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Determination of amino acids in biomass and protein samples by microwave hydrolysis and ion-exchange chromatography

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Abstract

A fast method for protein hydrolysis based on controlled heating in a microwave oven is described. The samples are heated to 150°C for 10–30 min where conventional methods use 110°C for 24 h. The method was tested with pure protein samples and "real" protein samples with carbohydrates, fats, nucleic acids and minerals. The microwave method showed similar or better results than the conventional method. The effect of degassing and stabilising agents was also tested. Degassing and thioglycolic acid stabilise methionine and partly tryptophan. Degassing and phenol stabilise tyrosine, phenylalanine and histidine in performic acid oxidised samples.

1. Introduction

Ion-exchange chromatography (IEC) combined with post-column derivatization with ninhydrin and detection at 570 nm is a sturdy method widely used for amino acid measurement. Determinations of amino acids bound in proteins require a hydrolysis of the peptide linkages before measurement. This is typically done by a 24 h acid (6 *M* HCl) hydrolysis at 110°C. Hydrolysis can be performed by (a) open reflux method under an atmosphere of nitrogen, (b) hydrolysis in evacuated sealed tubes, or (c) hydrolysis in screw cap tubes in an atmosphere of nitrogen [1]. Similar results were found for the screw cap method and for the reflux method, but the screw cap method gave an increased loss of methionine [1]. Cyst(e)ine and tryptophan are lost during a common acid hydrolysis, and asparagine and glutamine are converted to aspartic acid and glutamic acid, respectively [2,3].

A much faster hydrolysis of proteins can be obtained by microwave hydrolysis [4,5]. Samples are rapidly heated in closed vessels to 150°C and a total protein hydrolysis can be obtained in less than 30 min. Péter et al. [4] used a commercial household microwave oven for hydrolysis of small peptides. They found that the microwave hydrolysis gave reduced racemization and higher recovery of sensitive amino acids than hydrolysis by conventional heating. Grimm [5] used a pressure controlled microwave oven and gas phase hydrolysis of pure proteins and peptides. He found a slightly higher loss of serine and threonine than by common gas phase hydrolysis at 110°C.

It is well known that hydrolysis of pure polypeptides and protein samples can behave very differently from "real" protein samples. The presence of iron or copper in protein samples can cause serious difficulties in production of unusual artefacts and in the loss of certain amino

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acids [2], and the recovery of methionine and tryptophan has been reported to depend on the carbohydrate level of the sample [1,6].

This paper compares fast microwave hydrolysis of proteins with the traditional hydrolysis. The effect of degassing and stabilising agents is also described. Two "pure" protein sources, casein and gelatine, and a "real" protein source, BioProtein, were used as test protein. BioProtein is biomass produced from natural gas by microorganisms as described previously [7]. It contains about 70% raw protein, 10% carbohydrates, 10% fat, 7% nucleic acids and 5% minerals. The copper content is about 100 mg/kg and the iron content is about 200 mg/kg. Thus, it represents a difficult mixture for protein hydrolysis.

2. Experimental

2.1. Chromatographic system

The amino acids were separated by the sodium-based Pickering system for protein and collagen hydrolysates, the Pickering 150 × 4 mm I.D., 7-μm column ("high-efficiency") and 20 × 3 mm guard column being used (Pickering Labs., Mountain View, CA, USA). The system was combined with an Dionex Model 4500i ion chromatographic gradient pump equipped with a Dionex UV–Vis variable-wavelength detector, a Dionex autosampler, a Rheodyne injector and an IBM AT compatible computer with the Dionex AI-450 Model II data system.

The column was placed in a CHX650 column heater from Pickering Labs. and maintained at 55°C. A Dionex reagent pump and autoion reagent controller was used for post-column ninhydrin addition. The ninhydrin-eluent mixture was heated to 130°C in a Pickering post-column reactor.

The eluent flow-rate was 0.4 ml/min, the ninhydrin flow-rate was ca. 0.66 ml/min and the post-column reaction temperature 130°C. Absorption was monitored at 570 nm. Trione ninhydrin was used as post-column reagent.

2.2. Eluents and gradient programme

Eluent A: sodium citrate, pH 3.15 (40 g trisodium citrate dihydrate, 30 ml 30% HCl plus water to 2000 ml). Eluent B: sodium acetate, pH 7.40 (16.4 g sodium acetate, 105.2 g sodium chloride, water to 2000 ml). Eluent C: sodium regenerant pH 12.4 [0.7445 g EDTA, Titraples, 4.0 g sodium chloride, 10.2 ml sodium hydroxide (50% w/w), water to 1000 ml].

Gradient programme: 0–6 min 100% A; 6–28 min 100 to 0% A, 0 to 100% B; 28–42 min 100% B; 42–54 min 100 to 85% B, 0 to 15% C; 54–72 min 0 to 100% A, 100 to 0% B; 72–75 min 100% A.

2.3. Microwave system

A CEM (Matthews, NC, USA) MDS-2000 microwave sample preparation system equipped with temperature and pressure measuring and control device was used for microwave hydrolysis of protein samples. The power output was 630 ± 50 W. Samples were hydrolysed in CEM single-wall PTFE vials with double port cap. A CEM capping station was used for capping of the vials (torque 16.3 Nm). One port cap in one of the vials was fitted with a glass thermowell for the fiber-optic temperature probe. One port cap in another vessel was connected to the pressure sensing tube. The head spaces of the vials were connected through PTFE tubes fitted to the port caps. Normally two vials were operated together, but the system could handle up to eight vials simultaneously. Parameters for microwave acid hydrolysis are shown in Table 1.

2.4. Pre-hydrolysis oxidation

Some samples were oxidized by performic acid before hydrolysis in order to determine cystine/cysteine [8,9]: 20–50 mg protein sample was accurately weighed into the CEM vial and 5 ml ice-cold freshly prepared performic acid (0.5 ml 30% H_2O_2 and 4.5 ml formic acid, mixed at room temperature, after 30 min placed on ice), 250 μl 200 m*M* norleucine and when added 250 μl 10% phenol solution was mixed into it. The

Table 1
Typical parameters for the microwave acid hydrolysis

Parameter	Stage		
	1	2	3
Power (%)	100	100	0
Pressure	90	90	90
Run time (min)	3	30	20
Time @P (min)	–	20	–
Temperature (°C)	150	150	150
Fans speed (%)	100	100	100

Stage 1: the frozen sample is thawed (the temperature control does not tolerate a negative temperature; this stage is not necessary for unfrozen samples). Stage 2: when the sample(s) reach the set point temperature (or the set point pressure, whatever comes first), the temperature is kept for the time given by "Time @P" or for the "Run time", whatever comes first. Stage 3: the cooling phase.

oxidation mixture was kept at 0°C for 18 h. Care was taken to ensure that protein particles were thoroughly wetted and immersed in the oxidising medium during incubation. Following oxidation, the contents of the vials were frozen and the reagents removed in vacuum using a freeze-dryer (Note: performic acid is very corrosive to vacuum pumps). After drying, 5 ml water and 10 ml 30% HCl were added to the sample and it was prepared for microwave hydrolysis as described below.

2.5. Sample preparation

A 20–50-mg protein sample, 2.5 ml water or 2.5 ml 60 μM thioglycolic acid, 2.5 ml 10 mM norleucine and 10 ml 30% HCl were added to CEM single-wall PTFE vials. The vials were capped with a double port cap. Samples for degassing were frozen (− 80°C) in order to avoid bumping during evacuation [2] and the gas phase was replaced by evacuating and filling with nitrogen (99.995%) to 15 p.s.i. (1 p.s.i. = 6894.76 Pa). The evacuating/filling process was repeated four times.

The samples were either heated in an oven at 110°C or in the microwave oven programmed as showed in Table 1. The "Time @P" at 150°C was varied from 10 min to 60 min.

Hydrolysed samples were "neutralised" to about pH 2.2 by adding 3.5 ml 50% NaOH, diluted to 50 ml with sodium citrate pH 2.2 solution (40 g trisodium citrate dihydrate, 30 ml 30% HCl plus water to 2000 ml) and filtered (0.45 μm) before IEC.

IEC of the samples was performed in a random order. Each sample was hydrolysed twice and each hydrolysate was analysed twice. Results given in tables and figures represent mean of four determinations.

2.6. Reagents and chemicals

Trione ninhydrin reagent was obtained from Pickering Labs. Chemicals for eluents were of Suprapur or "zur Aminosäureanalyse" grade from Merck. All other chemicals were of analytical-reagent grade. Ultra-pure water (18.2 MΩ), obtained by use of a Millipore Milli-Q water-purification system (Bedford, MA, USA), was used throughout.

3. Results and discussion

The microwave acid hydrolysis can be described by three phases: (1) a fast-heating phase, (2) a constant-temperature phase, and (3) a cooling phase. Fig. 1 shows typical temperature and pressure curves for a 30-min acid hydrolysis of a BioProtein sample. The set-point temperature was reached within 4 min from start. The pressure was not constant during the constant temperature period. It initially rose to about 65 p.s.i. (peak at about 7 min from start) and then fell down to about 45 p.s.i. This pressure pattern was observed for all the hydrolysis experiments, although the pressure level varied somewhat. The pattern is probably due to a reduction in the HCl concentration during the hydrolysis, as some HCl is used by the reaction. The absolute pressure level may well depend on the type and amount of sample. This observation indicates that temperature is a better control parameter than pressure for performing reproductive micro-

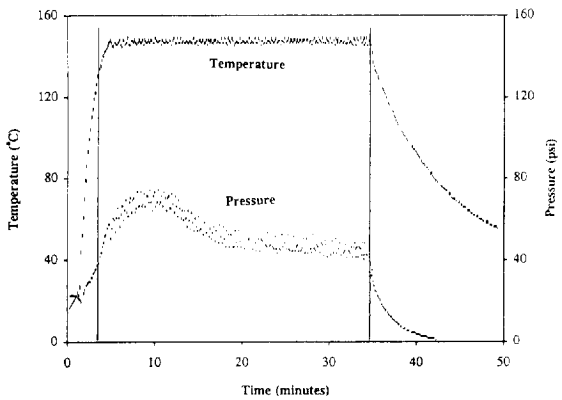

Fig. 1. Temperature and pressure curves for hydrolysis of a BioProtein sample in the microwave oven.

wave hydrolysis of protein samples; pressure control does not guarantee a constant hydrolysis temperature.

The effect of the hydrolysis time was initially tested in a series of experiments. The level of most amino acids increased when hydrolysis time was extended from 4 to 10 min and remained fairly constant when the hydrolysis time was further extended from 10 min and up to 60 min. Fig. 2 shows the measured amino acid level as a function of hydrolysis time for six selected amino acids. The amounts of serine and to a lesser extent threonine decreased when the hydrolysis time was extended. The level of isoleucine and valine increased when hydrolysis time was increased to 30 min. This is probably due to Leu–Leu, Ile–Leu, Ile–Val and Val–Gly peptide bindings in the protein that are difficult to hydrolyze [2,10]. A similar pattern was found for the same amino acids hydrolyzed at 110°C (Fig. 3).

Serine is known to be thermolabile under acid conditions and it has been suggested that serine is degraded due to first-order kinetics:

$$\ln A(t) = k_0 - k_1 t$$

where k_0 and k_1 are constants and $A(t)$ is the quantity of amino acid present after t h of hydrolysis; k_1 has been found to 0.0038–0.0043 h^{-1} [11]. We found that the serine degradation rate can be described by first-order kinetics with $k_1 = 0.84\ h^{-1}$ for hydrolysis times up to 20 min and $k_1 = 0.35\ h^{-1}$ for hydrolysis times between 20 and 60 min (Fig. 4). A similar biphasic behavior was not observed for hydrolysis at 110°C where k_1 was found to 0.0034 h^{-1} (results not shown).

Thioglycolic acid has been used to prevent the oxidation of methionine, tyrosine and carboxymethylcysteine during chromatography or acid hydrolysis of peptides and proteins [3,12]. Addition of thioglycolic acid has also been reported to improve tryptophan recovery [2]. Degassing can partly prevent the oxidation of methionine to methionine sulfoxide. We have tested the effect of degassing and thioglycolic acid on acid hydrolysis of BioProtein and two pure proteins, gelatine and casein, and compared traditional

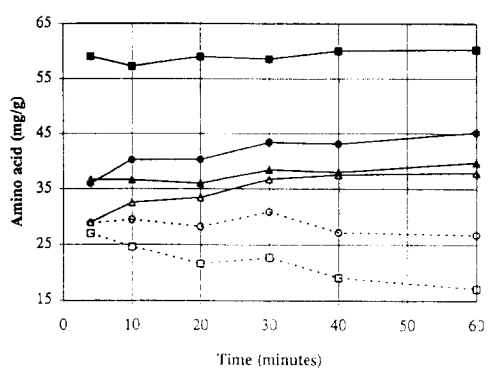

Fig. 2. Measured level of six different amino acids as a function of hydrolysis time. Hydrolyses were performed in a microwave oven at 150°C. ■ = Leucine; ● = valine; ▲ = glycine; △ = isoleucine; ○ = threonine; □ = serine.

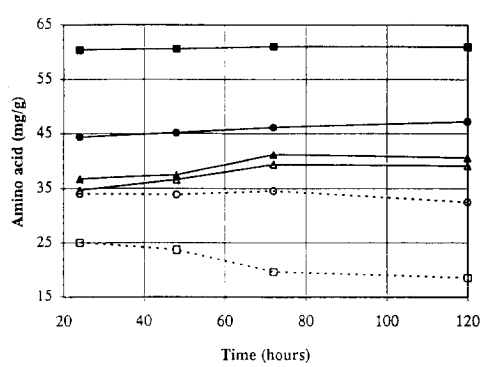

Fig. 3. Measured level of six different amino acids as a function of hydrolysis time. Hydrolyses were performed by heating to 110°C. ■ = Leucine; ● = valine; ▲ = glycine; △ = isoleucine; ○ = threonine; □ = serine.

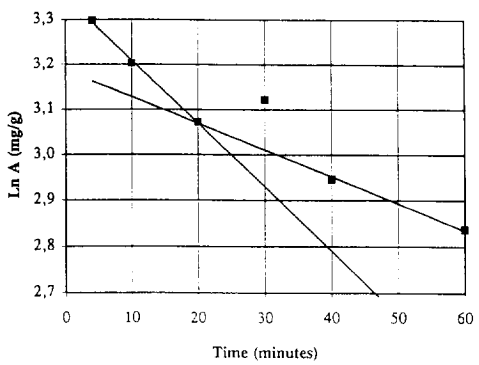

Fig. 4. Measured level of serine as a function of the hydrolysis time. Hydrolyses were performed in a microwave oven at 150°C. The lines represent the best fit for points at 4, 10 and 20 min [ln $A = 3.35 - 0.014t$ (min)] and at 20, 40 and 60 min [ln $A = 3.187 - 0.0059t$ (min)].

hydrolysis in closed vessels at 110°C for 24 h with hydrolysis in a microwave oven at 150°C for 10 and 30 min (Tables 2–5).

Methionine was found in the highest amount in degassed samples with thioglycolic acid (Table 2). However, the effect of degassing and addition of thioglycolic acid was small for the microwave hydrolyzed samples. In case of BioProtein the level increased from about 14 mg/g in the untreated sample to about 18.5 mg/g for the degassed and thioglycolic acid-treated sample. The effect was somewhat larger for gelatine whereas there was no effect at all for casein. This is in contrast to samples hydrolyzed at 110°C for 24 h. In that case there were some effect of degassing the samples and a large effect of adding thioglycolic acid to the samples. The observed differences between the microwave and the conventional method are probably due to the much longer treatment time for samples hydrolyzed at 110°C.

Tyrosine has been described to be seriously degraded during hydrolysis of certain proteins and hardly at all during others [2] and thioglycolic acid has been suggested as a stabilizing agent for tyrosine. We found that tyrosine was stable for all the sample types and conditions tested (Table 3).

Tryptophan is known to be acid labile and it is common to use basic hydrolysis to determine it. Thioglycolic acid has been reported to stabilize tryptophan during acid hydrolysis [6,12]. We found a higher level of tryptophan in the microwave-hydrolyzed samples but the level was much lower than expected from previous performed basic hydrolysis of the samples. The highest levels were found in degassed samples of casein with added thioglycolic acid. The low level found in BioProtein (expected level was 15.7 mg/g dry

Table 2
Measured level (mg/g) of methionine in samples hydrolyzed at 110°C for 24 h or at 150°C for 10 or 30 min

Sample	Treatment	No stabilisation	Degassed	Thioglycolic acid	Degassed + thioglycolic acid
BioProtein, dried	110°C, 24 h	0.1	6.7	14.0	16.3
	150°C, 10 min	14.5	15.8	16.5	18.3
	150°C, 30 min	13.5	16.0	18.2	18.5
BioProtein, fresh	110°C, 24 h	1.0	4.0	15.5	16.5
	150°C, 10 min	12.1	15.8	16.8	17.1
	150°C, 30 min	13.4	15.9	16.9	17.6
Gelatine	110°C, 24 h	2.0	2.5	8.6	9.0
	150°C, 10 min	2.9	4.4	8.8	9.6
	150°C, 30 min	1.9	4.6	9.0	9.6
Casein	110°C, 24 h	22.3	24.3	29.9	28.8
	150°C, 10 min	30.2	29.8	30.0	31.0
	150°C, 30 min	31.4	30.4	30.6	32.3

Table 3
Measured level (mg/g) of tyrosine in samples hydrolyzed at 110°C for 24 h or at 150°C for 10 or 30 min

Sample	Treatment	No stabilisation	Degassed	Thioglycolic acid	Degassed + thioglycolic acid
BioProtein, dried	110°C, 24 h	18.5	23.5	22.2	22.6
	150°C, 10 min	25.0	24.4	25.4	26.7
	150°C, 30 min	28.4	26.8	29.5	29.5
BioProtein, fresh	110°C, 24 h	20.9	22.3	23.4	23.5
	150°C, 10 min	25.2	25.6	24.4	24.7
	150°C, 30 min	24.3	27.7	27.2	27.3
Gelatine	110°C, 24 h	2.8	3.0	3.4	3.0
	150°C, 10 min	4.2	3.7	3.7	3.7
	150°C, 30 min	3.5	3.4	3.4	3.3
Casein	110°C, 24 h	55.5	52.7	57.3	54.9
	150°C, 10 min	52.5	55.8	54.8	56.3
	150°C, 30 min	56.3	57.1	56.1	56.8

matter) is probably due to its high content of carbohydrates as the recovery of tryptophan is negatively influenced by carbohydrates in the sample [6].

Cystine was unstable during acid hydrolysis, and the recovery was not increased by degassing (results not shown). The retention time of cysteine is similar to the retention time of proline and if the sample contains some cysteine it will not be measured correctly. Some of the cyst(e)ine was found as cysteic acid, especially in non-degassed samples. The cystine peak was absent in most samples where thioglycolic acid was added. Acid hydrolysis is known to cause very serious losses of cystine residues through severance of the disulfide bridges [2]. If oxygen

Table 4
Measured level (mg/g) of tryptophan in samples hydrolyzed at 110°C for 24 h or at 150°C for 10 or 30 min

Sample	Treatment	No stabilisation	Degassed	Thioglycolic acid	Degassed + thioglycolic acid
BioProtein, dried	110°C, 24 h	0.0	0.0	0.0	0.1
	150°C, 10 min	2.4	1.5	1.7	2.3
	150°C, 30 min	0.3	0.3	0.6	0.8
BioProtein, fresh	110°C, 24 h	0.2	0.3	0.6	0.3
	150°C, 10 min	1.8	1.5	2.8	2.9
	150°C, 30 min	1.2	0.4	0.8	1.1
Gelatine	110°C, 24 h	0.2	0.2	0	0.3
	150°C, 10 min	0.6	0.6	0.6	0.8
	150°C, 30 min	0.0	0.0	0.0	1.5
Casein	110°C, 24 h	0.1	0.1	3.4	4.2
	150°C, 10 min	7.5	6.4	5.6	11.8
	150°C, 30 min	7.4	3.6	10.9	7.9

Table 5
Measured level (mg/g) of tyrosine, phenylalanine, histidine, tryptophan and arginine in performic acid-oxidized samples of BioProtein

Amino acid	Hydrolysis time (min)	No stabilisation	Degassed	Phenol	Degassed + phenol	Non-oxidised sample
Tyrosine	10	0.7	1.0	2.0	6.8	27.0
	30	3.0	2.0	5.0	8.5	
Phenylalanine	10	2.1	1.9	24.0	31.6	32.4
	30	0.7	2.9	7.9	30.8	
Histidine	10	4.8	3.0	10.8	12.1	13.2
	30	1.6	5.5	13.5	12.7	
Tryptophan	10	7.7	8.1	0.9	5.3	15.7
	30	5.1	4.7	0.4	0.1	(basic hydrolysis)
Arginine	10	38.2	35.4	35.8	37.0	39.9
	30	39.8	39.4	39.6	40.1	

Samples were hydrolyzed in a microwave oven at 150°C for 10 or 30 min.

is present then the released thiol group will be oxidized, to a greater or lesser extent, to cysteic acid via cysteine and the sulfinic acid and sulfone. Cysteine suffers the same oxidative fate as cystine.

Cyst(e)ine can be quantitatively converted to cysteic acid by an oxidation with performic acid before the hydrolysis step [8,9]. However, performic acid oxidation is known to destroy tyrosine and tryptophan [10]. Loss of phenylalanine, histidine and arginine has also been reported [1,8]. Phenol can be added as a halogen scavenger to stabilize labile amino acids [8,13]. Table 5 shows the effect of degassing and adding of phenol on the detected level of tyrosine, phenylalanine, histidine, tryptophan and arginine. Degassing and phenol stabilize tyrosine, phenylalanine and histidine. The arginine level was not influenced by the peroxidation at all. The tryptophan level was highest in untreated samples, but the tryptophan quantitation was generally unreliable.

4. Conclusions

Microwave acid hydrolysis of protein samples is a fast method compared to conventional acid hydrolysis. A hydrolysis time of 10–30 min seems to be optimal as it cleaves most of the peptide linkages and shows no drastic loss of serine or threonine. Methionine was found to be fairly stable during microwave hydrolysis even when samples were degraded without degassing or addition of thioglycolic acid, although a higher level was found for degassed samples with thioglycolic acid added. Cyst(e)ine and tryptophan cannot be quantitatively determined after standard acid hydrolysis. Cyst(e)ine can be determined as cysteic acid after oxidation with performic acid. Phenol can be added to stabilise labile amino acid, but even then tyrosine and tryptophan cannot be determined correctly in oxidised samples. Thus, determination of all amino acids in a protein sample still requires three different hydrolyses: standard hydrolysis, peroxidation and hydrolysis and basic hydrolysis. However, the hydrolysis time can be reduced considerably by using the microwave technique.

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