ELSEVIER

Journal of Chromatography A, 795 (1998) 263–275

JOURNAL OF
CHROMATOGRAPHY A

Effect of the hydrolysis method on the determination of the amino acid composition of proteins

Martin Weiss, Michael Manneberg, Jean-François Juranville, Hans-Werner Lahm, Michael Fountoulakis*

F. Hoffmann-La Roche Ltd., Pharmaceutical Research–Gene Technologies, Building 93-444, 4070 Basel, Switzerland

Received 26 June 1997; received in revised form 18 September 1997; accepted 18 September 1997

Abstract

Fast and reproducible separation and determination of amino acids serves the economical and reliable characterization and quantification of peptides and proteins as well as the identification of proteins by amino acid composition analysis on a large-scale. A prerequisite of a successful compositional analysis is a complete hydrolysis of the peptides and proteins and a quantitative recovery of the residues in the hydrolyzate. We investigated the effect of different acid-hydrolysis methods on the compositional analysis of known proteins in solution and after blotting onto polyvinylidene difluoride membranes and worked out the conditions for the processing of large numbers of samples. The reliability of each method was studied by introducing the analysis data into the AACompIdent software and deducing the protein identification scores. All acid-hydrolysis methods delivered reliable analysis data. The most accurate data were provided by conventional, thermal hydrolysis of proteins in solution in the presence of methanesulfonic acid, closely followed by hydrolysis with hydrochloric acid and microwave radiation-dependent hydrolysis with hydrochloric or methanesulfonic acid, respectively. For blotted proteins, conventional hydrolysis delivered more accurate analysis data in comparison with the microwave radiation-induced hydrolysis. The extraction of the residues from the membrane hydrolyzate was a critical step for unambiguous protein identification. Microwave radiation-induced hydrolysis was responsible for a higher degree of racemization of the residues.
© 1998 Elsevier Science B.V.

Keywords: Hydrolysis; Amino acids; Proteins

1. Introduction

Amino acid composition analysis is a classical, rather complex, analytical method. It is essential for the detection of free amino acids and, in particular, in quality control studies for the investigation of the identity and the quantification of peptides and proteins. Moreover, data derived from amino acid compositional analysis are useful for protein identification by search in databases. Improvements in sample handling and the availability of user-friendly software contributed to the evolution of the technique as an efficient method for large-scale identification of proteins leading to the construction of two-dimensional (2D) protein maps. The latter are important in the characterization of the proteins of different organisms in healthy and diseased states [1–7].

Prior to analysis, the peptides and proteins have to be completely hydrolyzed to yield free amino acids.

*Corresponding author.

0021-9673/98/$19.00 © 1998 Elsevier Science B.V. All rights reserved.
PII S0021-9673(97)00983-7

Hydrolysis is a critical step and an amino acid analysis can only be successful if the preceding hydrolysis has been performed properly. Many hydrolysis protocols exist and the application of the proper method depends on the aim of the individual analysis. Hydrolysis is usually performed by heating the sample to be analyzed in the presence of high concentrations of acids using thermal or microwave radiation energy. Conventional liquid- or gas-phase hydrolysis is performed in constant boiling HCl in vacuo at 110°C for 24 h or multiples thereof depending on the frequency of amide bonds between aliphatic residue-containing amino acids [8,9]. For determination of methionine sulfoxide, the oxidation product of methionine, or of tryptophan, the hydrolysis is usually performed with methanesulfonic acid (MSA) [10,11]. Conventional heat-dependent hydrolysis at 110°C is time-consuming and occasionally is the bottleneck of the procedure. The hydrolysis time can be significantly reduced by using microwave radiation which produces high temperatures up to 180°C in a short time [12–17]. Following hydrolysis, the residues or their derivatives after reaction with fluorescent agents are separated by ion-exchange or reversed-phase high-performance liquid chromatography [18–20].

Critical issues in the identification of proteins, separated by 2D-polyacrylamide gel electrophoresis (2D-PAGE) and blotted onto polyvinylidene difluoride (PVDF) membranes, by amino acid analysis, are (i) the uniform extraction of all residues generated during hydrolysis so that the protein composition is not altered and (ii) a large sample throughput. The solubilization of the residues, after hydrolysis of proteins in solution, in the proper buffer for chromatographic separation does not represent a particular technical problem. However, the quantitative extraction of the residues, in particular the hydrophobic ones, from a PVDF membrane is associated with difficulties.

Because large numbers of diverse samples are often processed in an analytical laboratory, a high daily throughput largely depends on the availability of the required equipment and the application of the proper hydrolysis method. Here we studied the effect of different acid-hydrolysis methods on the quantification of the amino acids and the degree of residue racemization in the hydrolyzate, as well as the efficiency of the extraction methods on residue recovery from membranes. We also worked out the conditions for a large sample throughput required for efficient protein mapping. The results may be useful in the characterization and identification of proteins by amino acid compositional analysis for construction of protein maps.

2. Experimental

2.1. Materials

L- and D-Amino acid standards were purchased from Sigma and Hewlett-Packard. Hydrochloric acid and methanesulfonic acid were from Pierce. *N*-Isobutyryl-L-cysteine was from Novabiochem. Recombinant human interferon α_2 (IFNα_2) produced in *E. coli* was obtained from F. Hoffmann-La Roche (Basel, Switzerland) and bovine serum albumin (BSA) was from Serva.

2.2. Liquid-phase hydrolysis of proteins in solution at 110°C

All hydrolyses were performed in vials pyrolyzed at 490°C for 4 h. Conventional hydrolysis of proteins in solution was performed essentially as described by Manneberg et al. [21,22]. In short, to the protein solution (usually 500 μl) an equal volume of 12 *M* HCl was added (final HCl concentration 6 *M*) or alternatively the protein solution was evaporated to dryness and the precipitate was dissolved in 500 μl of 6 *M* HCl or 4 *M* methanesulfonic acid. To the HCl solution, 0.02% phenol was added (w/v, added as solid) and to the MSA solution, 0.2% 3-(2-aminoethyl)indole was included. The solution was frozen in dry ice and evaporated below 1.3 Pa for 3 min. The hydrolysis vial was closed by melting its neck under vacuum and was placed in an electrical oven at 110°C for 24 h.

2.3. Gas-phase hydrolysis of proteins in solution at 110°C

The protein solution was placed in 0.3-ml glass hydrolysis vials and evaporated to dryness in a speedvac evaporator. The vials were placed in a

PTFE holder consisting of a horizontal disk with holes to accommodate eight vials and carrying a vertical holding bar in the middle. The holder with the vials was placed in a hydrolysis vessel, consisting of two parts screwed together and forming an air-tight assembly with the help of a PTFE O-ring. The upper part was equipped with a vacuum tap. Two ml of 6 *M* HCl containing 0.02% phenol and 100 μl of β-mercaptoethanol were placed at the bottom of the lower part, and the vessel was assembled, evaporated and flushed with argon three times to remove oxygen. After one additional evacuation, the vacuum tap was closed and the vessel with the vials was placed in an electrical oven at 110°C for 24 h.

2.4. Gas-phase hydrolysis at 110°C of proteins blotted onto PVDF membranes

Following separation by 1D- or 2D-PAGE, the proteins were electrotransferred to PVDF membranes (Macherey–Nagel) in 10 m*M* 3-(cyclohexylamino)-1-propane sulfonic acid (CAPS), containing 10% methanol at 0.4 A for 2 h using a semi-dry transfer apparatus (Bio-Rad). The membranes were stained with 0.1% amido black in 10% acetic acid and 40% methanol for 1 h and destained with deionized water. Protein bands or spots were excised and placed in 0.3 ml hydrolysis vials. Gas-phase hydrolysis was performed as described above.

2.5. Large-scale hydrolysis at 110°C of proteins blotted onto PVDF membranes

The soluble protein fraction of the bacterium *Haemophilus influenzae* [23] was separated by 2D-PAGE as described [24–26], the proteins were electrotransferred to PVDF membranes and the membranes were stained as stated above. The protein spots were excised and the membrane pieces were placed in 0.3-ml hydrolysis vials. Gas-phase hydrolysis was performed in a specially designed hydrolysis vessel accommodating 56 vials. The vessel has a diameter of 10 cm and a height of 14 cm and it consists of two parts closing air-tightly with the help of an insulating O-ring. The two parts are held together with external, metallic rings, connected with six screws. The upper part carries an air tap for evacuation. The PTFE holder, which carries the horizontal disk for the accommodation of the vials, is placed in the lower part. Three centimeters above the disk carrying the vials, there is another disk functioning as a ‘roof’ to prevent liquid drops, formed on the internal surface of the upper half of the vessel during cooling, from falling into the vials and eventually contaminating the hydrolyzate. At the bottom of the vessel were placed 20 ml of 6 *M* HCl, containing 0.02% phenol and 100 μl of β-mercaptoethanol. The evaporation and hydrolysis were performed at 110°C as stated for proteins in solution.

2.6. Liquid-phase hydrolysis of proteins in solution by microwave radiation

The protein solution was placed in the hydrolysis vials together with the hydrolysis agent and the vials were sealed under vacuum as described for the conventional liquid-phase hydrolysis. The HCl solution contained 0.02% phenol, and the methanesulfonic acid solution 0.2% 3-(2-aminoethyl)indole. The vials in a special pressure-withstanding high-density PTFE reactor were put in their specific positions in a high-performance microwave unit (MLS-1200 MEGA 240, equipped with a suction unit EM5). A beaker with 200 ml water was placed in the microwave oven as it is important that a constant fluid volume be present in order to avoid different energy absorption by different liquid volumes. Hydrolysis was performed at 155°C (900 W) for 4 min.

2.7. Gas-phase hydrolysis of proteins in solution by microwave radiation

The protein solution in the 300 μl hydrolysis vials was evaporated to dryness and the vials were placed in a holder accommodating 50 probes. The holder with the vials was placed in a pressure-stable hydrolysis reactor provided with the equipment [15]. Fifty ml of 6 *M* HCl containing 0.02% phenol were placed in the reactor, and the reactor was closed and put in a second safety container. The container with the reactor were placed in the oven, degassed and flushed with argon three times. Hydrolysis was performed at 1000 W for 5 min and 500 W for 15 min, followed by a ventilation step for 15 min.

During hydrolysis the temperature reached 155°C and remained constant until the ventilation step.

2.8. Gas-phase hydrolysis of blotted proteins by microwave radiation

The membrane pieces carrying the protein spots were placed in the 300-μl hydrolysis vials and the same operations were repeated as described above for the gas-phase hydrolysis of proteins in solution. The hydrolysis was performed at 155°C for 30 min, reached by programming 1000 W for 5 min, 500 W for 25 min and ventilation for 15 min.

2.9. Extraction of the hydrolysis products

Following hydrolysis, the residues were dissolved in the proper buffer for liquid chromatography analysis. During the liquid-phase hydrolysis with HCl, the hydrolyzate was in solution, and after cooling down to room temperature, the hydrolyzing agent was evaporated over NaOH. The precipitate, as well as the precipitate after the gas-phase hydrolysis, was dissolved in 25 μl of 0.25 *M* borate buffer, pH 8.8 (Hewlett-Packard). Methanesulfonic acid is not volatile, so that the hydrolyzate was diluted 2-fold with 4 *M* NaOH and, where necessary, the pH was adjusted to approximately 8.8. The extract was further diluted 4-fold with water before chromatography.

For the extraction of the hydrolyzate from the membranes, three approaches were followed: (i) extraction with 100 μl of 0.1 *M* HCl; (ii) extraction with 100 μl of 60% acetonitrile (Merck), containing 0.1% trifluroacetic acid (Pierce); and (iii) extraction first with 100 μl of 60% acetonitrile, containing 0.1% trifluroacetic acid and then with 100 μl of 0.1 *M* HCl. The membranes were extracted either directly or after wetting with methanol. The membrane pieces were soaked in the extraction solution and sonicated for 10 min. The liquid was removed and the extraction step was usually repeated once again (except for the third approach). The combined extracts were evaporated to dryness in a speedvac evaporator and the residues were dissolved in 25 μl of 0.25 *M* borate buffer, pH 8.8.

2.10. Chromatography of amino acids

One μl of the hydrolyzate extract in the borate buffer from the proteins in solution (4% of total) or 5 μl of hydrolyzate extract from the blotted proteins (20% of total) were automatically injected into an AminoQuant amino acid analyzer (Model 1090A, Hewlett-Packard) equipped with pre-column *o*-phthaldialdehyde (OPA) and 9-fluorenylmethyl chloroformate (FMOC) derivatization. The modified residues were chromatographed on a reversed-phase HPLC column (AminoQuant column, 200×2.1 mm, Hewlett-Packard) equilibrated with 0.20 *M* sodium acetate, pH 7.2. The column was developed with a linear gradient of 0–60% acetonitrile in 0.20 *M* sodium acetate, pH 7.2, at 0.5 ml/min in 23 min. Amino acid standards, representing 100, 25 and 10 pmol (Hewlett-Packard) were run with the samples.

2.11. Separation of D- and L-residues

The hydrolyzate extract was derivatized with OPA and *N*-isobutyryl-L-cysteine prior to chromatography. The residues reacted with a 1:1 mixture (v/v) of 85 m*M* OPA and 110 m*M* *N*-isobutyryl-L-cysteine both in 1 *M* potassium borate [27,28]. The chiral derivatives were applied on a Hypersil ODS C_{18} column (250×4.0 mm) equilibrated with 23 m*M* sodium acetate, pH 6.0, at 35°C. The column was developed with a linear gradient of 0–53.5% methanol containing 8.3% (v/v) acetonitrile at 1 ml/min in 75 min.

2.12. Identification of the proteins by search in databases

The peak summary tables were transferred to an office program (RagTime, MacVONK) and the amino acid percentages were calculated. These values together with the theoretical pI and M_r values of the proteins were subjected to a database query (Swiss-Prot) using the AACompIdent software via the WorldWideWeb (http://expasy.hcuge.ch/ch2d/aacompi.html) on the ExPASy server [1,29]. The AACompIdent software compares the percent determined composition of a query protein with the theoretical percent compositions of all proteins in the database. The search can proceed on the basis of

various amino acid constellations, depending on the number of the residues determined [1]. We usually used the free constellation mode with no restrictions in the number of the amino acids required for protein search. The program normalizes the values of the amino acid percentages introduced, so that the total makes 100. Theoretically, the determination of only one amino acid is sufficient for protein search [30]. The degree of difference between determined and theoretical composition of a protein is given by a score number. The score is calculated for each database entry by the sum of the squared difference between the percent amino acid composition for the determined amino acids of the query protein and database entry. The database matches are ranked according to ascending score numbers [1]. The No. 1 ranking protein has simultaneously the lowest score.

3. Results

3.1. Liquid- and gas-phase hydrolysis of proteins with HCl

We compared the effect of different acid-hydrolysis methods on the quantification of amino acids using recombinant IFNα_2 and BSA. The proteins were subjected to conventional liquid- and gas-phase hydrolysis in an electrical oven at 110°C for 24 h and to liquid-phase hydrolysis for 4 min and gas-phase hydrolysis for 20 min in a microwave oven at 155°C. The residues were determined using an AminoQuant amino acid analyzer equipped with pre-column OPA- and FMOC-derivatization. The results from the four hydrolysis modes are shown in Fig. 1. The ratios determined using the four approaches were comparable in all cases except for the quantification of Ser and Thr following liquid-phase microwave radiation-induced hydrolysis, which resulted in higher losses for these residues (Fig. 1A,B). In control experiments, in which the addition of phenol into the hydrolysis reagent was omitted, higher losses of Ser and Thr (up to 80%) were observed during conventional liquid- and gas-phase hydrolysis and, in particular, during microwave hydrolysis (data not shown).

The percentages of the residues determined by the various methods were matched by the AACompIdent software against the theoretical compositions of the two proteins from the Swiss-Prot database. Each matching was characterized by a rank and a score number. The ranking in the protein matching list was according to increasing score numbers. The score represented the degree of difference between the theoretical and observed composition of the protein. The lower the score the higher the similarity between the two compositions. The confidence of the protein identification was high if the matching protein ranked No. 1 and the score was low, usually below 30 [30] (in the case of a complete agreement between determined and theoretical composition the score would be 0). IFNα_2 and BSA ranked No. 1 with very low scores, when conventional liquid- or gas-phase hydrolysis was used (Table 1). From the analysis data of the microwave radiation-induced hydrolysis, IFNα_2 ranked No. 1. BSA, although it showed low scores, ranked No. 2. The No. 1 ranked protein was serum albumin from sheep which showed similar or the same scores (Table 1, column Remarks).

3.2. Liquid-phase hydrolysis with methanesulfonic acid

IFNα_2 and BSA were further subjected to liquid-phase hydrolysis in the presence of 4 *M* MSA. A gas-phase hydrolysis was not possible because of the high boiling point of the reagent. Conventional hydrolysis at 110°C delivered a more accurate quantification of most residues in comparison with hydrolysis using microwave radiation (Fig. 2). The latter hydrolysis mode resulted in higher losses of Ser and Thr. Interestingly, also tryptophan could be determined following this hydrolysis procedure. Protein search with the determined residue percentages from the conventional hydrolysis data identified both proteins as rank No. 1 entries with almost theoretical scores (Table 1). Hydrolysis using microwave radiation delivered results which allowed the identification of IFNα_2 as rank No. 1 protein, however, with slightly higher scores as compared to conventional hydrolysis, whereas BSA ranked No. 3 with comparable scores (Table 1).

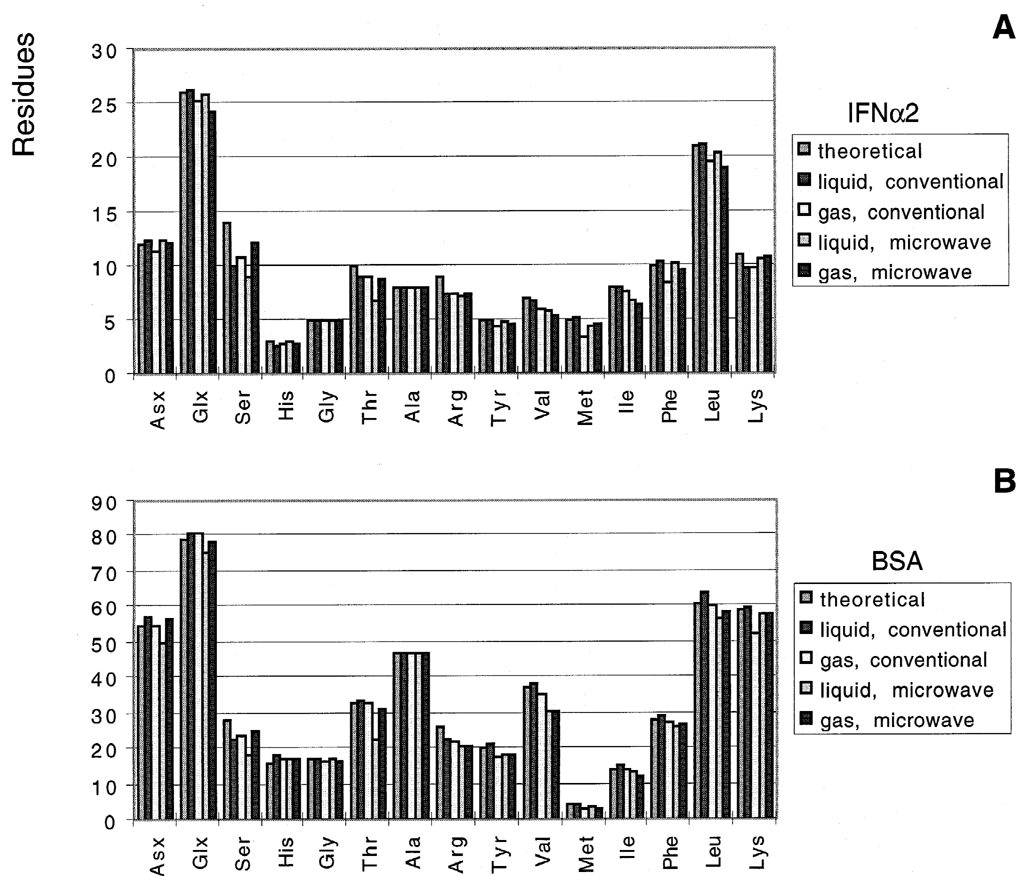

Fig. 1. Comparison of the amino acid compositions of human IFNα_2 (A) and BSA (B) following hydrolysis with 6 *M* HCl. The proteins were subjected to conventional liquid- (liquid, conventional) and gas-phase (gas, conventional) hydrolysis at 110°C for 24 h and to microwave radiation-induced hydrolysis at 155°C in liquid-phase for 4 min (liquid, microwave) and in gas-phase for 20 min (gas, microwave). The results were normalized to the Ala residues.

3.3. Gas-phase hydrolysis of blotted proteins

Gas-phase hydrolysis is often preferred to liquid-phase hydrolysis in order to reduce contamination introduced by the hydrolyzing agent, in particular, when only small protein amounts are available. Using recombinant IFNα_2, we compared conventional gas-phase hydrolysis at 110°C and microwave radiation-induced hydrolysis at 155°C with HCl (MSA cannot be applied in gas-phase hydrolysis). IFNα_2 was electroblotted onto PVDF membranes and the bands were excised and subjected to gas-phase hydrolysis. The hydrolyzate was extracted from the membranes with 0.1 *M* HCl and analyzed on the AminoQuant amino acid analyzer. We initially tested the proper hydrolysis time. For the conventional gas-phase hydrolysis, we used times of 24 and 30 h. The 30-h treatment appeared to deliver slightly better ratios for the hydrophobic residues. We therefore used this hydrolysis time for the blotted proteins. For microwave hydrolysis, we applied three times of 20, 30 and 45 min. The shorter time was insufficient, whereas hydrolysis for 45 min resulted in a significant loss of certain residues such as histidine (data not shown). Therefore, in subsequent experiments a hydrolysis time of 30 min was routinely used. We also determined the amount of protein recovered in the residue extract. This was 15–30% of the starting quantity (1 μg) after conventional gas-phase hydrolysis and 5–15% after microwave-

Table 1
Identification of proteins on the basis of amino acid composition analysis

Hydrolysis method	Rank of protein (score)	Remarks
$IFN\alpha_2$		
1. HCl, liquid, conventional	1 (9)	rank 2 $IFN\alpha_{17}$, score 14
2. HCl, gas, conventional	1 (6)	rank 2 $IFN\alpha_{17}$, score 10
3. HCl, liquid, microwave	1 (16)	rank 2 $IFN\alpha_{17}$, score 21
4. HCl, gas, microwave	1 (2)	rank 2 $IFN\alpha_{17}$, score 14
5. MSA, liquid, conventional	1 (4)	rank 2 $IFN\alpha_{21}$, score 16
6. MSA, liquid, microwave	1 (9)	rank 2 $IFN\alpha_{21}$, score 16
$IFN\alpha_2$, blotted onto PVDF membranes		
7. HCl, gas, conventional	9 (35)	rank 1 $IFN\alpha_{16}$, score 22
8. HCl, gas, microwave	9 (193)	rank 1 stathmin, score 152
BSA		
9. HCl, liquid, conventional	1 (2)	rank 2 sheep albumin, score 2
10. HCl, gas, conventional	1 (3)	rank 2 sheep albumin, score 4
11. HCl, liquid, microwave	2 (8)	rank 1 sheep albumin, score 6
12. HCl, gas, microwave	2 (3)	rank 1 sheep albumin, score 3
13. MSA, liquid, conventional	1 (1)	rank 2 sheep albumin, score 2
14. MSA, liquid, microwave	3 (10)	rank 1 sheep albumin, score 7
		rank 2 horse albumin, score 10

$IFN\alpha_2$ and BSA were hydrolyzed under different conditions as described in Section 2 and in Section 3. With the residue percentages a data query was performed using the AACompIdent software on the ExPASy server via the WorldWideWeb [1]. Conventional hydrolysis was performed at 110°C for 24 h (for blotted proteins 30 h). $IFN\alpha_2$, interferon α_2; PVDF, polyvinylidene difluoride; MSA, methanesulfonic acid.

induced hydrolysis. During the microwave treatment, the PVDF blots were disformed and they became hard which significantly impaired the extraction yield.

The composition resulting from the analysis of blotted $IFN\alpha_2$ showed a greater deviation from the theoretical composition in comparison with the protein in solution (Fig. 3). In general, the microwave-mediated hydrolysis yielded higher ratios for Glu and lower ratios for His, Ser and Thr. The percentages of the residues derived from the hydrolyses (Fig. 3) were used for protein search with the AACompIdent software. $IFN\alpha_2$ was included in the list of the first 20 entries and ranked No. 9 with a score of 35, and from the gas-phase hydrolysis in the microwave oven ranked No. 9 with a score of 193 (Table 1). In a parallel experiment, lyophilized $IFN\alpha_2$ subjected to liquid-phase hydrolysis ranked No. 1 with a score of 8.

We further tested whether the reliability of the microwave radiation-induced hydrolysis of blotted proteins was dependent on the amount of protein hydrolyzed. Different amounts of blotted $IFN\alpha_2$ and BSA, from 0.25 to 2 μg, were hydrolyzed for 30 min. We observed a protein quantity-dependent variation in the composition of the blotted proteins. The best results were obtained with protein amounts of 1 μg or higher. Below that amount the composition variation was large and in some cases less abundant residues such as His and Met could not be detected at all (data not shown).

3.4. Extraction of the hydrolyzate from PVDF blots

The rank and score found for blotted $IFN\alpha_2$ using conventional gas-phase hydrolysis at 110°C (Table 1) were unsatisfactory making impossible a protein identification by database searching. In order to improve the identification score, we modified the conditions of the hydrolyzate extraction using 60% acetonitrile, containing 0.1% trifluoroacetic acid. BSA and $IFN\alpha_2$ from PVDF blots were subjected to gas-phase hydrolysis and the hydrolyzates were extracted with either 0.1 *M* HCl or 60% CH_3CN containing 0.1% CF_3COOH. In a protein search, both proteins ranked No. 1 with low scores when data from the extraction with the organic solvent

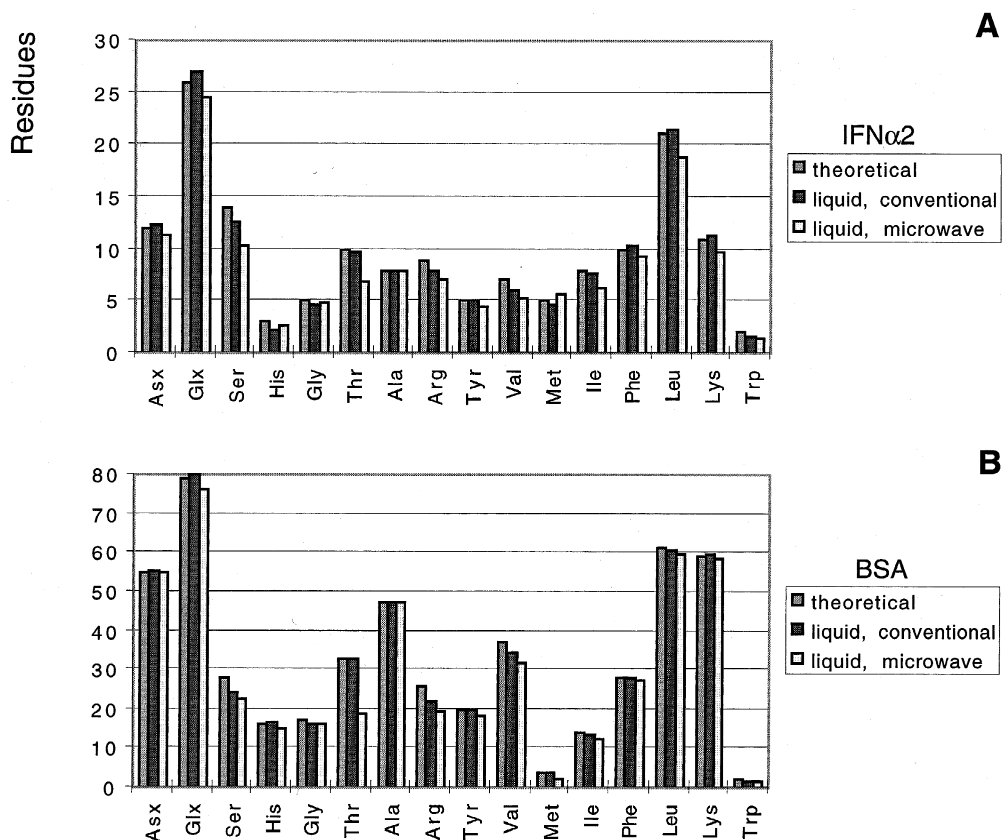

Fig. 2. Comparison of the amino acid compositions of human IFNα_2 (A) and BSA (B) following hydrolysis with 4 *M* methanesulfonic acid. The proteins were subjected to conventional liquid-phase hydrolysis at 110°C for 24 h (liquid, conventional) and to microwave liquid-phase hydrolysis at 155°C for 4 min (liquid, microwave). The results were normalized to the Ala residues.

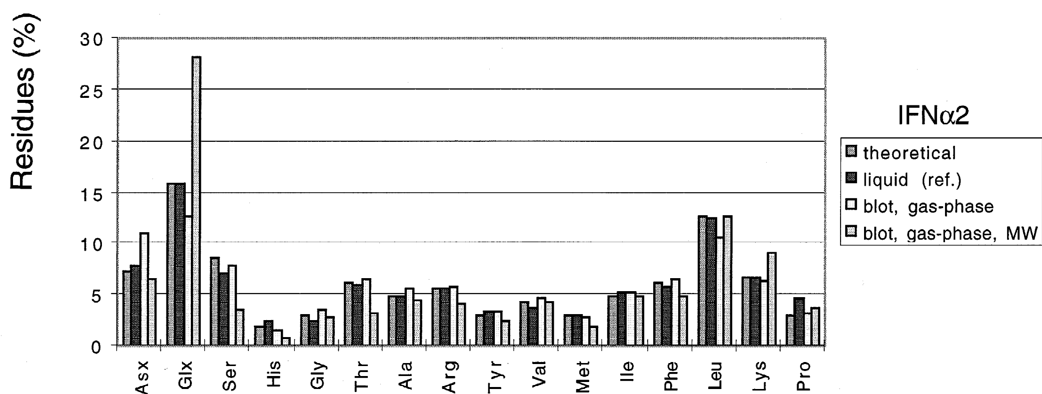

Fig. 3. Comparison of the amino acid composition of blotted human IFNα_2 following hydrolysis with 6 *M* HCl. After 1D-PAGE, the protein (1 μg) was blotted onto a PVDF membrane and the membrane was stained with amidoblack. The band was subjected to conventional gas-phase hydrolysis at 110°C for 30 h (blot, gas-phase) and to microwave radiation-induced gas-phase hydrolysis for 30 min (blot, gas-phase, MW). For comparison, IFNα_2 in solution was hydrolyzed at 110°C for 24 h (liquid (ref.)). The amino acids are expressed as percentage of total residues.

Table 2
Effect of the conditions of hydrolyzate extraction on protein identification

Protein	Extraction method	Protein identification	
		Rank	Score
1. IFNα_2	0.1 *M* HCl	N.I.	
2. IFNα_2	60% CH_3CN+0.1% CF_3COOH	1	13
3. BSA	0.1 *M* HCl	N.I.	
4. BSA	60% CH_3CN+0.1% CF_3COOH	1	16

IFNα_2 and BSA from PVDF blots were subjected to gas-phase hydrolysis with HCl, at 110°C for 30 h and the hydrolyzates were extracted as indicated. The proteins were identified using the AACompIdent software on the ExPASy server assessed via the WorldWideWeb [1]. N.I., not included in the 20 first entries; IFNα_2, interferon α_2.

were used (Table 2). Neither protein was included in the 20 first entries when data from the HCl extract were used. Because in control experiments HCl was efficient for extraction of hydrophilic residues, we performed the extraction of the hydrolyzate of unknown proteins in two steps with 100 μl each of 60% acetonitrile, containing 0.1% TFA, and 0.1 *M* hydrochloric acid.

3.5. Effect of the hydrolysis method on the degree of racemization of the amino acids

Treatment of the proteins in high concentrations of acids at elevated temperatures results in partial racemization of the residues. In order to investigate the degree of residue racemization introduced by hydrolysis, D- and L-amino acids were separated after derivatization with the chiral reagent *N*-isobutyryl-L-cysteine. We first investigated the chromatographic conditions in order to achieve a complete separation of the chiral derivatives. Fig. 4A shows the elution profile of a mixture of D- and L-amino acid standards. The separation was performed on a reversed-phase C_{18} column developed with a linear gradient of methanol containing 8.3% acetonitrile. All enantiomeric residues were efficiently separated, including D- and L-Trp and Gly (Fig. 4A).

The hydrolyzates derived from the hydrolysis of IFNα_2 and BSA using the various methods were additionally analyzed after reaction with *N*-isobutyryl-L-cysteine and the percentages of D-amino acids generated during the corresponding hydrolysis steps were determined. As an example, Fig. 4B shows the analysis of the hydrolyzate of IFNα_2 after liquid-phase hydrolysis with HCl using microwave radiation. The results of the racemization analyses from the various hydrolysis approaches are shown in Fig. 5. In most cases, the percentages of the D-forms were 0–10% of the total residue amount. Interestingly, conventional hydrolysis with MSA resulted in generation of lower amounts of D-forms in comparison with the HCl hydrolysis. Liquid-phase microwave radiation-induced hydrolysis produced larger amounts of D-residues compared to the conventional hydrolysis. Certain residues, such as histidine, methionine and lysine, showed a higher tendency to racemization (Fig. 5). In control experiments, in which no phenol was added to the HCl hydrolysis mixture, significantly higher percentages of racemate were detected (D-form percentages for most residues higher than 30%, data not shown). The percentages of total D-isoforms produced by the hydrolysis methods are given in Table 3.

4. Discussion

Amino acid analysis, although a classical technique, remains indispensable for quality control studies in biochemistry and biotechnology. Nowadays, it enjoys a renaissance based on its value in the construction of 2D protein maps which find a wide application in clinical diagnosis and drug discovery in the pharmaceutical industry [1,25,26]. A successful amino acid analysis largely depends on the proper performance of the hydrolysis. Collaborative control studies have shown that the influence of the hydrolysis conditions represent a major source of error in the analysis [31]. Our aim was to investigate the effect of acid-hydrolysis methods on the compositional analysis and the reliability of protein identification from databases on the basis of the amino acid composition analysis, as well as to optimize the conditions for large-scale protein identification.

Conventional hydrolysis with MSA delivered the most reliable protein identification scores and resulted in lower racemization in comparison with HCl hydrolysis. Hydrolysis with MSA allows the quantification of sensitive residues such as tryptophan. Determination of low abundance amino acids, like

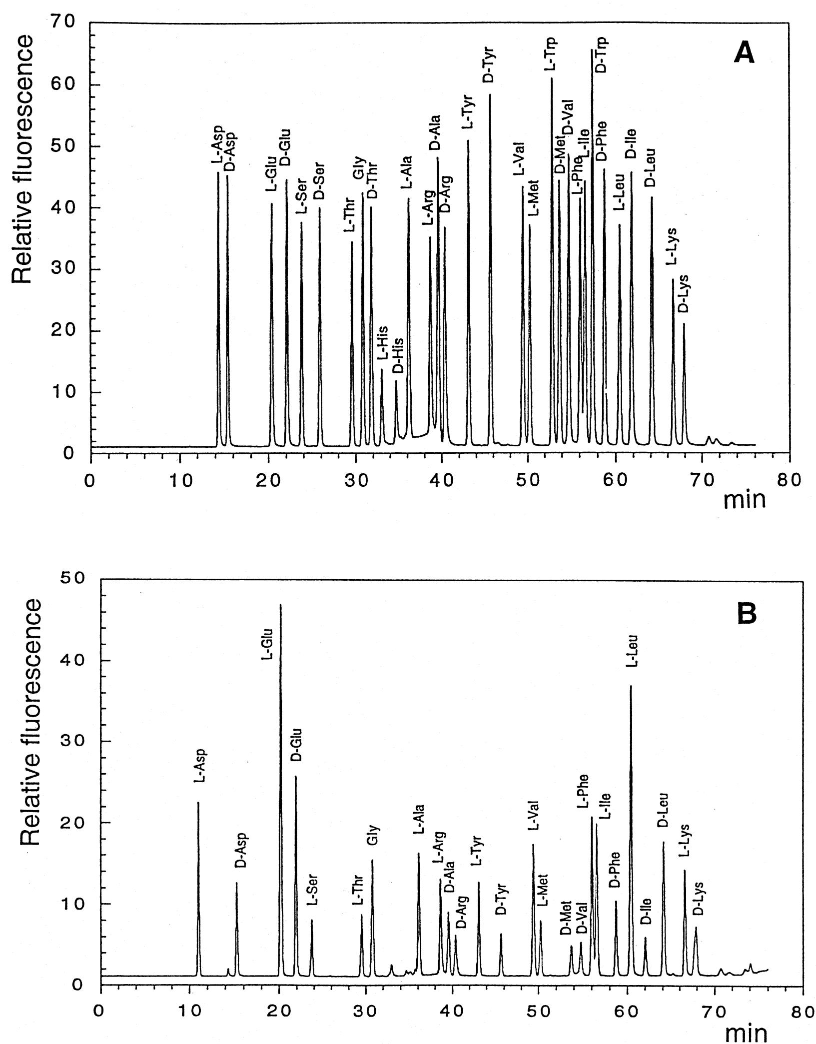

Fig. 4. Separation of D- and L-amino acid standards (A) and of the hydrolysis products of human IFNα_2, following liquid-phase hydrolysis with HCl using microwave radiation (B). The residues reacted with *N*-isobutyryl-L-cysteine and OPA and the chiral derivatives were analyzed as stated in Section 2. All enantiomeric residues were efficiently separated. (A,B) A difference in the elution times of the first two residues was occasionally observed.

tryptophan, increases the probability of an unambiguous protein identification. However, MSA cannot be evaporated. Only after pH adjustment and dilution, can a MSA-hydrolyzate be used for chromatographic analysis. Therefore, large amounts of protein are required for analysis, which restricts application of this method.

A prerequisite of a successful protein identification on the basis of the compositional analysis is a proper hydrolyzate extraction method from PVDF membranes [1–4,32]. Extraction with a solution containing acetonitrile was more efficient than extraction with HCl only. Our results showed that more accurate compositions were obtained from proteins in solution in comparison with blotted proteins (Tables 1 and 2). This is probably due to the unequal extraction of all residues of the hydrolyzate from the membranes.

Hydrolysis using microwave radiation energy is very fast, it can be accomplished in only 4 min, and

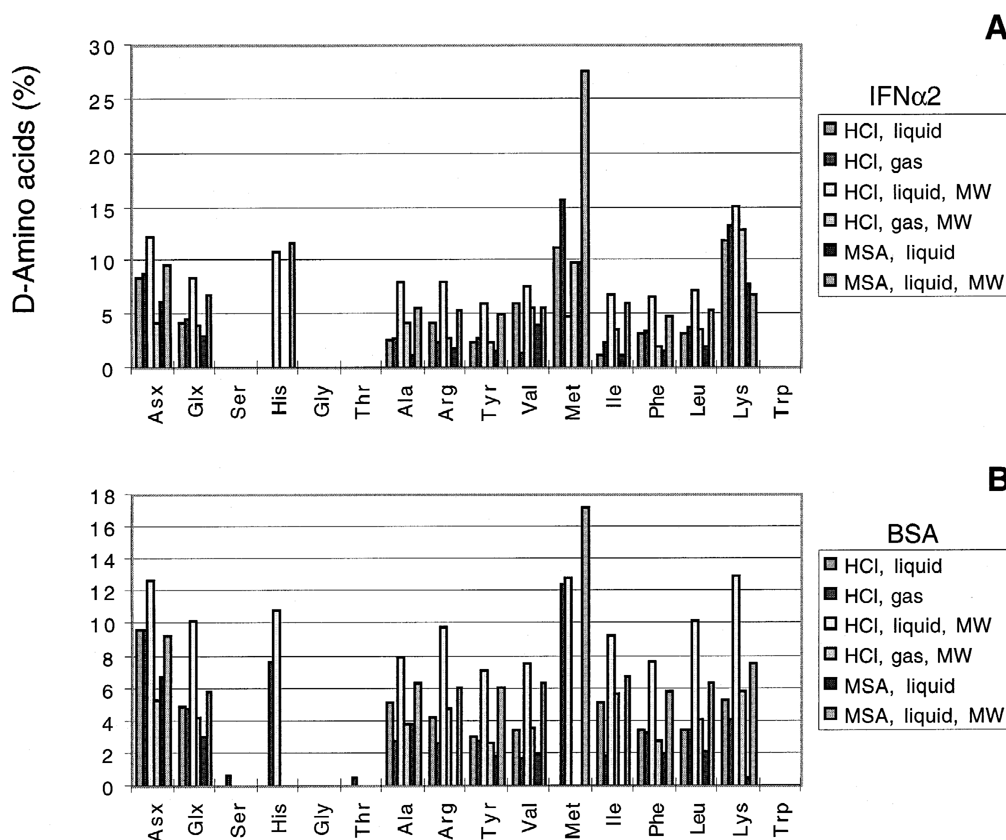

Fig. 5. D-Amino acid percentages in the hydrolyzates of IFNα_2 (A) and BSA (B) produced during acid hydrolysis. Conventional hydrolysis with HCl and methanesulfonic acid (MSA) and microwave (MW) hydrolysis were performed in the presence of phenol as described in Section 2. The residues were analyzed as stated in the legend of Fig. 4. Higher percentages of D-forms were found in the hydrolyzates generated using microwave radiation energy. In some cases no detectable peaks corresponding to D-isoforms were present in the chromatograms.

for proteins in solution it delivered very reliable results comparable to that of the conventional hydrolysis, except for slightly higher losses of serine and threonine (Figs. 1 and 2). Due to its short performance time, hydrolysis in the microwave oven would be the method of choice for processing large numbers of protein spots separated by 2D-PAGE. However, during microwave hydrolysis, the blots became rigid and the extraction of the hydrolyzate was incomplete. This usually resulted in a variation in the amino acid composition and irreproducibility of the analysis data. In addition, on account of the low recovery of hydrolyzate from PVDF membranes, identification of proteins represented by weak spots appears rather unlikely. Therefore, we chose the gas-phase hydrolysis at 110°C and prolonged the hydrolysis time to 30 h in order to efficiently hydrolyze bonds between hydrophobic residues. The relatively long duration of the hydrolysis did not affect the overall performance because several hydrolysis vessels were used at the time, each containing 56 hydrolysis vials. The bottleneck of the analysis were the chromatographic steps and, in particular, the interpretation of the protein search data from the databases. Using this approach we successfully identified 110 proteins of the bacterium *H. influenzae* applying pre-column and post-column residue derivatization with OPA [30]. A drawback of the identification of unknown proteins by compositional analysis is that the compositions of proteins

Table 3
Total D-amino acids generated during hydrolysis

Hydrolysis method	Protein			
	$IFN\alpha_2$ Phenol		BSA Phenol	
	+	−	+	−
HCl, liquid, conventional	4.1	4.4	4.1	3.8
HCl, gas, conventional	4.2	4.0	4.9	4.7
HCl, liquid, microwave	7.4	29.5	8.9	32.4
HCl, gas, microwave	3.8	9.0	3.6	9.6
MSA, liquid, conventional	2.8		2.2	
MSA, liquid, microwave	6.3		6.4	

$IFN\alpha_2$ and BSA were hydrolyzed under different conditions, in the presence or absence of 0.02% phenol, as described in Section 2 and in Section 3. Total D-amino acids are expressed as percentage of total residues. Conventional hydrolysis was performed at 110°C for 24 h. $IFN\alpha_2$, interferon α_2; MSA, methanesulfonic acid.

truncated at the N-terminal end (many of which were found in the *H. influenza* proteome, unpublished results) would significantly deviate from the theoretical composition.

From the analysis data of proteins in solution, our model protein $IFN\alpha_2$ always ranked No. 1 with very low scores. The data would be sufficient for an unambiguous identification of the protein in a database in spite of its high similarity with the other interferons (Table 1). In a correct identification, the ratio of the score of rank No. 2 protein divided by the score of rank No. 1 protein is usually 2 or higher [1]. On the other hand, identification of BSA, without species exclusion, would be ambiguous. The scores obtained, although very low and close to the theoretical values, were similar to the scores of albumins from other species because of the high similarity of these proteins. BSA identified on the basis of data derived from the microwave hydrolysis ranked No. 2 or No. 3. The scores of the higher ranked proteins were similar or identical with that of BSA. BSA did not rank as No. 1 entry probably on account of the inaccurate quantification of the Ser and Thr, following the microwave digestion.

Treatment of the proteins with high concentrations of acids at elevated temperatures resulted in partial racemization of certain residues. The degree of racemate formation was dependent on the hydrolysis method applied and the hydrolysis agent used and in very few cases it was higher than 10%. In general, microwave radiation-dependent hydrolysis yielded higher percentages of D-isomers in comparison with the conventional hydrolysis, in particular, liquid-phase hydrolysis with HCl (Fig. 5). Phenol did not exert an influence in the formation of racemates during conventional hydrolysis, whereas it significantly reduced the generation of racemates during microwave hydrolysis (Table 3). When microwave energy was used, gas-phase hydrolysis generated lower amounts of D-forms compared to the liquid-phase. This is probably because in the latter case the samples were in direct contact with the liquid reagent. Certain residues such as Asx, His, Met and Lys appeared to have a higher tendency for racemization. His and Met are usually represented by small peaks and it is possible that the quantification of the corresponding D-forms was not very accurate.

In summary, we used human $IFN\alpha_2$ and BSA as model proteins to investigate the effect of acid hydrolysis methods on the accuracy of compositional analysis and the formation of racemates. Conventional hydrolysis of proteins in solution with HCl and MSA yielded very accurate composition data. Microwave radiation-induced hydrolysis delivered essentially comparable results with slightly higher losses of hydrolysis-sensitive residues. More accurate compositions were obtained from proteins in solution in comparison with blotted proteins. The percentage of D-forms generated, with few exceptions, ranged between 0 and 10%. The microwave approach was responsible for higher racemization.

Acknowledgements

We thank Dr. H. Langen for helpful discussions and Drs. H. Lötscher and J. Mous for their support.

References

[1] M.R. Wilkins, C. Pasquali, R.D. Appel, K. Ou, O. Golaz, J.-C. Sanchez, J.X. Yan, A.A. Gooley, G. Hughes, I. Humphery-Smith, K.L. Williams, D.F. Hochstrasser, Bio/Technology 14 (1996) 61.
[2] C.H. Wheeler, S.L. Berry, M.R. Wilkins, J.M. Corbett, K. Ou, A.A. Gooley, I. Humphery-Smith, K.L. Williams, M.J. Dunn, Electrophoresis 17 (1996) 580.

[3] O. Golaz, M.R. Wilkins, J.-C. Sanchez, R.D. Appel, D.F. Hochstrasser, K.L. Williams, Electrophoresis 17 (1996) 573.
[4] J.X. Yan, M.R. Wilkins, K. Ou, A.A. Gooley, K.L. Williams, J.-C. Sanchez, O. Golaz, C. Pasquali, D.F. Hochstrasser, J. Chromatogr. A 736 (1996) 291.
[5] U. Hobohm, T. Houthaeve, C. Sander, Anal. Biochem. 222 (1994) 202.
[6] G. Shaw, Proc. Natl. Acad. Sci. USA 90 (1993) 5138.
[7] P. Jungblut, M. Dzionara, J. Klose, B. Wittman-Leibold, J. Prot. Chem. 11 (1992) 603.
[8] D.H. Spackman, W.H. Stein, S. Moore, Anal. Chem. 30 (1958) 1190.
[9] C.H.W. Hirs, W.H. Stein, S. Moore, Biol. Chem. 211 (1954) 941.
[10] S. Moore, in: J. Meienhofer (Ed.), Chemistry and Biology of Peptides, Ann Arbor Publishers, Ann Arbor, MI, 1972, p. 629.
[11] S. Blackburn, in: S. Blackburn (Ed.), Amino Acid Determination, 2nd ed., M. Dekker, New York, 1978, p. 8.
[12] L.B. Fischer, Anal. Chem. 58 (1986) 261.
[13] A. Pecavar, M. Prosek, D. Fercejtemeljotov, J. Marsel, Chromatographia 30 (1990) 159.
[14] H.-W. Lahm, W. Lergier, M. Manneberg, R. Knorr, J. Prot. Chem. 7 (1988) 258.
[15] M. Manneberg, H.-W. Lahm, in: H.-R. Höpker (Ed.), Proccedings of the 4th Würzburger Chromatographie Gespräche, Pharmacia, Freiburg, 1990, p. 124.
[16] A. Peter, G. Laus, D. Tourwe, E. Gerlo, G. Vanbinst, Peptide Res. 6 (1993) 48.
[17] L. Joergensen, H.N. Thestrup, J. Chromatogr. A 706 (1995) 421.
[18] J.V. Benson, J.A. Patterson, in: A. Niederwieser, G. Pataki (Eds.), New Techniques in Amino Acid, Peptide and Protein Analysis, Ann Arbor Science Publishers, Ann Arbor, MI, 1971, p. 1.
[19] I. Molnarperl, M. Pinterszakacs, M. Khalifa, J. Chromatogr. 632 (1993) 57.
[20] G. Georgi, C. Pietsch, G. Sawatzki, J. Chromatogr. 613 (1993) 35.
[21] M. Manneberg, H.-W. Lahm, M. Fountoulakis, Anal. Biochem. 224 (1995) 122.
[22] M. Manneberg, H.-W. Lahm, M. Fountoulakis, Anal. Biochem. 231 (1995) 349.
[23] R.D. Fleischmann, M.D. Adams, O. White, R.A. Clayton, E.F. Kirkness, A.R. Kerlavage, C.J. Bult, J.-F. Tomb, B.A. Dougherty, J.M. Merrick, K. Kenney, G. Sutton, W. FitzHugh, C. Fields, J.D. Gocayne, J. Scott, R. Shirley, L.I. Liu, A. Glodek, J.M. Kelley, J.F. Weidman, C.A. Phillips, T. Spriggs, E. Hedblom, M.D. Cotton, T.R. Utterback, M.C. Hanna, D.T. Nguyen, D.M. Saudek, R.C. Brandon, L.D. Fine, J.L. Fritchman, J.L. Fuhrmann, N.S.M. Geoghagen, C.L. Gnehm, L.A. McDonald, K.V. Small, C.M. Fraser, H.O. Smith, J.C. Venter, Science 269 (1995) 496.
[24] B. Bjellqvist, C. Pasquali, F. Ravier, J.-C. Sanchez, D.F. Hochstrasser, Electrophoresis 14 (1993) 1357.
[25] H. Langen, C. Gray, D. Roeder, J.-F. Juranville, B. Takacs, M. Fountoulakis, Electrophoresis 18 (1997) 1184.
[26] M. Fountoulakis, H. Langen, S. Evers, C. Gray, B. Takacs, Electrophoresis 18 (1997) 1193.
[27] H. Brückner, R. Wittner, H. Godel, J. Chromatogr. 476 (1989) 73.
[28] H. Brückner, T. Westhauser, H. Godel, J. Chromatogr. A 711 (1995) 201.
[29] R.D. Appel, A. Bairoch, D.F. Hochstrasser, Trends Biochem. Sci. 19 (1994) 258.
[30] M. Fountoulakis, J.-F. Juranville, P. Berndt, Electrophoresis (1997) in press.
[31] K.U. Yuksel, T.T. Andersen, I. Apostol, J.W. Fox, R.J. Paxton, D.J. Strydom, in: J.W. Crabb (Ed.), Techniques in Protein Chemistry VI, Academic Press, San Diego, CA, 1995, p. 185.
[32] F. Lottspeich, C. Eckerskorn, R. Grimm, in: Cell Biology: A Laboratory Handbook, Academic Press, San Diego, CA, 1994, p. 417.