic peptides were eluted from the column using a gradient of acetonitrile and water containing 0.05% trifluoroacetic acid. Alternate mass (MS) and intensity-dependent tandem (MS/MS) spectra were acquired in the centroid mode. Tandem MS/MS spectra were interpreted manually and in conjunction with searches of protein databases using the SEQUEST algorithm (*17*).

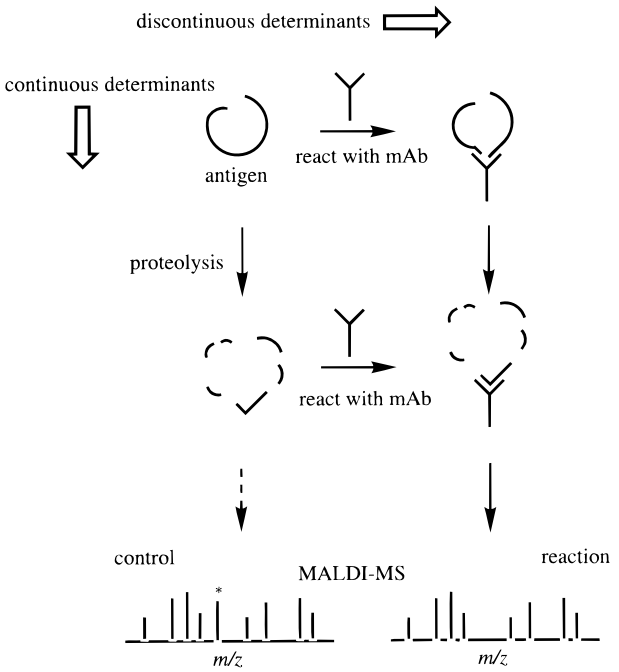


FIGURE 1: Schematic representation of the mass spectrometric-based immunoassay.

## RESULTS AND DISCUSSION

*Mass Spectrometric-Based Immunoassay.* A schematic representation of the mass spectrometric-based immunoassay for identifying the determinants of protein antigens is illustrated in Figure 1. To identify continuous determinants,

Table 1: Relative Peak Ion Intensities from the MALDI Mass Spectra of Tryptic Digestion Products of the Viral Proteins from a Type A Influenza Strain

| *m*/*z* (mono.) $[M + H]^+$ measured | *m*/*z* (mono.) $[M + H]^+$ calculated | aligned residues of the protein | relative peak intensity before reaction with IgG (%)[a] | relative peak intensity after reaction with IgG (%)[a] | antigen type[b] |
|---|---|---|---|---|---|
| 544.6 | 546.2 | 239−243 | 14 | 14 | HM |
| 622.9 | 622.3 | 205−208 | 47 | 42 | NC |
| 676.4 | 676.4 | 331−336 | 19 | 20 | HM |
| 708.9 | 708.4 | 385−389 | 43 | 42 | NC |
| 805.3 | 805.4 | 227−233 | 23 | 21 | HM |
| 849.4 | 849.4 | 244−250 | 27 | 31 | MT |
| 863.1 | 863.4 | 211−217 | 27 | 34 | MT |
| 962.1 | 961.5 | 226−233 | 28 | 42 | HM |
| 1022.5 | 1021.5 | 353−361 | 33 | 29 | NR |
| 1086.8 | 1086.4 | 462−470 | 19 | 26 | NC |
| 1125.7 | 1125.7 | 48−57 | 100 | 100 | MT |
| 1273.7 | 1273.7 | 164−174 | 37 | 39 | MT |
| 1448.9 | 1448.7 | 231−242 | 13 | 13 | MT |
| 1676.3 | 1675.9 | 401−416 | 19 | 11 | NC |
| 1755.1 | 1753.8 | 447−461 | 56 | 46 | NC |
| 1876.2 | 1875.9 | 79−95 | 44 | 34 | MT |
| 2033.3 | 2031.9 | 78−95 | 9 | 8 | MT |
| 2056.7 | 2056.1 | 189−206 | 10 | 5 | HM |
| **2210.1** | **2210.1** | **207−225** | **59** | **3** | **HM** |
| 2313.9 | 2313.2 | 187−206 | 8 | 5 | HM |
| 2383.7 | 2383.0 | 188−210 | 39 | 48 | MT |

[a] Relative peak intensities represent an average of values from two separate mass spectra. [b] HM, hemagglutinin; NC, nucleocapsid protein; MT, matrix protein; NR, neuraminidase.

digestion of the antigen with a site-specific protease precedes its reaction with antibody. Discontinuous determinants are identified after limited proteolysis of the antigen−antibody complex. Antibodies have been found to be generally resistant to proteolysis so that only unbound portions of the antigen are freed into solution. An unreacted proteolysis mixture serves as a control for identifying the peptides in the mixture and for determining their relative ion intensities by MALDI-MS (*16*). The proteolysis products after reaction with monoclonal antibody are also analyzed by MALDI-MS, and the differences in their relative ion intensities are correlated to identify the epitopic peptides (*13*). It is important to note that many factors influence the detection of peptides by MALDI-MS, including the nature of the matrix, the size, structure, and concentration of the peptide, the laser fluence, and the detection efficiency of the analyzer (*18*). Relative ion intensities, however, will remain constant where other experimental factors are unchanged. Thus, a diminished relative ion intensity or the absence of an ion signal for a proteolytic peptide of the reaction mixture that is present in the control can be attributed to the binding of the peptide to antibody. This analysis requires that reproducible mass spectra are obtained irrespective of the sample surface from which the peptides are desorbed. This criterion is met through electrospray (*19*, *20*) and heat-assisted deposition which produces uniform coatings of analyte and matrix for MALDI-MS analysis.

Critical to the success of the method is the observation that noncovalent antibody−peptide complexes are resilient to both the sample deposition approaches and the MALDI ionization event (*13*). Several groups have now reported that noncovalent complexes can be generated and preserved by MALDI mass spectrometry (*21*−*24*). We have shown that antibody−peptide complexes can be preserved under certain experimental conditions, and importantly that this feature can be exploited in the context of a mass spectrometric-based immunoassay which has significant advantages over other approaches (*12*, *13*).

*Antigenic Determinant of a Single Surface Antigen in a Complex Mixture from a Type A Influenza Strain.* The immunoassay has been applied to a mixture of viral proteins isolated from a type A influenza strain to assess its viability to screen the antigenic identity of the virus through direct analysis of complex biological mixtures. Mixtures of viral proteins have been analyzed directly without purification.

The MALDI mass spectra of a tryptic digest of the mixture of the viral proteins from the type A strain before and after reaction with monoclonal antibody are shown in Figure 2. Approximately 50 peptide ion signals are evident in the spectra; the mass-to-charge ratios are labeled for the most abundant ions which are shown together with a measure of the relative ion intensities in Table 1. Examination of the data of Table 1 reveals that the spectra of Figure 2 closely resemble one another; the major difference between the two spectra is the presence of an intense ion at *m*/*z* 2210 in the spectrum of the control mixture (Figure 2a) that appears as a weak ion after reaction of the digest with the antibody. The relative ion intensity for this peptide decreases by 56% in absolute value after reaction of the digestion mixture with the antibody (Table 1). In contrast, the remainder of the peptide signals are relatively unaffected by the presence of antibody. Repeated experiments have revealed that differences in the relative ion intensities for the peptide are typically no greater than ±5−10% in absolute value. Thus, peptides whose ion signals are found to decrease in relative peak intensity by a value greater than this difference after reaction with the monoclone are predicted to bind antibody. These results are consistent with our earlier observations for a model protein (*13*).

*Sequence of the Determinant As Determined by Tandem Mass Spectrometry and Database Searching.* The identity of the peptide determinant, and the viral protein from which

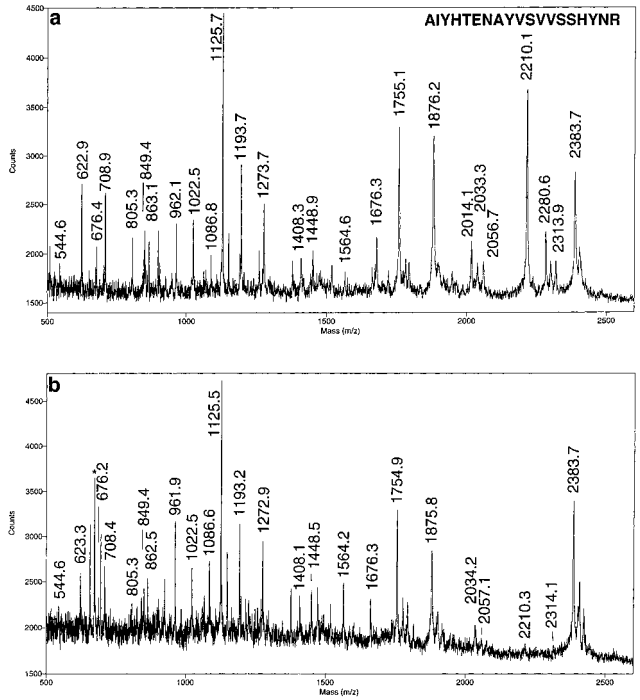


FIGURE 2: MALDI mass spectra of the tryptic digest of the viral proteins from a type A influenza strain (a) before and (b) after reaction with monoclonal antibody. The peak labeled with an asterisk is attributed to a matrix cluster ion.

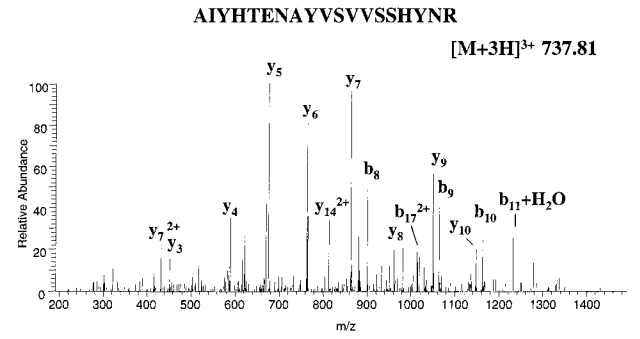


FIGURE 3: ESI tandem MS/MS spectrum for the triply protonated $[M + 3H]^{3+}$ ion (*m*/*z* 737.8) of the binding peptide.

it originates, can be determined by searching protein databases using the masses for the proteolytic peptides from the tryptic digestion mixture (*17*, *25*, *26*). This is obviously most successful when the sequence of the protein already appears in the database. Several factors, however, influence the success of database searches with mass spectral data, including the number and accuracy of the input masses. In instances where the identity of the protein is unknown, such searches can still prove useful where homologous proteins appear in the database. This is indeed the case for the viral proteins from influenza where considerable homology has been observed across strains. Variations in the sequences of the antigenic determinants are, however, characteristic of the virus. Thus, the most reliable identification of the determinant of an influenza viral antigen is obtained by a search of the protein database in conjunction with tandem mass spectrometry. Tandem MS/MS mass spectral data can be manually interpreted or correlated directly with hypothetical MS/MS spectra predicted for the proteolysis products of database entries, or some predefined subset, to derive the peptide sequence (*27*, *28*).

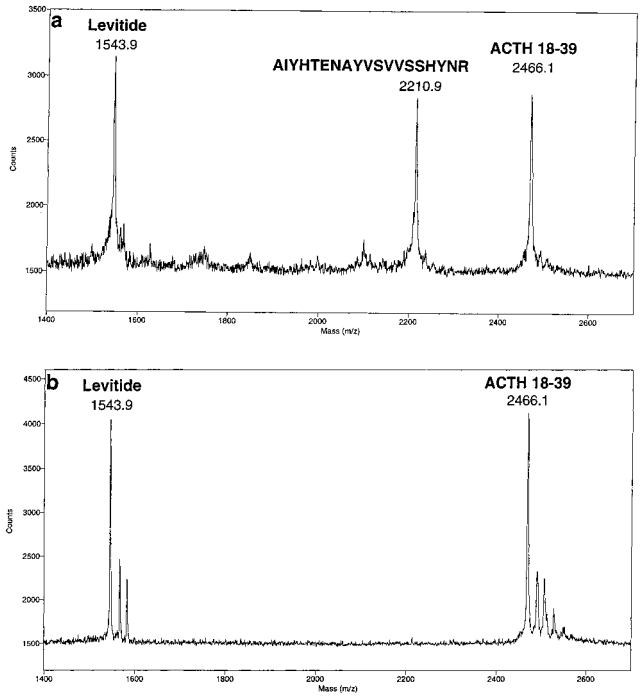


FIGURE 4: MALDI mass spectra of the synthetic form of the hemagglutinin determinant in the presence of two nonbinding controls (a) before and (b) after reaction with 1 mole equiv of monoclonal antibody.

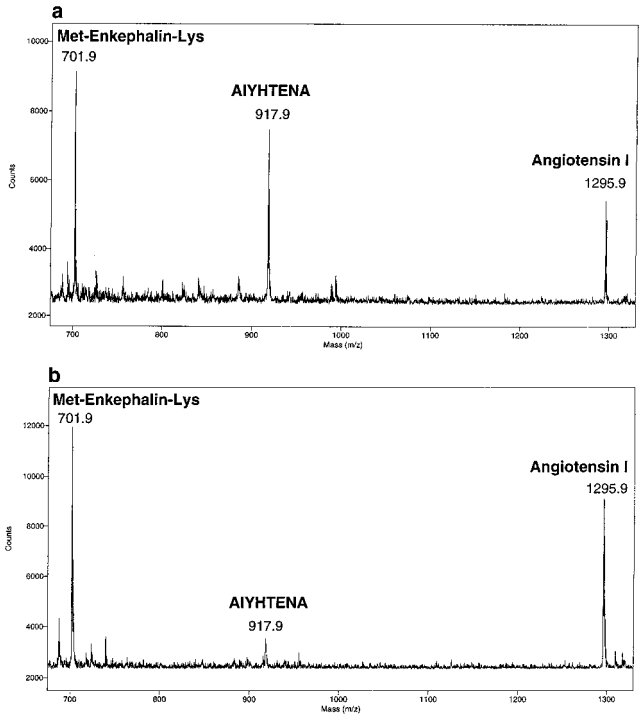


FIGURE 5: MALDI mass spectra of the synthetic peptide AIYHTENA in the presence of two nonbinding control peptides (a) before and (b) after reaction with 1 mole equiv of monoclonal antibody.

On-line LC−ESI-MS was performed on the complete tryptic digest mixture, and intensity-dependent MS/MS spectra were obtained for the most abundant peptide ions that were detected. The MS/MS spectrum for the triply protonated $[M + 3H]^{3+}$ ion of the binding peptide (*m*/*z* 737.8) is shown in Figure 3. The sequence of the peptide AIYHTENAYVSVVSSHYNR was derived from the MS/MS spectrum and an alignment of the sequence data with proteins of

Table 2: Summary of the Output from a Search of the NCBI Nonredundant Protein Database with the BLAST Algorithm for Homologous Sequence Entries Using the Derived Sequence of the Peptide Determinant

| protein identification numbers | segment of hemagglutinin sequence | homology within the peptide (%) |
|---|---|---|
| P12590, CAA91083, BAA00722, 497730, BAA00308, JQ1643, 554678, 305186, 305147, 495805, 305194, 305131, 305125, P28730, BAA00309, 305192, 305178, 305137, A35788, HMIVTA, 495809 | AIYHTENAYVSVVSSHYNR | 100 |
| 1401166A | IYHTENAYVSVVSSHYNR | 94.7 |
| JQ2372, 305173, BAA05874, BAA06719, 305143, BAA01027, 495799, BAA02768, 305129, 2554951, 2554955, 495811, 497710, 2554957, 2554961, 305133, 497722, 497726, 497728, 305121, 305119, 305123, 497716, 497712, 305157, 305139, 305141, 495803, 305161, 497708, 305127, 305159, 305180, 305188, 305182, 305176, 305190, S16785, JQ2371, JQ2370, BAA02769, JQ1437, 3065727 | AIYHTENAYVSVVSSHYSR | 94.7 |

Table 3: Relative Peak Ion Intensities from the MALDI Mass Spectra of Chymotryptic Digestion Products of the Viral Proteins from a Type A Influenza Strain

| *m*/*z* (mono.) $[M + H]^+$ measured | *m*/*z* (mono.) $[M + H]^+$ calculated | aligned residues of the protein | relative peak intensity before reaction with IgG (%)[a] | relative peak intensity after reaction with IgG (%)[a] | antigen type[b] |
|---|---|---|---|---|---|
| 524.3 | 523.3 | 171−174 | 32 | 36 | NR |
| 566.2 | 565.3 | 162−165 | 100 | 100 | HM |
| 702.5 | 703.4 | 324−329 | 13 | 11 | HM |
| 720.5 | 721.4 | 282−289 | 22 | 20 | NC |
| 729.4 | 729.4 | 23−28 | 12 | 11 | MT |
| **735.4** | **734.3** | **210−215** | **12** | **8** | **HM** |
| 760.5 | 760.4 | 116−121 | 34 | 36 | NR |
| 778.4 | 779.3 | 359−365 | 25 | 24 | HM |
| 793.4 | 793.5 | 104−109 | 61 | 60 | MT |
| 823.1 | 823.4 | 437−443 | 9 | 7 | HM |
| 872.4 | 871.3 | 416−422 | 24 | 22 | NR |
| 896.4 | 897.4 | 401−407 | 15 | 18 | HM |
| 914.4 | 914.5 | 336−343 | 24 | 25 | HM |
| 1024.5 | 1024.7 | 47−55 | 85 | 81 | MT |
| 1086.5 | 1086.6 | 444−452 | 15 | 11 | HM |
| 1125.4 | 1125.6 | 49−58 | 14 | 12 | HM |
| 1137.5 | 1136.3 | 46−55 | 12 | 8 | MT |
| 1155.4 | 1156.5 | 369−378 | 11 | 9 | HM |
| 1171.5 | 1171.7 | 513−522 | 13 | 14 | HM |
| 1220.4 | 1220.5 | 78−88 | 11 | 10 | HM |
| 1249.3 | 1248.9 | 223−233 | 22 | 18 | HM |
| 1265.3 | 1265.6 | 113−122 | 56 | 51 | HM |
| 1320.3 | 1320.7 | 230−240 | 13 | 9 | MT |
| 1351.3 | 1351.6 | 453−463 | 20 | 15 | HM |
| 1358.3 | 1358.6 | 502−515 | 19 | 19 | HM |
| 1418.3 | 1418.8 | 21−32 | 10 | 13 | MT |
| 1450.5 | 1449.7 | 425−436 | 5 | 5 | HM |
| 1503.4 | 1503.8 | 316−328 | 7 | 8 | NC |
| 1585.4 | 1584.8 | 310−323 | 6 | 4 | HM |
| **1663.3** | **1663.8** | **195−209** | **11** | **9** | **HM** |
| 1730.3 | 1729.8 | 109−122 | 11 | 11 | HM |

[a] Relative peak intensities represent an average of values from two separate mass spectra. [b] HM, hemagglutinin; NC, nucleocapsid protein; MT, matrix protein; NR, neuraminidase.

the nonredundant NCBI (National Center for Biotechnology Information, National Institutes of Health, Bethesda, MD) database (*17*). A search of the NCBI database using the BLAST (Basic Local Alignment Search Tool) algorithm (*29*) revealed that there are 21 unique known strains in which the hemagglutinin protein contains precisely the same determinant (Table 2). A further 45 contain hemagglutinin in which one residue within the determinant is substituted or absent. An additional 34 influenza strains were also shown to contain hemagglutinin with some sequence homology in the vicinity of this portion of the protein. As such, a synthetic immunogen comprising this peptide sequence would be predicted to offer a degree of protective immunity across 100 known strains of the virus.

*Verification and Refinement of the Hemagglutinin Determinant.* The binding of hemagglutinin peptide AIYHTENAYVSVVSSHYNR to the monoclonal antibody was independently corroborated by the reaction of the synthetic form of the peptide and two nonbinding controls (Figure 4). Figure 4a shows a substantial ion signal for the hemagglutinin peptide of residues 207−225 at *m*/*z* 2210. This peptide ion is absent from the spectrum of the same mixture after reaction with the monoclonal antibody. Peptides which comprise residues 214−225 and 215−225, formed during the synthesis of the complete determinant, are shown similarly not to bind antibody (data not shown). Thus, the critical binding region appears to occupy the N-terminal portion containing residues 207−225 of the protein. In support, the ion signal for peptide

AIYHTENA constituting residues 207−214 (*m*/*z* 918) is shown to exhibit a 64% decrease in relative intensity after reaction with monoclonal IgG relative to a nonbinding control peptide (Figure 5).

This entire region appears to be critical for the binding of antibody. The MALDI mass spectral data for a chymotryptic digest of the same mixture of viral proteins are shown in Table 3. The use of a second protease with a different specificity is sometimes useful in these studies where a determinant contains or flanks a proteolysis site (*13*). The relative ion intensities for the most abundant peptide ions are shown to be unchanged after the reaction of the chymotryptic digest with the monoclonal antibody. Of particular note is the fact that the hexapeptide of residues 210−215 of the hemagglutinin sequence does not bind antibody on the basis of the relative peak intensity measurements. The same is true of the peptide of residues 195−209 that overlaps with the N-terminal portion of the hemagglutinin determinant that has been identified. Thus, the complete peptide sequence appears to be required to fit tightly within the binding pocket.

*Location of the Determinant in the Hemagglutinin Antigen.* A three-dimensional model for the hemagglutinin antigen was constructed using the ProMod algorithm (*30*) based on the sequence derived by tandem mass spectrometry and database searches. The derived sequence is first aligned with four homologous template sequences from the Brookhaven Protein Data Bank (PDB). The algorithm then computes the weighted average position of each atom based on the template sequences. This generates an initial framework on which to assemble the three-dimensional protein structure. Loop regions whose sequences are not similar to those of the templates are constructed through insertion of homologous fragments of other database entries with resolutions that are better than 2.5 Å. Backbone amide groups are then incorporated using a library of pentapeptide backbone fragments of the database of the highest resolution (≤2.0 Å). Finally, amino acid side chains are inserted with the most probable orientations based on the backbone conformation.

A three-dimensional backbone representation of the HA1 chain of the hemagglutinin antigen identified is shown in Figure 6. In the native form, this chain is attached to a smaller HA2 subunit through a disulfide bridge. A long fibrous region containing residues of both HA1 and HA2 is centered about a helical colied-coil domain extending from the membrane surface that is attached to the HA2 subunit (*31*). The peptide determinant containing residues 207−225 sits atop the HA1 chain that comprises part of a globular region consisting of eight strands of $\beta$-sheet. This region has previously been implicated in the antigenicity of the protein (*32*). The residues face the central cavity of the homotrimeric complex formed on the surface of the viral particle by three identical hemagglutinin molecules (see Figure 7).

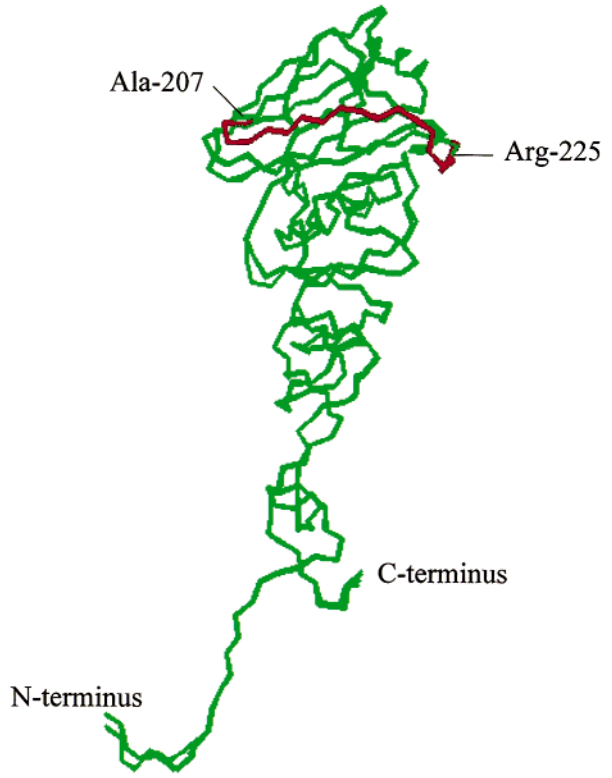


FIGURE 6: Backbone representation of a theoretical model for the HA1 chain of the hemagglutinin antigen from coordinates obtained by the ProMod algorithm.

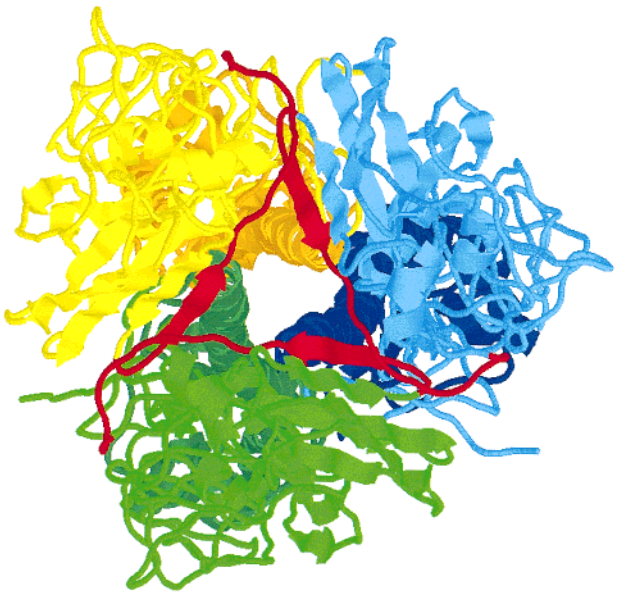

FIGURE 7: Ribbon representation of the top view of the hemagglutinin homotrimer showing the location of the peptide determinant (red) about the central cavity.

## CONCLUSIONS

A new experimental approach has been reported for the identification of antigenic determinants of a single component antigen in complex biological mixtures using mass spectrometry. The basis for the success of this new immunoassay is the observation that antibody−peptide complexes can be preserved during the mass spectrometric analysis under certain experimental conditions. This finding, previously reported for a model protein (*13*), is advantageous in the context of identifying determinants rapidly and with high sensitivity. Since the antibody is not immobilized before its reaction with antigens and the antibody−peptide complexes are not isolated from solution prior to mass spectrometric analysis, the approach affords optimal sensitivity with minimal sample handling. It is also amenable to high sample throughput and automation. The method can detect a peptide determinant of a single hemagglutinin antigen in a mixture

of viral proteins from a type A influenza strain. Mass spectral data can be used to search protein databases, and in this manner, the sequence and antigenic identity of a viral strain of influenza can be surveyed. The high reaction specificity of monoclonal antibodies should permit multiple determinants to be identified in a single analysis. We are presently exploring the applicability of the method to measuring immunoaffinity binding constants directly by mass spectrometry.

## ACKNOWLEDGMENT

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