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Isolation of individual amino acids from various microbiological sources using reversed-phase high-performance liquid chromatography

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Abstract

A new method for the preparative isolation of individual amino acids on a milligram scale based on reversed-phase high-performance liquid chromatography (RP-HPLC) after pre-column derivatization with carbobenzoxychloride (Z-Cl) has been developed. The chromatographic procedure was tested by the investigation of jack bean urease hydrolysate. The method has been applied to the preparative separation of Z-amino acids (from 10 up to 16) obtained from protein hydrolysates of various sources (green microalgae, blue-green algae, halophilic and methylotrophic microorganisms) and was proved to be reliable by the separation of deuterated amino acids (enrichment 97–99%) from *Methylobacillus flagellatum* (due to the bioconversion of CD_3OD and D_2O). Independent of the biological source of the protein, the amino acids were isolated with high recovery (from 68% up to 89%) and chromatographic purity (from 96% up to 99%). The method was also applied for the isolation of phenylalanine and leucine excreted by amino-acid overproducing microorganisms.

1. Introduction

Amino acids, labelled with stable isotopes (^2H, ^{13}C, ^{15}N, ^{18}O) are becoming an indispensable tool for protein structure research and the investigation of various metabolism mechanisms using detection methods suitable for stable isotope labelled compounds, such as mass spectrometry (MS), nuclear magnetic resonance (NMR), infrared (IR), Raman spectroscopy and neutron diffraction [1–5].

There are several approaches reported for the preparation of amino acids labelled with stable isotopes. Introduction of the label into the molecule is often carried out by long multi-step organic synthesis. Biotechnology offers attractive alternatives to the organic methods of preparation. One approach is to label whole organisms by growing them on labelled precursors and subsequent fractionation of the biomass into the various compounds [6]. The labelled amino acids are then isolated from the protein hydrolysate of the total labelled biomass.

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For the separation of amino acids there are two basic liquid chromatographic methods. The first method is based on ion-exchange chromatography, originally developed by Stein and Moore [7], using post-column derivatization for detection of the amino acids. The second approach uses HPLC after pre-column derivatization. Additionally, RP-HPLC has become more and more popular in amino acid analysis [8–10]. Compared with the analytical chromatography of amino acids far less work has been directed toward refining preparative chromatography.

Up to now the preparative separation of amino acids is achieved most of all by using ion-exchange chromatography with available chromatographic systems, in which the eluent can be removed by simple evaporation. The use of volatile buffer constituents, such as ammonium formate, acetate buffers or pyridine has the advantage that the amino acid can be obtained by simple concentration of the appropriate fraction [11–16]. The strategy for the preparation of amino acids labelled with stable isotopes based on ion-exchange chromatography has been reviewed by LeMaster and Richards [17].

It is of great scientific but also practical interest to realize the advantages of preparative RP-HPLC for the separation of amino acids and their derivatives in quantities needed for further biochemical and biomedical investigations.

Recently we reported some preliminary experiments in preparative-scale RP-HPLC of amino acids as their benzyloxycarbonyl (Z-) derivatives from protein hydrolysates [18,19]. We prefer to use carbobenzoxychloride (Z-Cl), a widely used reagent in the synthesis of peptides, for pre-column derivatization. Z-amino acid derivatives are sufficiently stable and are easy to obtain, and it is easy to cleave this group under acidic and even neutral conditions to obtain optically active amino acids. Moreover, the price of Z-Cl is much lower than the other derivatization reagents. Isotopically labelled Z-amino acids, themselves or after cleavage of the Z-group, are of scientific interest and can be used directly for peptide synthesis.

The objective of the present study was to evaluate the application of RP-HPLC to the preparative separation of amino acids and their derivatives from various protein hydrolysates and fermentation broth. The derivatization of amino acids has been developed in a way that only one single derivative of each amino acid is formed. The method has been checked by investigation of jack bean urease hydrolysate and has been applied to the isolation of amino acids from various biomasses.

2. Experimental

2.1. Equipment and material

All unlabelled L-amino acid standards and Z-amino acid standards came from Reanal (Budapest, Hungary) and Sigma (St. Louis, MO, USA). Jack bean urease (EC 3.5.1.5) was from Reachim (Moscow, Russian Federation). Biomasses of green algae (*Chlorella sp. K., Dunaliella salina Teod.*), cyanobacteria (*Spirulina pl.*) and halobacteria (*Halobacterium halobium*), were cultivated as described in the literature [20–23]. Methylotrophic microorganisms (*Methylobacillus flagellatum KT1, Brevibacterim methylicum VGS 1*) were cultivated using the synthetic medium M9 [24]. Carbobenzoxychloride (Z-Cl) was purchased from the Protein Research Foundation (Japan). Sodium sulphate, sodium hydroxide, sodium dodecylsulphate (SDS), hydrochloric acid, hydrobromic acid, glacial acetic acid, and trifluoracetic acid (TFA) were purchased from Reachim. Acetonitrile and methanol were obtained from Aldrich (Milwaukee, WI, USA). All compounds were of analytical reagent grade. All solvents were of HPLC grade. Buffer components were from Reachim. All buffers used in HPLC were degassed by sonification for 7 min at 22 kHz. Deionized water was provided by the Milli-Q Plus water-purification system (Millipore, Bedford, MA, USA).

For centrifugation of cell biomasses (or protein) a Beckman centrifuge J2-21 (Palo Alto, CA, USA) and a Beckman ultracentrifuge L7-55 were used. To control the growth of cells the biophotometer Bonet-Maury-Jouan Biolog-2

(Paris, France) was used. Cell walls were destroyed mechanically and by sonification at 22 kHz (or 40 kHz), 70 W for 3–10 min using a Sonifier B-12 (Branson, Germany).

2.2. High-performance liquid chromatography

The analytical and preparative separation of Z-amino acids were carried out using a Knauer chromatograph (Berlin, Germany), equipped with a Model 64 HPLC pump, a Knauer injector, and an LCD 2563 UV-absorbance detector. Peak areas and retention times were recorded using a Shimadzu C-R 3A Chromatopac integrator (Tokyo, Japan). Z-amino acids were detected at 254 nm by UV-absorbance detection. The injection system was equipped with a 20-μl, 50-μl, 200-μl or 1-ml sampling loop. Various columns were used, including a 150 × 15 mm I.D. Lichrospher 100, 5 μm RP 18, obtained from Merck (Darmstadt, Germany); a 250 × 4.6 mm I.D. Lichrosorb, 5 μm RP 18, obtained from Knauer; a 150 × 4 mm I.D. Dyasorb 130, 7 μm C_8; a 250 × 4.6 mm I.D Econosil, 5 μm C_{18}; and a 250 × 3 mm I.D. Hypersyl, 5 μm ODS, obtained from Elsico (Moscow, Russian Federation); a 250 × 10 mm I.D. Silasorb, 12 μm C_{18} and a 150 × 3 mm I.D. Separon SGX, 7 μm C_{18}, obtained from Kova (Prague, Czech Republic).

Standard solutions for HPLC were prepared by dissolving the Z-amino acids in methanol. Samples were dissolved in methanol and the volume of injected sample was 20–350 μl. The mobile phase was composed of a mixture of two solvents: (A) deionized water–trifluoracetic acid (100:0.1–0.5, v/v), (B) HPLC-grade acetonitrile–trifluoracetic acid (100:0.1–0.5, v/v) or HPLC-grade methanol–trifluoracetic acid (100:0.1–0.5, v/v). Different gradients were used to achieve separation of the Z-amino acids. The optimized gradient started from 0% B to 60% B in 60 min, from 60% to 100% B in 5 min, for 5 min with 100% B, from 100% to 0% B at 5 min, for 10 min with 0% B. The flow-rate was 1 ml/min for the analytical separation of amino acids and 7 ml/min for the preparative separation. For preparative chromatography a steeper gradient was used: from 10% B to 80% B in 30 min, from 80% B to 100% B in 5 min, for 5 min with 100% B, in 1 min to 0% B, for 9 min with 0% B. Preparative re-chromatography was carried out in the isocratic mode with different proportions of A and B. All separations were performed at ambient temperature.

Quantitative estimation of the purity of the preparatively isolated HPLC fractions was carried out by amino acid analysis after cleavage of the Z-group using HBr/CH_3COOH [25]. Amino acid analysis was carried out using a Beckman HPLC (USA) (Model Gold system) in accordance with the Picotag system of Waters (MA, USA), which is based on the RP separation of PITC derivatives, applying UV-absorbance detection at 254–269 nm. For the isolation of amino acids excreted by amino-acid overproducing microorganisms Silicagel L 40/100 from Chemapol (Prague, Czech Republic) was used for adsorption column chromatography.

2.3. Mass spectrometry

Mass spectrometry has been used for the determination of the amount of isotopic incorporation when labelled amino acids were isolated from uniformly labelled biomasses. Electron-impact mass spectrometry was performed on a Finnigan MAT-144 S mass spectrometer (San Jose, CA, USA) using electron-impact ionization at 70 eV, (current 0.8 mA, source temperature 250°C, temperature programme gradient 10°C/min). Each measurement was repeated three times.

2.4. Hydrolysis of proteins

Proteins were hydrolyzed in 6 *M* HCl (or 6 *M* DCl). The solutions were gassed with nitrogen and sealed in hydrolysis tubes under nitrogen, then incubated at 110°C for 24 h. For the hydrolysis of the proteins, obtained from total labelled biomass, 6 *M* DCl (Isotope, St. Petersburg, Russian Federation) was used. The samples to be separated by RP-HPLC were dried, derivatized and directly used for processing.

2.5. Derivatization procedure

To a standard solution, containing an equimolar mixture of 20 amino acids (0.004 mmol) in 2 ml of 2 *M* NaOH (0.004 mmol), 1.36 g Z-Cl (0.008 mmol) and 0.25 ml 4 *M* NaOH were simultaneously added over a 40-min period. The reaction mixture was kept at 0°C under vigorous stirring. The pH was maintained between 10 and 12, by adjusting the flow-rate of the Z-Cl and NaOH. After the reaction was completed, the solution was extracted with 0.25 ml of pentane to remove the excess of Z-Cl, followed by cooling and acidification of the aqueous layer to congo red with 4 *M* HCl. Then, the Z-amino acids were extracted with ethyl acetate (3×0.2 ml). The combined extracts were dried over sodium sulphate and concentrated in vacuo. The yield of the Z-amino acid mixture obtained was 89%. Z-derivatives of protein hydrolysates from various microbiological sources were obtained in the same manner as described above for the standard mixture of amino acids.

2.6. Isolation of amino acids from jack bean urease

Jack bean urease (300 mg) was hydrolyzed at 110°C in 1.5 ml of 6 *M* HCl for 24 h in sealed ampules. The hydrolysate was evaporated to dryness and the resulting mixture was derivatized using 5-fold excess of Z-Cl. The yield of Z-amino acid mixture was 87%. The preparative separation of Z-amino acid derivatives from protein hydrolysate of jack been urease was made using gradient elution as described for the standard amino acid mixture, followed by re-chromatography of mixed fractions using isocratic elution at 21, 29, 33, 43, 47, and 52% B.

2.7. Isolation of amino acids from Spirulina pl

Phycobiliproteins from the biomass of *Spirulina pl.* were obtained as described in Ref. [26] or by dissolution in water from destroyed cells. Shortly, to 5 g of cells 10 ml of water was added, followed by homogenization in a glass homogeniser with a PTFE tube. After 24 h the aqueous phycobiliprotein solution was separated by centrifugation (30 min, 7000 *g*). After lyophilization 3.4 g of phycobiliproteins was obtained and subjected to hydrolysis. Phycobilines (pigments) were separated from the hydrolysate by extraction with chloroform, followed by derivatization of the hydrolysate and RP-HPLC as described above for jack bean urease hydrolysate.

2.8. Isolation of amino acids from bacteriorhodopsin of the purpur membranes of Halobacterium halobium[1]

A 100 mg amount of the purpur membranes were suspended in 30 ml of a 5% sodium dodecylsulfate solution. After the apoprotein was precipitated with 15 ml of methanol, the pellet was separated and washed two times with 15 ml of water and centrifuged (30 min, 10 000 *g*). The resulting delipidized protein was hydrolyzed, followed by derivatization and RP-HPLC separation as described for jack bean urease.

2.9. Isolation of amino acids from biomasses of the green algae Chlorella sp. K., Dunaliella salina

A 1-g amount of green algae *Chlorella sp. K.*, was suspended in chloroform–methanol–water (65:25:10, v/v), the suspension obtained was mechanically destroyed, and then sonificated (3×3 min); a 650-mg amount of total delipidized protein fraction was subjected to hydrolysis, then derivatized and separated using RP-HPLC. Protein separation from the green algae *Dunaliella salina* was carried out as for *Chlorella sp. K.*

[1] The method developed was applied for the analysis of biosynthetic incorporation of site-specific isotopically labelled amino acids in bacteriorhodopsin and for mass spectrometric analysis.

2.10. Isolation of perdeuterated amino acids from methylotrophic microorganisms Methylobacillus flagellatum, Brevibacterium methylicum, cultivated on medium with 2% CD_3OD (99.9% D) and 98% of D_2O (99.9% D)

A 150-mg amount of biomass of *Methylobacillus flagellatum* was suspended in 20 ml of water, destroyed by sonification at 40 kHz 3 min (6 × 30 s) cooling on ice. After removing the cells by centrifugation (30 min, 10 000 *g*), the lipids were removed by extraction with chloroform–methanol (1:2, v/v, 3 × 10 ml). The remaining material, obtained after delipidation, was dried. A 95-mg amount of total proteins were hydrolysed in 6 *M* DCl at 110°C during 24 h, then derivatized and separated using HPLC.

2.11. Isolation of Phe from the Phe overproducing microorganism Methylobacillus flagellatum

To 5 g of lyophylized cultural medium 30 ml of isopropanol was added, acidified to pH 2 using 5 *M* HCl. The salts were removed by centrifugation and the supernatant was evaporated, Phe was recrystallised from ethanol, $[\alpha]_D^{20} = 35°$(in water).

Phe was also isolated from the cultural medium as the Z-derivative with a yield of 87% and a purity of 99%.

3. Results and discussion

3.1. Precolumn derivatization and separation of the Z-amino acids derivatives

When HPLC is used for the separation of amino acids some problems arise connected with the large range of polarities and low molar absorptivities of the different amino acids in the UV region. To overcome these difficulties either pre- or post-column derivatization can be used [27,28]. The derivatization improves the chromatographic properties of the amino acids and makes the detection of amino acids more easy.

One of the possibilities to improve the resolution of the protein hydrolysate and to solve problems with respect to detection, is to use pre-column derivatization in RP-HPLC [29–31].

One major drawback of the Z-Cl derivatization is the occurrence of three functional amino acids [32]. As a consequence, multiple derivatives of these amino acids are obtained, which has been investigated under different chromatographic conditions. This multiple derivatization is dependent on the molar ratio of the derivatizing reagent and the amino acid. The formation of mono-, di- and tri- (Z) derivatives at the same time makes it difficult to identify all peaks in the chromatograms. Therefore, we developed a procedure for derivatization of an equimolar mixture of amino acids using 3–5 fold excess of Z-Cl to obtain amino acids with only one single Z-derivative for each (Fig. 1). Under these conditions N^{α},N^{G},N^{G}-tribenzyloxycarbonylarginine; α,e-dibenzyloxycarbonyllysine; im,α-dibenzyloxycarbonylhistidine; O,N-dibenzyloxycarbonyl-

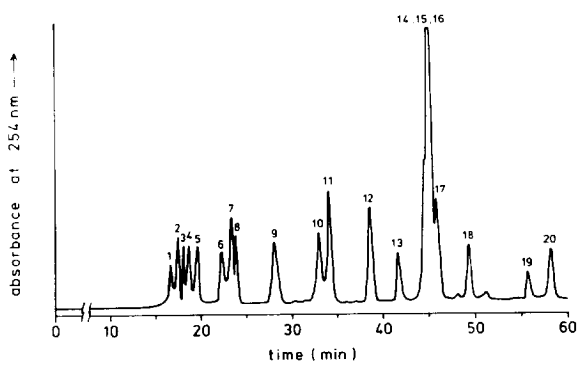

Fig. 1. Analytical RP-HPLC separation of the twenty Z-amino acids. Column: Econosil C_{18}, 5 μm, 250 × 4.6 mm I.D.; mobile phase: (A) water–TFA (100:0.5, v/v), (B) acetonitrile–TFA (100:0.5, v/v); gradient elution: 0 min 0% B, 60 min 60% B, 65 min 100% B; flow-rate: 1.5 ml/min; sample size 5 μmol/amino acid; injection volume: 10 μl. Peaks: 1 = Asn, 2 = Gln, 3 = His, 4 = Ser, 5 = Thr, 6 = Asp, 7 = Glu, 8 = Gly, 9 = Ala, 10 = Pro, 11 = Tyr, 12 = Val, 13 = Met, 14 = Leu, 15 = Ile, 16 = Trp, 17 = Phe, 18 = Lys, 19 = Cys, 20 = Arg.

serine; O,N-dibenzyloxycarbonylthreonine; O,N-dibenzyloxycarbonyltyrosine; N,S-dibenzyloxycarbonylcysteine were formed. When pre-column derivatization is used it is important to take into account the stability of the derivatives because of the time elapsing between derivatization and injection and the time needed for the chromatographic separation. Derivatives of histidine are very unstable and a delay between derivatization and injection of more then 20 min also complicates peak identification. Cysteine was previously oxidized to cystine and after derivatization we obtained di-(Z)-cystine which is stable under HPLC conditions.

Optimization of the chromatographic conditions with respect to the chromatographic resolution was carried out with respect to eluent composition and gradient profile. On an analytical scale satisfactory resolution was obtained for all Z-derivatives using a linear gradient from 0 to 60% B in 60 min. The problems dealing with the separation of Ser/Glu, Tyr/Pro, Met/Val on Lichrosorb RP-18, Separon SGX 8, were solved by using Hypersyl ODS or Econosil C_{18}. Resolution between Phe and Leu/Ile could be substantially improved on an Econosil C_{18} column. The optimal resolution is shown in Fig. 1.

Hydrolysis of the protein under alkaline conditions results in a racemic mixture of amino acids and destruction of some of the amino acids. Enzymatic hydrolysis gives additional amino acids due to autohydrolysis of the enzyme. In order to obtain optically active amino acids we used acid hydrolysis. It is known that under such conditions tryptophan, asparagine and glutamine are completely destroyed, while serine, threonine and tyrosine are only partly destroyed [33]. Preparative separation was made for 17 Z-amino acid derivatives: Asp, Glu, Ser, Thr, Gly, Ala, Pro, Tyr, Met, Val, Leu, Ile, Phe, Lys, Cys, Arg, and His. Applying the optimal analytical conditions to the preparative separation led to a very long separation time and high eluent consumption. Therefore, a steeper gradient profile, as a compromise between resolution and separation time, has been used as is shown in Fig. 2.

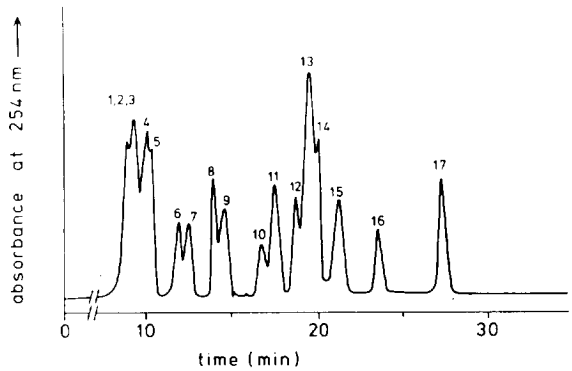

Fig. 2. Preparative RP-HPLC separation of the seventeen Z-amino acids. Column: Silasorb C_{18}, 12 μm, 250 × 10 mm I.D.; mobile phase: (A) water–TFA (100:0.5, v/v), (B) acetonitrile–TFA (100:0.5, v/v); gradient elution: 0 min 10% B, 30 min 80% B, 35 min 100% B; flow-rate: 5 ml/min; sample size: 100 mg; injection volume: 350 μl. Peaks: 1 = Asp, 2 = Glu, 3 = His, 4 = Ser, 5 = Thr, 6 = Gly, 7 = Ala, 8 = Pro, 9 = Tyr, 10 = Val, 11 = Met, 12 = Leu, 13 = Ile, 14 = Phe, 15 = Lys, 16 = Cys, 17 = Arg.

3.2. Investigation of jack bean urease hydrolysate

The developed chromatographic procedure was tested by investigation of jack bean urease as a model protein with a well-known amino acids composition [34]. Derivatization of jack bean urease hydrolysate was carried out in excess of Z-Cl as described for the standard mixture. Under these conditions tribenzyloxycarbonylarginine, dibenzyloxycarbonyllysine, dibenzyloxycarbonylhystidine, dibenzyloxycarbonyltyrosine were formed. The preparative separation of derivatized jack bean urease hydrolysate was accomplished in two steps: (i) gradient elution and (ii) one-step re-chromatography of the fractions obtained using isocratic elution [for fractions 1–6 (see Fig. 3) at 21, 29, 33, 43, 47, 52% B, respectively]. The results of the re-chromatography are represented in Table 1. Purity of amino acids was previously estimated by HPLC, and then was determined using amino acid analysis after cleavage of the Z-group using HBr/CH_3COOH. The extra peak in the chromatograms (Fig. 3, peak 8) corresponds to hydrophobic dipeptides containing valine,

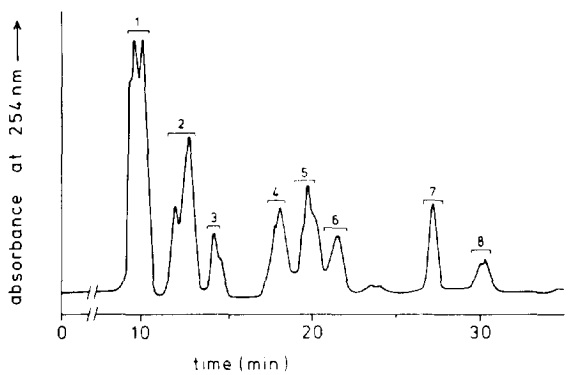

Fig. 3. Preparative RP-HPLC separation of the hydrolysate of jack bean urease. Column: Silasorb C_{18}, 12 μm, 250 × 10 mm I.D., mobile phase: (A) water–TFA (100:0.5, v/v), (B) acetonitrile–TFA (100:0.5, v/v); gradient elution: 0 min 10% B, 30 min 80% B, 35 min 100% B; flow-rate: 5 ml/min; sample size: 300 mg; injection volume: 500 μl. Identification of fractions 1–7 see Table 1.

Table 1
Amino acid composition of jack bean urease. Results of preparative separation

Amino acid	Content[a] (%)	Peak number[b]	Yield (%)	Purity (%)
Asp	5.8	1	78	98
Asn	4.6	–	–	–
His	3.0	1	68	97
Glu	6.3	1	78	98
Gln	2.0	–	–	–
Ser	5.5	1	67	96
Thr	6.7	1	67	97
Gly	9.4	2	82	98
Ala	8.8	2	87	99
Pro	4.9	3	78	96
Tyr	2.5	3	73	96
Met	2.5	4	66	98
Val	6.2	4	83	99
Phe	2.9	5	73	98
Leu	8.2	5	66	98
Ile	7.9	5	66	98
Trp	0.6	–	–	–
Cys	1.9	–	–	–
Lys	5.8	6	85	99
Arg	4.5	7	68	99

[a] Content (%) of amino acids per urease molecule.
[b] The peak numbers refer to Fig. 3.

leucine, and/or isoleucine, due to difficulties in hydrolysis of the peptide bonds formed by these amino acids during hydrolysis. Using the developed method we separated 350-mg samples of protein hydrolysates. Amino acids were separated with a purity of 96–99% and a yield of 68–87%.

3.3. Amino acid separation from protein hydrolysates of various sources

To demonstrate the applicability of the method to the isolation of amino acids, different types (in taxonomic respect) of microorganisms have been studied (green algae, cyanobacterium, halobacterium and methylotrophic microorganisms). Most of these microorganisms can be used as an appropriate source for the production of amino acids and other biologically active substances labelled with stable isotopes. Dependent on the specific biological source from which the proteins were obtained, different methods of protein purification were applied [35,36]. When total proteins were separated from the biomasses of microorganisms (green algae *Chlorella sp. K.*, *Dunaliella salina*, cyanobacterium *Spirulina pl.*) a protein-rich strain was used with a small carbohydrate content and the residue remaining after delipidation was hydrolyzed. In other cases the quantity of carbohydrates was taken into account [37] and proteins were precipitated to separate them from polysugars [8,36]. In the case of methylotrophic microorganisms (*Methylobacillus flagellatum* or *Brevibacterium methylicum*) the total protein was hydrolyzed after removal of the lipids by extraction.

A general procedure for amino acid isolation is presented in Fig. 4. The main stages are: cultivation of microorganism, disruption of cell biomass, protein separation, followed by hydrolysis, derivatization of protein hydrolysate using Z-Cl and separation of the mixture of Z-derivatives using RP-HPLC with the isolation of either the individual Z-amino acid derivatives or of the free amino acids after cleavage of the Z-group. Derivatization of the protein hydrolysate and of amino acids excreted from amino-acid over-

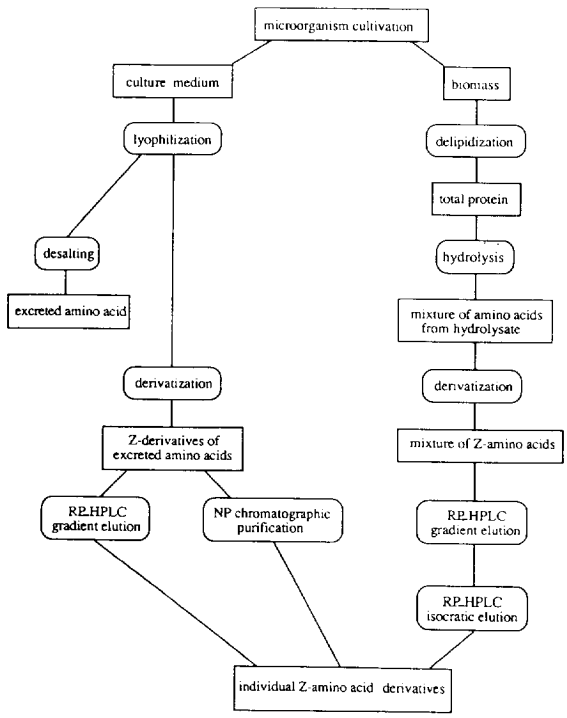

Fig. 4. General scheme of amino acids separation from protein hydrolysates and culture media.

producing microorganisms was accomplished in accordance with the method described above. Excreted amino acids were isolated as free amino acids or as their Z-derivatives after derivatization of the cultural medium that was previously lyophilized. This procedure simplifies the separation of the amino acid from other metabolites. The preparative separation of Z-amino acid derivatives was achieved on a 250 × 3 mm I.D. Hypersyl 5 μm ODS column as well as on a 250 × 10 mm I.D. Silasorb 12 μm C_{18} column, followed by re-chromatography of the mixed fractions. Data of amino acid isolation from protein hydrolysates of various sources are shown in Table 2. Little methionine and cystine were obtained, since they are only present in very low amounts in the algae. The yield of the individual amino acids is 68–89%, chromatographic purity 96–99%. The amino acids were isolated on a milligram scale (column loads: 100–500 mg). For larger loads, the cross-sectional area of each column as well as the volume of the effluent should be increased proportionally.

The method developed is suitable for the isolation of isotopically labelled amino acids from protein hydrolysates of various sources.

For instance, the method developed was proved to be reliable by the fact that the separation profile of perdeuterated amino acids from *Methylobacillus flagellatum*, cultivated on synthetic media with 2% CD_3OD (99.9% D) and 98% of D_2O (99.9% D) was similar to the profile obtained for the separation of unlabelled amino acids, because the isotope enrichment does not influence the chromatographic characteristics. The isotope enrichment of deuterium in the individual amino acids was estimated using MS. The extent of isotope enrichment of the amino acids was found to be in the range 96–99%.

4. Conclusions

A new method for the preparative isolation of individual amino acids on a milligram scale based on RP-HPLC after pre-column derivatization with carbobenzoxychloride (Z-Cl) was developed. The method was applied to the isolation of amino acids from various sources. Comparative analysis of the results obtained showed that independent of the biological source of protein the method developed allowed to isolate amino acids with high yield (68–89%) and chromatographic purity (96–99%). The method developed is suitable for the isolation of isotopically labelled ^{2}H-, ^{13}C-, ^{15}N-, ^{18}O-amino acids from protein hydrolysates of various sources.

Acknowledgements

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Table 2
Data of the isolation of amino acids from protein hydrolysates and culture media from various sources using RP-HPLC

Amino acid	Source	Content, (% abs. dry biomass)	Yield (%)	Purity (%)
Asp	*Spirulina*	5.3	73	97
	Chlorella	4.3	76	98
	Methylobacillus flagellatum	3.8	75	98
Glu	*Spirulina*	8.2	74	97
	Chlorella	3.6	73	96
	Methylobacillus flagellatum	4.6	75	98
Ser	*Spirulina*	2.9	67	97
	Chlorella	2.2	69	96
	Methylobacillus flagellatum	1.6	66	96
Thr	*Spirulina*	2.8	69	96
	Chlorella	2.2	68	98
	Methylobacillus flagellatum	2.0	67	98
Gly	*Spirulina*	2.8	84	98
	Chlorella	3.0	87	98
	Methylobacillus flagellatum	2.3	88	98
Ala	*Spirulina*	4.2	89	99
	Chlorella	4.1	87	99
	Methylobacillus flagellatum	2.7	86	97
His	*Spirulina*	0.7	68	96
	Chlorella	1.1	68	97
	Methylobacillus flagellatum	1.1	69	97
Pro	*Spirulina*	2.5	78	99
	Chlorella	3.1	79	98
	Methylobacillus flagellatum	1.5	77	98
Tyr	*Spirulina*	2.1	77	96
	Chlorella	1.5	68	97
	Methylobacillus flagellatum	1.5	68	97
Val	*Spirulina*	3.1	77	97
	Chlorella	2.7	73	96
	Methylobacillus flagellatum	1.8	76	98
Phe	Culture medium			
	Brevibacterium methylicum	0.6	87	99
	Spirulina	2.3	76	98
	Chlorella	2.3	75	98
	Methylobacillus flagellatum	1.8	77	96
Arg	*Spirulina*	3.7	69	97
	Chlorella	3.1	68	98
	Methylobacillus flagellatum	4.0	68	99
Lys	*Spirulina*	2.6	74	98
	Chlorella	3.3	73	99
	Methylobacillus flagellatum	3.1	75	98
Leu/Ile	*Spirulina*	4.9/2.7	68	97
	Chlorella	4.1/1.6	67	98
	Methylobacillus flagellatum	2.9/1.7	66	98

methyllicum and culture medium of *Brevibacterium methylicum*. The authors are grateful to J. Raap for assistance in preparing the manuscript. The authors are grateful to U.R. Tjaden for helpful discussions and valuable critical remarks.

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