2,4-Toluene diisocyanate adducted peptides in conjugates formed with human serum albumins *in vitro* and *in vivo*

Yin-Mei Chiung[1, 2], Pei-Shan Liu[3], Yin-Da Wu[4], Wei-Chau Chang[4], Yi-Yun Kao[3], Chiu-Jone Chen[1], Tung-Shen Shih[1]

[1] Institute of Occupational Safety and Health, Shijr City 221, Taipei, Taiwan

[2]Department of Microbiology and Immunology, National Defense Medical College, Taipei 114, Taiwan

[3]Department of Microbiology, Soochow University, Shihlin, Taipei 111, Taiwan

[4]Genomics Research Center, Academic Sinica, Taipei 115, Taiwan

[5]Department of Occupational Medicine and Envioronmental Hygiene, National Chenkun University, Tainan 600, Taiwan

Correspondence: *Dr. Yin-Mei Chiung, Division of Medicine, Institute of Occupational Safety and Health, Shijr City 221, Taipei, Taiwan. Tel: 886-2-26607600; Fax: 886-2-26607731; Email: chiun@mail.iosh.gov.tw*

Abstract

The protein adducts of isocyanates in human plasma were reported. In this study, the adducted albumin of long term exposed workers were isolated with specific antiserum by affinity chromatograph, and the peptides obtained from trypsin digestion were analyzed by electrospray ionization mass spectrometry (ESI-MS), matrix-assisted laser desorption/ionization time-of-flight mass spectrometric (MALDI-TOF MS) analysis, and tandem mass analysis by MALDI-TOF/TOF. The results were compared with the spectra of peptides obtained from digests of conjugates synthesized in vitro. Conjugates formed in vitro were digested by protease K and trypsin in solution. The fragments of TDI-HSA tryptic digests distinct different from HSA tryptic digests, were identified on Maldi-TOF/TOF experiments, by the appearance of an ions of m/z175 or m/z190 in the MS/MS spectra, otherwise, the possible sequences were elucidated also. By comparison of these spectra, common sites adducted by TDI were found on Lys 249 in conjugates synthesized in phosphate buffer and diluted whole plasma. In the conjugates isolated from exposed workers, a lot of peptides with strong intensity could not assigned to albumin when submitted. These speculated adductive peptides found in experimental samples were verified by MS/MS experiments.

Key words: albumin conjugates, 2,4-Toluene Diisocyanate, worker, ESI-MS, MALDI-TOF-MS

1 Introduction

As for hazardous chemicals, the relevant protein change may serve as an outcome marker for preclinical or clinical biological effects of exposure to chemicals during occupational disease induction [1]. The occupational asthma induced by diisocyanates in exposed worker populations has been estimated about 5 to 10% [2]. In Taiwan, about 3% of exposed workers with asthma related syndromes and 28% with respiratory allergic diseases were estimated in prevalence [3]. Diisocyanate-containing compounds are widely used in many products including surface coatings, water proofing coatings, paints, polyurethane foams, insulation, adhesives resins, elastomers, binders and sealant. The role of specific humoral response against TDI showed contradicts and ambiguous in several studies using synthesized antigens [4-6]. Animal models showed

an elevation of specific IgG and IgE, parallel to an enhancement of lymphocyte proliferation in vitro stimulation by TDI albumin conjugates [7] with mitogens, and Protein adducts of TDI is considered to involve in the etiology of sensitization reactions[8] .

TDI is very easy to form covalent bonds with amino groups. Its conjugates with HSA have been investigated by acid hydrolysis, and confirmed the objective amino acid is Lysine [9-10]. In TDI-treated albumin, globulin and DNA, covalent-linked conjugates were exhibited in dose dependent manner especially for –2,4 TDI [11]. The albumin adducts of these chemicals were reported not only in serum but also in nasal lavages, the chemical structure of albumin adducts existed in plasma proteins of workers exposed to TDI was reported [12], by sulfuric acid hydrolysis of fractions eluted from gel filtration and ion exchange chromatography, but the exact position where covalent bond formed were reported fragmentary [13-14]. In our previous study, the lysates of adducted proteins have been also reported from airway cells in asthmatic workers [15]. Hill et al. found that in experiments on guinea pigs exposed to 14C labeled TDI vapor, [16] an irreversible binding of isocyanates to albumin and hemoglobin has also been reported in workers [17-20]. Blood protein adducts or N-terminal adducts are suggested to be potential dosimeters for biomornitoring markersfor exposed people [21]. *In vitro* experiments, lysyl residues were found to react with the isocyanates [22], adducted on the ε- amine. Our previous studies suggested ten binding sites from conjugates form in vitro under physiological condition [23]. By the shifted MW, TDI adducted peptides were screen out and proved via peptide sequence analysis using MALDI-TOF/TOF mass spectrometry [24]. In this study, the adducted albumin of long term exposed workers were isolated, after digestion with trypsin, the peptides were analyzed by MALDI-TOF MS analysis. The results were compared with the spectra of peptides obtained from digests of conjugates synthesized in vitro.

2 Materials and methods

2.1 Chemicals

HPLC-grade trifluoro-acetic acid and acetonitrile were obtained from J. T. Baker. HSA and trichoroacetic acid were obtained from Sigma Chemical Co. NaOH was obtained from Baker analyzed. HPLC-grade Methanol and NaCl were obtained from Merck Co.

TDI (solution, purity 97%) was purchased from the Tokyo Kasei Kogyo company. Ammonium carbonate was purchased from Acros Co. TPCK Trypsin was obtained from Pierce Biotechnology Inc. Glycopeptidase F (EC. 3.5.1.52) was obtained from Takara Bio Inc. All chemicals and reagents were of the highest purity. Water was purified using a Milli-Q system.

2.2 Electrophoresis on Polyacrylamide Gel.

Samples of TDI-HSA conjugates, untreated and control standards were reduced with 2% SDS, at 95℃ for 5min, then separated on 4%-12% Tris Glycin SDS-PAGE; the digested peptides were reduced then separated on 16% SDS-PAGE. TDI-HSA and its lysate samples were also applied on 3-10 IEF-Gels respectively after mixed with ampholyte. Samples were visualized following electrophoresis using silver stain or Comassiae blue stain. The conjugates and unconjugated HSA were evaluated by its different behavior of mobility compared with control and untreated samples.

2.3 TDI-HSA conjugates synthesize in vitro

TDI-HSA conjugate is prepared according to a described method [23]. The reaction solution is mimic human body fluid under 25 ℃ for 15 minutes, and terminated by adding ammonium carbonate or monoethanoamine (MEA) for ESI-MS analysis; and using ammonium carbonates to stop the reaction for samples of MALDI-TOF-MS analysis. After centrifugation, dialysis, conjugates were digested by protease K or trypsin, respectively. The control experiments were carried out using untreated HSA that was prepared as in the above steps but without adding TDI.
Conjugation reaction with reference serum sample was done following the same procedure, in which non-exposed serum was diluted ten times, stopped by ammonium carbonate, TDI adducted albumin, digested with trypsin in solution after the isolation by affinity chromatograph.

2.4 Isolation of TDI-albumin conjugates from sera

Albumin in pooled sera of exposed workers was removed previously. The TDI adducted albumin was isolated by affinity chromatograph, in which the columns were precoated with anti TDI-HSA antiserum. Anti TDI-HSA antiserum was prepared through a serious

immunization on rabbits, after absorption with albumin; the specificity was identified by negative immunological reactions with human serum albumin and positive results with adducted albumin.

2.5 Enzymatic digestion of TDI-HSA conjugates

TDI-HSA conjugates for MALDI-TOF analysis were applied on a 8 % PAGE, the separated bands were cut into 3 portions, and digested with trypsin at 37℃ for 16hrs. Peptides samples were prepared following the method of tryptic digestion. The peptide digests were then stored in -30℃. Following enzymatic digestions, the resultant peptides were checked on polyacrylamide gel electrophoresis (PAGE) to ensure the reaction completed and then applied onto ESI-MS analysis. The amino acid sequence of HSA used in peptides prediction was described to contain 609 amino acids, noted as CRC 64: F88FF64DD242E818.

2.6 LC-ESI-MS instrumentation

Peptides of HSA and TDI-HSA were separated by reversed-phase HPLC (Waters alliance 2795) on a C4 column (4.6 mm × 50 mm), respectively. The solvent system consists of 20 mM formic acid in 90/10 and 20/80 water/acetonitrile solutions, the flow rate was set at to 1 ml/min, 80%of the flow was split off before it was inducted into the electrospray probe. ESI-MS data were acquired on a Micromass® Quattro Ultima triple quadruple mass spectrometer. The spray voltage was 3.0 kV, cone voltage was 150 V, source temperature was 80℃, and the corn voltage, source temperature and desolvation temperature were set at 150V, 80℃ and 300℃, respectively.

2.7 MS experiments on MALDI-TOF and MALDI-TOF /TOF

Tryptic digests were desalted by Zip-Tips. Eluted peptides were mixed with equal volume of matrix solution (10mg/ml □-cyano-4-hydroxycinnamic acid (CHCA) dissolved in 1:1 acetonitrile/water with 1% TFA) and then spotted on MALDI target plate and vacuum dried. The MALDI-TOF mass spectra were acquired on Applied Biosystems 4700 Proteomics Mass Spectrometer. Tryptic peptides isolated from in-gel digestion were analyzed on Analyzer in both MS and MS/MS modes.

3 Results

3.1 SDS-PAGE Electrophoresis of TDI-HSA and digested peptides formed in vitro

The mobility of HSA and the TDI-HSA conjugates can not solved on Tris Glycin SDS-PAGE system because of the rather small difference in molecular mass, but significantly different on IEF-PAGE systems because of mask reaction on positively charged moieties caused by covalent bonding. The recognition of fragments poses antigenic determinants of conjugates was shown as Fig. 1. After digestion, the digested fragments are smaller than control sample. The major components of peptides digested by protease K were similar evaluated from the Rf value on SDS-PAGE and directly ESI.

Ten major bands from TDI-HSA were estimated to have molecular mass about 4.9Kda, 3.7kDa and 2.7kDa, with a range from 27.4~2.1kDa on 16% SDS-PAGE. Control samples parallel synthesized without adding of TDI, exhibited to have molecular mass about 6.8Kda, 5.7kDa and 4.1kDa, with a range from 40.0~6.9 kDa.

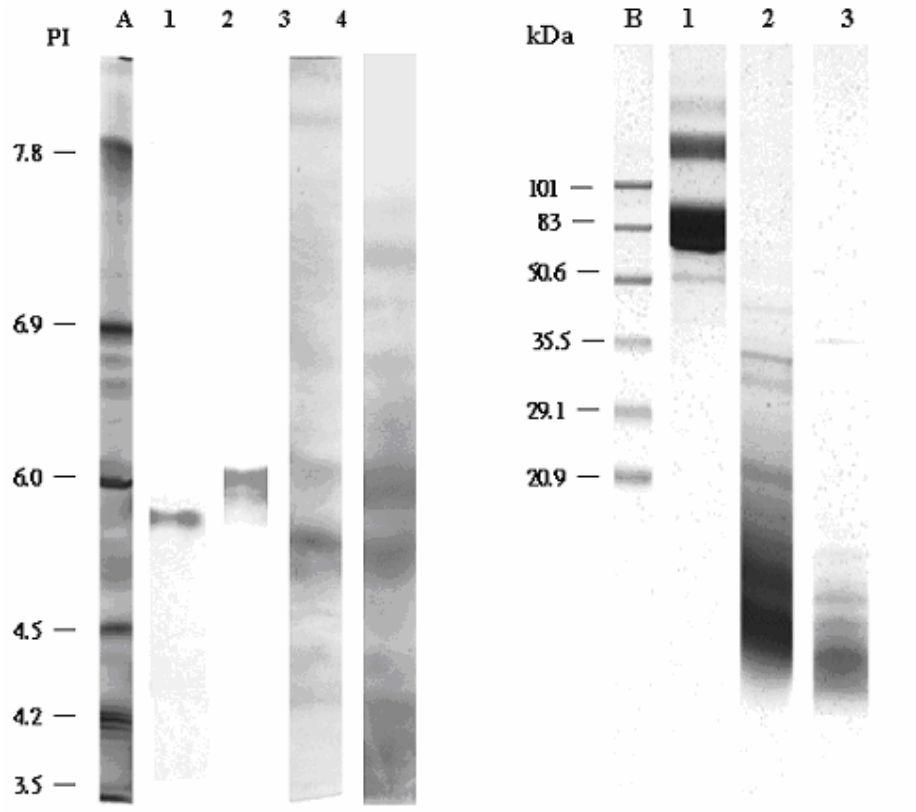

Figure 1. Gel electrophoresis of TDI-HSA and peptides obtained by protease K digestion. A1-A4: 3-10 IEF-PAGE; A: Marker of IEF standards; A1: HSA, A2: TDI-HSA; A3: peptides of HSA; A4: peptides of TDI-HSA. B1-3: 16% Tris-Glycin PAGE; B: Marker of MW standards; B1: TDI-HSA; B2: peptides of HSA; B3: peptides of TDI-HSA conjugates.

3.2 ESI-MS analysis of peptides digested from TDI-HSA adducts

Abundant signals of protonated molecular ions were observed in ESI-MS spectra. The suspected peaks not appeared in albumin lysates were observed, and after transformation calculation from multiple charged spectra, those peptides where covalent bonds formed were elucidated as shown in Table 1. The low molecular mass signal 785 m/z was donated as FKAW fragment plus a covalently bound TDI-monoethanoamine (TDI-MEA) moiety (234 mass unit), that is calculated two miss cuts, which were a casuals results of TDI addition. Two corresponding peptides with

1949 Da and 3403 Da in molecular mass after transformation was considered as a non-cleaved peptide linked with MEA-TDI-Lys moiety at 437 or 438. Another two corresponding peptides were assigned to contain that moiety, located at 413, with two or three miss cuts with 2756 Da and 3098 Da, respectively. The low molecular mass signal 785 m/z was assigned as a short fragment with two miss cuts, which is adducted on Lys 236, plus a covalently bound TDI-MEA moiety that is single charged, exhibited a possible fragmentation.

Table 1 ESI-MS spectrometry analysis on peptide fragments from TDI conjugates which digested by protease K synthesized *in vitro* with a buffered saline.

Adducted site	Sequence of fragments	Miss cut number	Calculated* (Da)	Observed (Da)
44 (36-51)	KDLGEENFKALVLIAF	4	2040.9	2042
214 (202-218)	LLPKLDELRDEGKASSA	4	2147	2150
236 (235-238)	FKAW	2	784	785
249 (245-252)	QRFPKAEF	2	1391	1392
375 (367-377)	VVLLLRLAKTY	1	1522.8	1525
413 (402-425)	KPLVEEPQNLIKQNCELFEQ LGEY	3	3095.4	3098
413 (396-416)	KVFDEFKPLVEEPQNLIKQNC	2	2752.3	2756
437 (431-459)	LLVRYTKKVPQVSTPTLVEV SRNLGKVGS	5	3402.8	3403
437 (436-451)	TKKVPQVSTPTLVEVS	1	1946.9	1949
565 (539-570)	TLSEKERQIKKQTALVELVKHKPKATKEQLKA	3	3935.1	3938
581 (578-591)	FVEKCCKADDKETC	4	1852.7	1853

* Molecular weight of peptides plus TDI modified by one monoethanoamine

3.3 MS analysis of peptides from TDI-HSA conjugates in buffered saline

A search of the albumin sequence for subsequences produced several hits, both

spectra reveals a number of $(M+H)^+$ ions that could be readily matched. Those predicted peptides appeared in albumin spectra but not found in the adducted ones, present the corresponding signals by the addition with 175 or 190 Da, which were designated as the addition of TDI moieties for stop reagent ammonium bicarbonate.

The initial mass fingerprinting on MALDI-TOF mass spectra of albumin and its TDI conjugate are different obviously as shown in Figure 2A. Both spectra reveal a number of $(M+H)^+$ ions that could be readily matched to the sequence of albumin. However, in the tryptic digest of TDI-HSA samples, peptides with relevant molecular masses and strong intensities are recognized for their increase in the mass of the TDI moieties. For some adducted peptides the difference of the observed mass and the calculated mass and were 2 or 3 amu, that can assigned for the additive proton number for the those peptides in the condition of sample treatment and analysis after TDI conjugation. The location sites of seven ions and the associated sequences were marked in Figure 2A. The ion at m/z 1443.7 was attributed to that on Lys^{249}(247-257), which is also a relevant ion on ESI mass spectrometry. The ion at m/z 1499.6 is a corresponding ion with TDI adducted on the same Lys^{565} with two misscuts. The observed ions deduced to adductive peptides in tryptic digest are m/z, 1210.6, 1443.7, 1471.8, 1499.6, 1532.7, 2090.1, and 2207.2 in Maldi-ToF mass spectrometry.

3.4 Peptides of TDI-HSA isolated from control serum and pooled sera of exposed workers

The initial mass fingerprinting on MALDI-TOF mass spectra of TDI conjugates isolated from adductive control serum and pooled sera of exposed workers are different obviously from that formed in buffered saline as shown in Figure 2B and Figure2C. Both spectra reveal a number of $(M+H)^+$ ions that could be readily matched to the sequence of albumin. However, peptides with relevant molecular masses and strong intensities are recognized for their increase in the mass of the TDI moieties could not assigned as previous. The ion at m/z 1187.7 was attributed to that on Lys^{249}, the ion at m/z 1323.6 is a corresponding ion with TDI adducted on the same Lys^{27}. One ion observed was deduced to peptides in the tryptic digests of these two TDI conjugated albumin is m/z 1467.8. The sequence of this peptide is the portion on which TDI was not adducted on both i*n vitro* and *in vivo* conditions. It also provided a strong evidence for the source of those other peptides, come from albumin molecule.

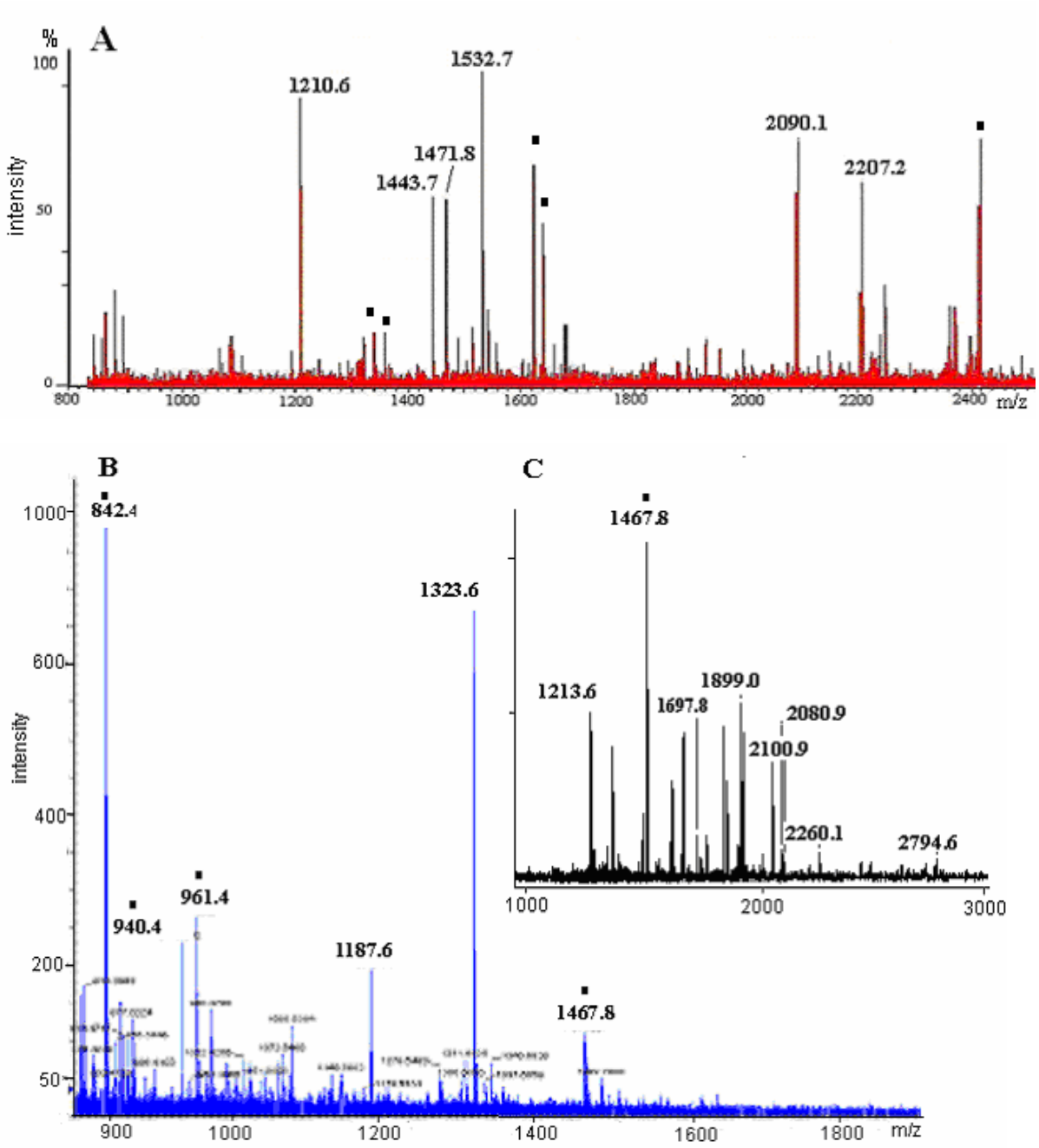

Figure 2. Peptides found in the tryptic digests of TDI-conjugated albumins isolated by affinity columns from control serum and exposed workers. A: TDI conjugated albumin synthesized in buffered saline; B: TDI conjugated albumin synthesized with control serum; C:TDI conjugated albumin isolated from exposed workers. (▪**:** ions deduced to albumin)

3.5 Determination of binding sites of TDI-HSA by MS/MS experiments

Because of appearance of the ion at m/z 1443.6 found in the Maldi mass spectrometric experiment, we designate the adducted site is on Lys^{249}, assigned as the peptide FP(T)KAEFAEVSK (247-257). The results also support that TDI –Lysine residues always cause key miss cutting on lysine during trypsin digest treatment. Other responsible sequences were predicted as $HPYFYAPELLFFAKR^{184}$ and $AACLLPKLDELR^{210}$, at m/z 2090.1 and 1532.7, respectively. In the spectra of adducted peptides, the signals were neither found for both of the Mr of albumin peptides and its adducted forms, showed in matrix assistant ionization at m/z 2090.1 and 1532.7. Combine with the data from tryptic digests in Maldi-TOF/TOF and protease K digests in ESI-MS, we suppose for short time conjugate reaction, under physiological condition, TDI binding sites on albumin were verified.

3.6 MS/MS analysis of conjugated albumin in serum

In prove of the TDI moieties in the conjugated albumin in serum, tryptic digests of isolated adductive albumin, the suspected peptides, were identified on Maldi-TOF/TOF experiments, by the appearance of an ions of m/z175 in the MS/MS spectra. A representative CID spectrum of the ion at m/z 1187.7 is shown in figure 3. All the fragments in the positive ion spectrum are primarily b- and y- series, from which the peptide sequence can be easily deduced. The adducted TDI moieties at the □-NH_2 groups of lysine are noted for the whole molecule (175.1 Da) or its saturated isocyanate side chain fragments.

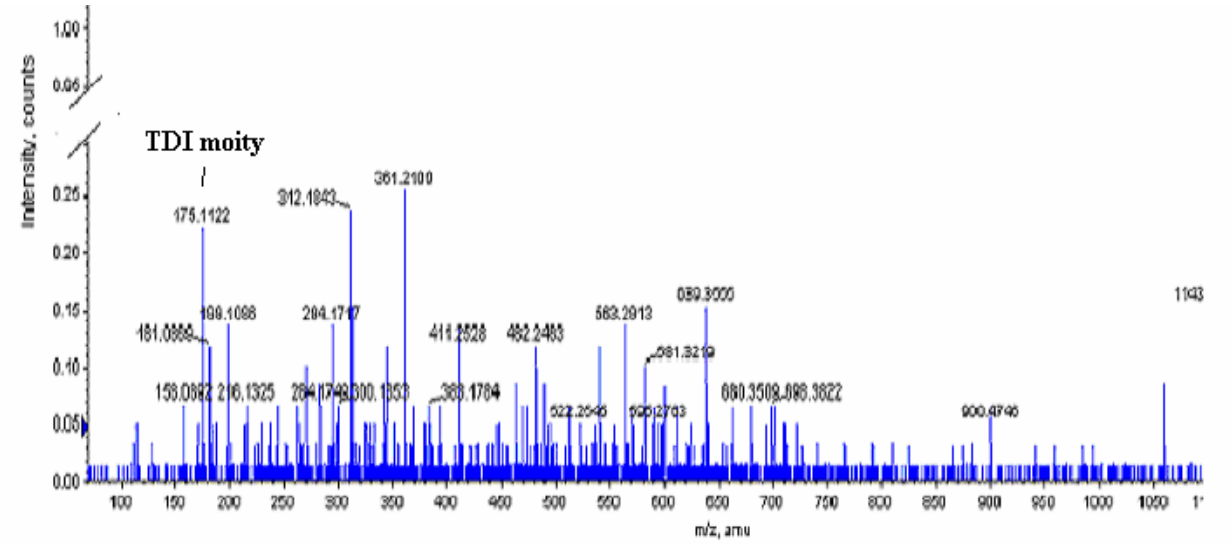

Figure 3. MS/MS identification of TDI-adducted sites on albumin synthesized with control serum. MS/MS spectrum corresponding to TDI-peptides: attached on Lys249, indicated are the parent ion (MH^{+1}=1187.8 Da), as well as the observed a, b, c cleaved ion.

4 Discussion

The positions of binding sites were proved from the expected MW of TDI conjugating peptides by ESI-MS, matrix-assisted laser desorption/ionization time-of-fly mass spectrometric (MALDI-TOFMS) analysis and several ions were identified by collision induced decay (CID) on MALDI-TOF/TOF. *In vitro* system, we use whole human serum albumin as a substrate, which was the enzymatic deglycosylated after the reaction, in order to reduce the disturbance of ionization. In the batches of different blocking agents and proteolytic enzymes, we have observed similar results of binding sites. The mass shift of +175 Da is considered corresponding to TDI residues with or without an ammonium ion when using ammonium carbonate as a block agent. The adducted peptide at Lys^{249} with 1392Da and 1443.7 Da in MW is easily ionization in both ESI and Maldi ionization methods, respectively. Besides of this, in the conjugates of control serum isolated by affinity columns coated TDI specific antiserum, two adductive peptides were also fit the increments of TDI moieties and fit the albumin sequence. But in the in the MALDI-TOF spectrum of suspected TDI conjugates, which was isolated from sera of the exposed workers, most of the tryptic peptide ions could not be assigned following the same analytic rules. Because in the same spectrum, the relevant ion at 1467 m/z, is a well assigned peptides for human serum albumin, there is no doubt for

the protein isolated. It was confirmed on SDS-PAGE and immunoblot, a single band with the similar MW of albumin recognized by anti-HSA antiserum. We speculate in human serum condition, there have other natural small molecules, which can stop the active isocynate groups. Other reasons can be considered, the duration of contact with TDI in exposed workers, that is proportion to the reaction time in vivo, may be much longer than in vitro systems. The reaction mode and components in the reaction system between the two situations are quite different. All the results support Lys 249 is an important TDI binding site. It might be the possible adducted location in the albumin molecule of exposed workers, because of the preexisted small nucleophilic molecules in body fluid, obviously possessing blocking effects on free isocyanate groups, might competitive with positively charged residues in albumin.

5 Concluding remarks

We suppose there are about ten TDI binding sites on human serum albumin molecule. Those sequences containing TDI-Lys moieties might play pathophysiological roles in allergic responses. Our study provides evidences in precise structure changes repeatedly. By comparison of these spectra on adductive peptides, we suggest Lys 249 might be is an important site adducted by TDI both *in vivo* and *in vitro*.

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