Utilization of newly developed immobilized enzyme reactors for preparation and study of immunoglobulin G fragments

Lucie Korecká a,∗, Zuzana Bílková b, Michal Holeápek a, Josef Královský a, Milan Beneš c, Jiří Lenfeld c, Nicolas Minc d, Roxana Cecal e, Jean-Louis Viovy d, Michael Przybylski e

a Department of Analytical Chemistry, University of Pardubice, nám. Čs. legi 565, 53210 Pardubice, Czech Republic
b Department of Biological and Biochemical Sciences, University of Pardubice, nám. Čs. legi 565, 53210 Pardubice, Czech Republic
c Institute of Macromolecular Chemistry, Academy of Sciences of the Czech Republic, Heyrovského nám. 2, 16206 Prague, Czech Republic
d Laboratory of Physical-Chemistry, Institut Curie (UMR CNRS/IC 168), Paris Cedex 05, France
e Laboratory of Analytical Chemistry and Biopolymer Structure Analysis, University of Konstanz, Fach M639, 78457 Konstanz, Germany

Available online 2 June 2004

Abstract

The newly developed immobilized enzyme reactors (IMERs) with proteolytic enzymes chymotrypsin, trypsin or papain were used for specific fragmentation of high molecular-mass and heterogeneous glycoproteins immunoglobulin G (IgG) and crystallizable fragment of IgG (Fc). The efficiency of splitting or digestion were controlled by RP-HPLC. The specificity of digestion by trypsin reactor was controlled by MS. IMERs (trypsin immobilized on magnetic microparticles focused in a channel of magnetically active microfluidic device) was used for digestion of the whole IgG molecule. The sufficient conditions for IgG digestion in microfluidic device (flow rate, ratio S:E, pH, temperature) were optimized. It was confirmed that the combination of IMERs with microfluidic device enables efficient digestion of highly heterogeneous glycoproteins such as IgG in extremely short time and minimal reaction volume.

© 2004 Elsevier B.V. All rights reserved.

Keywords: Immobilized enzyme reactors; Immunoglobulin G

1. Introduction

Peptide mapping is a convenient and widely used method for the identification and characterization of proteins. This technique is used for studying primary structure of proteins and can be employed for the detection of any post-translational modifications (glycosylation, etc.) [1–3].

Conservative oligosaccharide structure in glycoproteins is not random under the normal physiological conditions and the changes in the content or in the sequence of oligosaccharide chains may result in the immunopathological response, such as auto-immune diseases [4].

Peptide mapping consists of site-specific enzymatic (or chemical) digestion of the protein into a number of smaller peptide fragments followed by separation and detection of the acquired peptides [1–3]. Studying of protein macromolecules is very difficult due to their complex and heterogeneous structure. Resolution is in the isolation of signature peptides which facilitated the detection of target post-translational modifications, e.g. glycosylation, and study of selected specific diagnostically important part [5]. The first step of protein digestion into peptide fragments is critical for the fingerprint quality in peptide mapping. The use of immobilized enzymes for cleavage of proteins is advantageous in comparison with their soluble forms. The main advantages of immobilized enzyme reactors (IMERs) are the complete elimination of the enzyme from the reaction mixture and short digestion time without risk of the
2. Materials and methods

2.1. Chemicals

Chymotrypsin (EC 3.4.21.1), Trypsin (EC 3.4.22.2), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC), human IgG-reagent grade, dithiothreitol (EDAC), human IgG-reagent grade, dithiothreitol (DDT), iodoacetic acid (IAA), acrylamide, N,N′-methylenebis-acrylamide, N,N,N′,N′-tetramethylethylene-diamine (TEMED), molecular mass marker kit (50,000–14,300 relative molecular mass units), polyoxyethylsorbitane monolaurate (Twem 20) were products of Sigma-Aldrich (St. Louis, MO, USA); trifluoroacetic acid and N-hydroxysulfosuccinimide sodium salt (S-NHS) were obtained from Fluka (Buchs, Switzerland); acetonitrile and water for HPLC gradient grade were from Lab-Scan (Dublin, Ireland), macroporous bead cellulose Perloza MT 200 was supplied by Lovochemie (Lovosice, Czech Republic); magnetic macroporous bead cellulose L1459-3 (125–250 μm) and alginate coated form of ferrite microparticles A59 (0.2–2 μm) were supplied by Institute of Macromolecular Chemistry (Academy of Sciences, Prague, Czech Republic); ImmunoPure IgG Purification Kit (PIERCE, Rockford, USA); hemoglobin from Serva (Heidelberg, Germany), and Reanal (Budapest, Hungary) was the supplier of sodium periodate. The remaining chemicals were supplied by Lachema (Brno, Czech Republic) and were of analytical reagent grade.

2.2. Purification of IgG and isolation of Fc fragment using affinity chromatography on a protein A

Lyophilized polyclonal human IgG was used for purification of IgG and isolation of Fc fragment by affinity chromatography on a column with protein A (ImmuNoPure IgG Purification Kit, PIERCE, Rockford, USA). Column was equilibrated with 5 ml of binding buffer (0.1 M phosphate buffer pH 7.5 with 0.15 M NaCl). One milliliter of IgG (10 mg/ml) was applied and column was washed by 15 ml of binding buffer. Pure IgG was eluted with 5 ml of elution buffer (0.1 M glycine buffer pH 2.8 with 0.15 M NaCl). Collect fractions were neutralized by addition of 50 μl of neutralization solution (0.1 M Tris–HCl buffer pH 9.5). Purity of the fractions was determined by SDS-PAGE. The column was regenerated with 8 ml of citric acid pH 3.0.

The same procedure was used for isolation of Fc fragment of IgG. 1 ml of IgG split by immobilized papain (see Section 2.7), i.e. mixture of Fc and Fab fragments, was used. Fc fragment was retained on a column and Fab fragment was washed out with a binding buffer. Fc fragment was then eluted with elution buffer and purity of the fractions was determined by SDS-PAGE.

2.3. Immobilization of trypsin and chymotrypsin on a non-magnetic macroporous bead cellulose (according to ref. [17]—slightly modified)

Five milliliters of macroporous bead cellulose was activated using 5 ml of 0.2 M NaOH. Mixture was stirred 90 min at room temperature. Cellulose was washed five times with distilled water. Ten milligrams of trypsin or chymotrypsin, dissolved in 0.1 M carbonate buffer pH 9.0, was added and mixture was stirred for 2 h at room temperature. Celulose with immobilized enzyme was washed five times with 0.1 M carbonate buffer pH 9.0. After centrifugation (2590 x g, 3 min), natrium borohydride (15 mg/5 ml) was added and mixture was stirred 20 min at room temperature. Cellulose was washed five times with 0.1 M carbonate buffer pH 9.0, five times with 0.1 M carbonate buffer pH 9.0 with 1 M NaCl, five times with 0.1 M carbonate buffer pH 9.0 with 1 M NaCl, five times with 0.1 M carbonate buffer pH 9.0 with 1 M NaCl, five times with 0.1 M carbonate buffer pH 9.0 with 1 M NaCl, five times with 0.1 M carbonate buffer pH 9.0. The same buffer was used as a storage buffer. Activity of the enzyme reactor was determined using high molecular-mass substrate hemoglobin (according to the method of Anson and Mirskij, 1938) [18].
2.4. Immobilization of papain on a magnetic macroporous bead cellulose

Five milliliters of magnetic macroporous bead cellulose was activated using 5 ml of 0.2 M NaOCl. Mixture was stirred 90 min in darkness at room temperature. Cellulose was washed five times with 0.1 M phosphate buffer pH 7.0 with 0.002 M EDTA. One milligram of enzyme papain dissolved in the same buffer was added and mixture was stirred 10 min at room temperature. Three milligrams of natrium cyanoborohydride was added and it was stirred at 4 °C over the night. Immobilized papain was washed five times with 0.1 M phosphate buffer pH 7.0 with 0.002 M EDTA, five times with 0.1 M phosphate buffer pH 7.0 with 0.1 M NaCl and five times with 0.1 M phosphate buffer pH 7.0 with 0.002 M EDTA. As a storage buffer 0.1 M phosphate buffer pH 7.0 with 0.002 M EDTA and benzamidine (0.1 mg/ml 1 ml storage buffer) was used. Activity of the enzyme reactor was determined using highmolecular-mass substrate hemoglobin (according to the method of Anson and Mirskij, 1938) [18].

2.5. Immobilization of trypsin on alginate coated form of ferrite microparticles A59

One milliliter of 0.5% (m/v) suspension of magnetic particles A59 was washed five times with 0.1 M phosphate buffer pH 7.3 with 1% (v/v) Tween 20. Three hundred microliters of EDA( and 100 cal 5-NHS was added. Three hundred microliters of trypsin solution (1 mg of trypsin and 0.2 mg of benzamidine in 300 µl of 0.1 M phosphate buffer pH 7.3) and 300 µl of 0.1 M phosphate buffer pH 7.3 with 1% (v/v) Tween 20 was added and it was stirred at 4 °C over the night. Immobilized trypsin was washed 10 times with 0.1 M phosphate buffer pH 7.3 with 1% (v/v) Tween 20. As a storage buffer 0.1 M phosphate buffer pH 7.3 with 1% (v/v) Tween 20 and benzamidine (0.1 mg/ml 1 ml storage buffer) was used.

2.6. Digestion of IgG with trypsin and chymotrypsin immobilized on a non-magnetic macroporous bead cellulose

Ten milligrams of IgG was dissolved in 1 ml of 0.4 M NH4HCO3 with 8.0 M urea and 100 µl of 45 mM DTN was added. Mixture was incubated 15 min at 50 °C. After cooling to room temperature, 100 µl of 100 mM IAA was added. After incubation (10 min at room temperature), 2.8 ml of distilled water was added along of the dilution of urea to concentration 2 mol/l.2 ml of this mixture was added to 200 µl of trypsin (chymotrypsin) immobilized on a macroporous bead cellulose. Mixture was stirred at 37 °C for 3 h (24). During incubation a small fractions of mixture were collected and analyzed by SDS-PAGE and RP-HPLC.

2.7. Splitting of IgG to Fc and Fab fragments using immobilized papain

One milliliter of immobilized papain was washed five times with 0.1 M phosphate buffer pH 7.0 with 0.002 M EDTA and 0.01 M cystein. Cellulose in this buffer was incubated at 37 °C for 35 min. Immobilized papain was washed five times with 0.1 M phosphate buffer pH 7.0 with 0.002 M EDTA. Then 1 ml of IgG dissolved in 0.1 M phosphate buffer with 0.002 M EDTA (10 mg IgG/ml) was added. Mixture was stirred at 37 °C overnight.

2.8. Cleavage of Fc fragment IgG with trypsin immobilized on a non-magnetic macroporous bead cellulose

One milliliter of Fc fragment isolated on a protein A (see Section 2.2) was unfolded according the protocol 2.6. Mixture was concentrated from 4 to 1 ml using ultracentrifugation (4800 g, 15 min). Mixture was added to a 100 µl of immobilized trypsin and stirred at 37 °C for 3 h. Sample was analyzed by SDS-PAGE and RP-HPLC.

2.9. Digestion of IgG using microfluidic device

Unfolded IgG (see Section 2.6) (1.2 mg/ml) was applied into a channel of magnetically active microfluidic device [10] (polydimethylsiloxane-PDMS, channel parameters: 20 mm (l) × 0.4 mm (h) × 3 mm (w)) with 0.56 mg of alginate coated form of ferrite microparticles A59 with immobilized trypsin (see Section 2.5) (activity was 136.8 µg (0.56 mg of particles)). The ratio of S/E was 1:20, flow rate of mobile phase (0.1 M phosphate buffer pH 7.3) was 15 µl/h. According to plug parameters (6 mm (l) × 0.4 mm (h) × 3 mm (w)), amount of active particles and flow rate the Residential time (Tres) defined as contact time of enzyme with substrate (Tres (min) = V/2Q × flow rate) was determined. Vplug is defined as volume of the carrier with the enzyme inside of channel.

2.10. SDS-PAGE

SDS-PAGE was performed according to the method of Laemmli [19]. Peptide fragments were separated on 10% polyacrylamide gel and detected with Coomassie blue staining. To confirm the identity of proteins (molecular mass) standard of molecular mass was applied. Protein mobilities were determined relative to the mobility of the tracking dye Coomassie blue (relative mobility %). For densitometric evaluation ElfoMan software (Semecky, Prague, Czech Republic) was used.

2.11. HPLC equipment

Chromatographic separations were performed using two chromatographic systems. The first HPLC system was from Waters (Milford MA, USA) and comprised Model 616 pump, 717 autosampler and 996 Diode Array Detector. Separations were performed on 250 mm × 2 mm i.d. reversed-phase column of 5.29 µm Luna C18 from Labio (Prague, Czech Republic). Second HPLC system was from GBC (Dandenong, Australia) and comprised LC 1150 HPLC.
pump, ERC-3415 Degasser, LC 1650 advanced autosampler, LC 1210 UV/Vis detector and LCO1M column oven from Ecom (Prague, Czech Republic). Separations were performed on 250 mm × 4.6 mm i.d. reversed-phase column of 5 μm Supelcosil LC-18-DB (Supelco, Sigma–Aldrich, St. Louis, MO, USA).

2.12 RP-HPLC separation

Solvent A was water (Lab-Scan, Dublin, Ireland) containing 0.1% (v/v) trifluoroacetic acid (Fluka, Buchs, Switzerland). Solvent B was acetonitrile (Fluka, Buchs, Switzerland) containing 0.085% (v/v) trifluoroacetic acid. Different gradients were used for the chromatographic separations of peptide fragments.

(1) Separation of peptide fragments of IgG: HPLC system waters: 4–30% (v/v) solvent B from 0 to 40 min; 30–80% (v/v) solvent B from 40 to 53 min; 80–100% (v/v) solvent B from 53 to 60 min; continuing at 100% (v/v) solvent B an additional 7 min and then 0–4% (v/v) solvent B from 67 to 69 min. Flow rate of mobile phase was 0.3 ml/min, injection volume 20 μl, column temperature 40 °C. UV-absorption detection was performed at 215 nm.

(2) Separation of Fc and Fab fragments and H (heavy) and L (light) chains of IgG: HPLC system GBC: 0% (v/v) solvent B from 0 to 5 min; 0–100% (v/v) solvent B from 5 to 40 min; 100–0% (v/v) solvent B from 40 to 45 min. Flow rate of mobile phase was 1.0 ml/min, injection volume 150 μl, column temperature 25 °C. UV-absorption detection was performed at 230 and 280 nm. Method of standard addition was used.

(3) Separation of peptide fragments of IgG digested in microfluidic device: HPLC system GBC: 4–40% (v/v) solvent B from 0 to 60 min; 40–100% (v/v) solvent B from 60 to 65 min; 100–4% (v/v) solvent B from 65 to 67 min. Flow rate of mobile phase was 0.8 ml/min, injection volume 80 μl, column temperature 40 °C. UV-absorption detection was performed at 220 nm.

2.13 Mass spectrometric (MS) analysis

The FT-ICR measurements were performed with a Bruker (Bruker Daltonik, Bremen, Germany) Apex II FT-ICR mass spectrometer equipped with a 7T superconducting magnet, a cylindrical infinity ICR analyser cell and an external Scout 100 fully automated X–Y target stage Maldi source with pulsed collision gas. A 100 mg/ml solution of 2,5-di-dihydroxybenzoic acid (DHB) in acetonitrile: 0.1% (v/v) trifluoroacetic acid in water (2:1) was used as matrix for sample preparation. Calibration was performed with a standard peptide mixture with an m/z range of approximately 5000.

The β-amyloid peptide-βA(1–40) peptide was synthesized by solid phase peptide synthesis (SPPS) on a
NovaSyn TGR resin according to Fmoc/tBu chemistry, using a semi-automated economy peptide synthesizer EPS-221 (ABIMED, Germany). The crude peptide was purified by reversed phase HPLC using a semipreparative Vydac C4 column. As mobile phases, 80% (v/v) acetonitrile, 0.1% (v/v) TFA in MilliQ (solvent B) and 0.1% (v/v) TFA in MilliQ (solvent A) were used. The experimental conditions depend on the hydrophobicity and the molecular weight of the peptides.

3. Results and discussion

Immobilized enzyme reactors with proteolytic enzymes trypsin, chymotrypsin or papain were used for digestion of polyclonal human IgG. These enzymes were immobilized on nonmagnetic and magnetic carriers (see Table 1). Using IMERs we could reduce the digestion time without the risk of contamination of the resulting peptide mixture with autoproteolytic fragments of the enzyme.

Proteolytic activity of IMERs was monitored by SDS-PAGE and by more sensitive separation technique RP-HPLC. We have determined the essential time to complete digestion of protein, the ratio of substrate:enzyme (S:E) performed in certain reaction volume. Kinetics of the reaction, characterized by Michaelis constant ($K_M$), is significantly influenced by the availability of active sites of the enzyme for the analyte. Consequently high molecular-mass substrate hemoglobin was used for the evaluation of IMERs activity.

Chymotrypsin digestion time (24 h) is sufficient for the commonly used ratio of substrate and soluble form of enzyme from 50:1 [16] to 25:1 [2]. The application of IMER with immobilized chymotrypsin (the ratio S:E was 30:1; see Table 1) has not influenced essentially the digestion time for unfolded polyclonal human IgG (Fig. 1). Due to the many potential digestion sites the chymotrypsin is not suitable for

![](image)
Fig. 2. Densitometric evaluation of SDS-PAGE analysis of IgG digested with trypsin IMER in time. Ten percent polyacrylamide gel, detection with Coomassie blue staining. Protein mobilities were determined relative to the mobility of the tracking dye Coomassie blue (relative mobility %), OD: arbitrary units. For densitometric evaluation ElfoMan software (Semecky, Czech Republic) was used.

Fig. 3. RP-HPLC peptide fingerprint of time course of IgG digestion with trypsin IMER. HPLC system Waters. Flow rate 0.3 ml/min, injection volume 20 μl, column temperature 40°C, detection at 215 nm. Gradient: 4–30% (v/v) B/0–40 min, 30–50% (v/v) B/40–53 min, 50–80% (v/v) B/53–60 min, 80–4% (v/v) B/64–67 min.
the peptide mapping of high molecular glycoproteins such as IgG. Trypsin digestion time (3 h) is sufficient for commonly used ratio of substrate and soluble form of enzyme 50:1 [16].

Using IMERs with immobilized trypsin reactor in batch-wise arrangement, the same digestion efficiency of unfolded IgG was obtained in 1 h even at the ratio S:E 100:1. Digestion efficiency was confirmed by SDS-PAGE (Fig. 2) and RP-HPLC (Fig. 3).

Acquired finger prints of IgG are very complex as a consequence of its high molecular mass and heterogeneity. Studying of posttranslational modifications derived from the modification of side chains of one or a few amino acids, e.g. by glycosylation or phosphorylation is difficult. In certain cases it is possible to analyse only a specific part of complex molecule. Heavy chains (H) or Fc fragments of IgG molecule include key C1c2 domain with pathologically important glycosylation. The ideal case would be to reduce...
Fig. 8. Chromatographic separation of Fc and Fab fragments of human polyclonal IgG at 230 nm. HPLC system GBC. Flow rate 1.0 ml/min, injection volume 150 μl, column temperature 25 °C, detection at 280 nm. Gradient: 0% (v/v) B/0–5 min, 0–100% (v/v) B/5–35 min, 0% (v/v) B/35–45 min. Peaks in 27 min correspond to Fab fragment, in 28 min to Fc fragment. In 38 min is a peak of a residue of whole molecule of IgG (confirmed by method of standard addition).

the number of peptides that must be separated or to isolate a signature peptide with corresponding posttranslational modification such as glycosylation. The sequence of these peptides is closely related to the parent proteins from which they are derived [20].

Simple preparation of heavy (H) chain is advantageous but this polypeptide is heterogeneous due to the presence of Vc1 domain of the molecule. Preparation of Fc fragment is time consuming but Cβ2 and Cγ3 domains are conservative in respect of amino acid sequence.

The efficiency of splitting of IgG to H and L chains was controlled by SDS-PAGE and RP-HPLC analysis (Figs. 4 and 5). The conditions of separation of H and L chains derived from monoclonal IgG by RP-HPLC were already published [1], so we optimized the conditions for polyclonal human IgG. Double peaks of both chains of IgG on Fig. 5 are presented due to the polyclonality of IgG and higher sensitivity of RP-HPLC of peptides at 230 nm. Due to the variability

CLEAVAGE REPORT

Sequence: APEB771-711

N-C terminus: Hydroxyl (L01 Da) / Amidation (16.02 Da)

Enzyme: Trypsin Parameters: / IncR-P

Peptide mass values are: Mono

<table>
<thead>
<tr>
<th>Num</th>
<th>M+H</th>
<th>M+2H</th>
<th>M+3H</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>135 637 7048</td>
<td>137 637 7048</td>
<td>254 775 8096</td>
<td>GAPE</td>
</tr>
<tr>
<td>2</td>
<td>137 637 7048</td>
<td>137 637 7048</td>
<td>254 775 8096</td>
<td>LFFFAEDV0SNK</td>
</tr>
<tr>
<td>3</td>
<td>137 637 7048</td>
<td>137 637 7048</td>
<td>254 775 8096</td>
<td>DAEKRDR0YTEVERQ</td>
</tr>
<tr>
<td>4</td>
<td>137 637 7048</td>
<td>137 637 7048</td>
<td>254 775 8096</td>
<td>1954 8796 977 8423 652 2394</td>
</tr>
<tr>
<td>5</td>
<td>137 637 7048</td>
<td>137 637 7048</td>
<td>254 775 8096</td>
<td>2391 3110 1136 1594 797 7736</td>
</tr>
<tr>
<td>6</td>
<td>137 637 7048</td>
<td>137 637 7048</td>
<td>254 775 8096</td>
<td>2643 2392 1322 3332 801 7838</td>
</tr>
<tr>
<td>7</td>
<td>137 637 7048</td>
<td>137 637 7048</td>
<td>254 775 8096</td>
<td>3261 5354 1631 2716 1087 8593</td>
</tr>
<tr>
<td>8</td>
<td>137 637 7048</td>
<td>137 637 7048</td>
<td>254 775 8096</td>
<td>3709 8961 1084 9519 1236 9706</td>
</tr>
<tr>
<td>9</td>
<td>137 637 7048</td>
<td>137 637 7048</td>
<td>254 775 8096</td>
<td>4327 1722 2164 0990 1443 0626</td>
</tr>
</tbody>
</table>

Fig. 9. RP-HPLC peptide fingerprint Fc fragment digested with trypsin IMER. HPLC system Waters. Flow rate 0.3 ml/min, injection volume 20 μl, column temperature 40 °C, detection at 215 nm. Gradient: 4–30% (v/v) B/0–40 min, 30–50% (v/v) B/40–53 min, 50–80% (v/v) B/53–65 min, 80–4% (v/v) B/64–67 min.

Fig. 10. Theoretical peptide Aβ1-40 fragments after trypsin digestion.
of H chains (see Fig. 5) and due to the time consuming iso-
lation of entire H chain using size exlusion chromatography
we have selected the Fc fragment for additional study.

The Fc fragment was prepared by enzymatic splitting of
human polyclonal IgG using IMERs with papain. The pa-
pain was immobilized to a magnetic form of macroporous
bead cellulose (see Table 1). The use of magnetic supports
prevents undesirable sample dilution and losses of the car-
rier during washing. The efficiency of splitting into Fc and
Fab fragments was controlled by SDS-PAGE (Fig. 6) and
RP-HPLC analysis (Figs. 7 and 8). Probable resistance of
IgG subclass to enzymatic splitting was confirmed only by
RP-HPLC (see Figs. 7 and 8). The whole molecule of IgG
was detected even after the splitting for 16 h [21,22]. Using
affinity chromatography on a column with immobilized pro-
tein A we isolated highly purified fraction of Fc molecules
with sufficient concentration of protein.

The first step of the peptide mapping is a site-specific
enzymatic digestion of the target protein into a number of
smaller peptide fragments. Isolated Fc fragment was di-
gested using IMERs with trypsin (see Table 1) without risk
of the contamination of resulting peptide mixture. Digestion
efficiency was confirmed by RP-HPLC. Acquired peptide
map of Fc fragment (Fig. 9) in comparison to a peptide map
of complete IgG (see Fig. 3) was less complicated and more
comprehensive for subsequent interpretation.

The specificity of trypsin reactor (alginate coated fer-
rite microparticles A59) was controlled by MALDI/FT-ICR
mass spectrometry (Figs. 10 and 11). Model protein low
molecular mass peptide β-amylode peptide Aβ(1-40) was
utilized. Conditions 10 μg of substrate, 10 μl reaction vol-
ume, particles with proteolytic activity equal to 15 μg of
tryptsin, digestion time 15 min, 37 °C and phosphate buffer
are sufficient for the specific fragmentation of protein with-
out miss-cleavages.

By miniaturization of the whole system we can signifi-
cantly reduce the digestion time and increase the sensitiv-
ity and specificity of fragmentation. IMERs with trypsin
immobilized on magnetic alginate coated form of ferrite

---

Fig. 11. MALDI FTICR/MS analysis of peptide Aβ1-40 after trypsin
digestion.
microparticles focused in a channel of magnetically active microfluidic device \cite{10} (PDMS, channel parameters: 20 mm (l) × 0.4 mm (h) × 3 mm (w)) was used for digestion of the whole IgG molecule (Fig. 12).

The conditions for IgG digestion (ratio S:E, pH buffer, temperature and flow rate of mobile phase) were optimized (see Table 1). The efficiency of digestion was controlled by RP-HPLC. According to plug parameters (6 mm (l) × 0.4 mm (h) × 3 mm (w)) and flow rate 15 μl/h and protein content 1.2 mg/ml the *Resident time* for complete digestion 15 min was determined. Finger print of the whole molecule IgG acquired by digestion in microfluidic device is shown in Fig. 13. It was confirmed that the combination of IMERs with microfluidic device enables efficient digestion of highly heterogeneous glycoproteins such as IgG in extremely short time and minimal reaction volume.

4. Conclusions

Newly developed IMERs with proteolytic enzymes chymotrypsin, trypsin or papaín are sufficient for the specific fragmentation of high molecular-mass and heterogeneous glycoproteins. IMERs were used for digestion of highly heterogeneous IgG molecule without the risk of contamination of resulting peptide mixture with autoproteolytic fragments of the enzyme. The integration of IMERs into μ-chip device enables minimalization of digestion time and increases the sensitivity and specificity of fragmentation. This approach provides an effective tool for qualitative analysis of high molecular-mass glycoproteins and even for identification of their *signature peptide* in order to demonstrate the changes in protein structure derived from glycosylation.

Acknowledgements

This work was supported by the Ministry of Education Czech Republic VZ 253100002 and Grant Agency of the Czech Republic 203/02/0023.

References


\[8\] J. Turková, Z. Kučerová, H. Vašková, M. Beneš, Int. J. Biochro-
matogr. 3 (1997) 45.


\[18\] M.L. Anson, J. Mirsky, Gen. Physiol. 22 (1938) 79.


