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Comparative study on microwave and conventional methods for protein hydrolysis in food*

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Summary. A rapid microwave hydrolysis procedure was developed for amino acid determination in food. The hydrolysis was performed with 6M HCl in sealed vessels using a microwave digestion system.

Bovine Serum Albumin was chosen as a model protein to compare its theoric amino acid sequence with the experimental results obtained after hydrolysis by both the traditional oven heating and the microwave methods. Furthermore the selected microwave methods were carried out on different food matrices (cheese and durum wheat) and the obtained data were compared with the traditional method results.

This comparative study shows that the rapid microwave hydrolysis is as accurate and precise as the traditional hydrolysis for determining amino acids in food.

Keywords: Amino acids – Protein hydrolysis – Oven heating – Microwave – Amino acids in food

Introduction

The rate-limiting step of an amino acid analysis, after the recent advances in chromatographic methods, is the preparation of protein hydrolysates. The traditional protocol (Moore et al., 1958) utilizes 6M hydrochloric acid at 110° C for 24 and 72 hours and has been the standard method for the past thirty years. This method is long and laborious and may require extended time to hydrolyse certain peptide bonds which are known to be more stable such as those involving valine, isoleucine and leucine (Woodward et al., 1990). Protein (lysozyme) hydro-

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lysis for amino acid analysis by means of a commercial microwave oven was first reported by Chen et al. (1987). Afterwards Gilman and Woodward (1989), to avoid a.a. contamination, suggested a microwave hydrolysis in vapor phase when sample amount is limited (methionyl human growth hormone).

Although microwave heating is frequently used to prepare samples for atomic absorption or emission analysis it is not yet widely used for the hydrolysis of proteins (in particular of food proteins) and currently there are limited automated techniques for standard protein hydrolysis which are convenient and reproducible.

In this research a microwave irradiation method of protein hydrolysis in liquid phase has been developed on Bovine Serum Albumin. This protein has been chosen as a model system since it contains more difficult to hydrolyse peptide linkages. The method has been also applied to two food matrices, different in composition and technology of production: a cheese (Pecorino romano) and a durum wheat semolina.

Materials and methods

Chemicals

Reagents, ninhydrin and methyl cellosolve were from Carlo Erba (Milan, Italy) analytical grade. Bovine Serum Albumine (BSA) was purchased from Sigma Chemical Co (St. Louis, MO) and amino acid standard from Beckman Instruments Inc. (Palo Alto, CA).

Samples

The samples chosen for hydrolysis were: standard BSA; italian cheese (Pecorino romano); durum wheat semolina. The BSA amino acid composition data, evaluated by different heating procedures, were compared with theoric values based on published sequence data for this protein (Brown, 1974; 1975; Mac Gillivray et al., 1979; Reed et al., 1980). For the other food proteins the microwave data were compared with the classic method results.

Equipment

The microwave system used was a Microwave Digestion System, Model MDS 2000 (CEM Corporation). The unit offers a wide range of hydrolysis conditions and can operate at 0–100 percent full power (630 $\pm$ 50 watts). The MDS 2000 is equipped with an inlet/outlet port to allow the tubing connected to the vessel and the Pressure Controller to pass through the cavity wall without allowing microwave leakage.

The amino-analyzer was a Beckman 118BL system equipped with a computer (Digital PC 380) for automatic integration.

Methods

The sample with a known amount of protein (30–50 mg) was placed inside a Teflon PFA digestion vessel (CEM Corporation) along with 6M HCl (8 ml). The vessels were connected to a purge trap and to a manifold for evacuation, nitrogen purge, pressure monitoring and controlling and a safety release mechanism.

Microwave hydrolysis were conducted following two different programs (Table 1). Full microwave power (about 650 W) was initially applied to the vessels. When pressure reached the desired value (80 or 100 psi) the power was lowered at 80% and the pressure was maintained constant by the Pressure Controller.

Table 1. Microwave hydrolysis programs

Program 1	1st step	2nd step
Power (%)	100	80
Pressure (psi)	100	100
Time at pressure (sec)	10	X

X = 10, 20 and 30 minutes

Program 2	1st step	2nd step
Power (%)	100	80
Pressure (psi)	80	80
Time at pressure (sec)	10	X

X = 5, 10 and 15 minutes

Upon completion of the digestion process the vessel was allowed to cool to room temperature before opening vent valve to release 1 Atm (15 psi) of nitrogen remaining. Afterwards the samples were removed from the vessels and placed in the vacuum evaporator to remove liquid, redissolved in 50 ml of pH 2.2 starting chromatographic buffer and then injected.

All amino acid analyses were performed at 50° C by a 32 cm–0.9 cm i.d. column packed with Beckman W2 resin according to a slight modification of the single-column procedure developed by Devenyi (1968). The mobile phases used for the multi-linear gradient were as follows: 0.2 N sodium citrate pH 3.5, 0.2 N sodium citrate pH 4.12 and 1 N sodium citrate pH 6.4. For automatic integration a calibration run with a standard amino acid mixture freshly prepared, was made to obtain elution time and response factor for each amino acid. All the experiments were carried out in triplicate.

Results and discussion

Bovine Serum Albumin, a protein of known primary structure and of particular importance in food science, has been used as a model system in this research. In Table 2 and Table 3 are reported its residue sequence and the relevant amino acid composition expressed as percent of the total amino acid weight. In particular, this protein is characterized by a large number of bonds involving valine, isoleucine and leucine, three difficult to hydrolyse amino acids. These linkages are underlined in Table 2.

Results for amino acid analysis of BSA as a function of hydrolysis time (10, 20 and 30 minutes), under a constant pressure of 100 psi (Program 1), are summarized in Table 4. The recoveries of valine, leucine and isoleucine, obtained after 10 minutes of microwave heating, remain nearly constant with increasing time at 20 and 30 minutes. On the other hand during these experiments a degradation of heat sensitive amino acids such as serine, threonine and tyrosine has been observed.

Therefore a second set of experiments has been performed using lower pressure values during microwave hydrolysis (80, 70 and 60 psi). The use of a digestion procedure at a pressure of 60 psi and 70 psi for 5 minutes (data not shown) seems not adequate to fully hydrolyse this protein. An incomplete

Table 2. Bovine Serum Albumin (BSA) sequence (Brown, 1974, 1975; MacGillivray et al., 1979; Reed et al., 1980)

Residues	Sequences of BSA residues				
1	DTHKSEIAHR	FKDLGEEHFK	GLVLIAFSQY	LQQCPFDEHV	KLVNELTEFA
51	KTCVADESHA	GCEKSLHTLF	GDELCKVASL	RETYGDMADC	CEKEQPERNE
101	CFLSHKDDSP	DLPKLKPDPN	TLCDEFKADE	KKFWGKYLYE	IARRHPYFYA
151	PELLYANKYN	GVFQECCQAE	DKGACLLPKI	ETMREKVLTS	SARQRLRCAS
201	IQKFGERALK	AWSVARLSQK	FPKAEFVEVT	KLVTDLTKVH	KECCHGDLLE
251	CADDRADLAK	YICBBZBTIS	SKLKECKDPC	LLEKSHCIAE	VEKDAIPEDL
301	PPLTADFAED	KDVCKNYQEA	KDAFLGSFLY	EYSRRHPEYA	VSVLLRLAKE
351	YEATLEECCA	KDDPHACYTS	VFDKLKHLVD	EPQNLIKQNC	DQFEKLGEYG
401	FQNALIVRYT	RKVPQVSTPT	LVEVSRSLGK	VGTRCCTKPE	SERMPCTEDY
451	LSLILNRLCV	LHEKTPVESK	VTKCCTESLV	NRRPCFSALT	PDETYVPKAF
501	DEKLFTFHAD	ICTLPDTEKQ	IKKQTALVEL	LKHKPKATEE	QLKTVMENFV
551	AFVDKCCAAD	DKEACFAVEG	PKLVVSTQTA	LA	

Linkages involving valine, leucine or isoleucine are underlined.

Table 3. Theoric amino acid composition of BSA calculated on the basis of protein residue sequences

Amino acid codes			Bovine Serum Albumin (BSA) standard			
1 letter	3 letters	Name	Number of residues n°	Molecular weight M.W.	Total weight M.W. × n°	Amino acid %
D	Asp	Aspartic acid	41	133.05	5455.05	7.11
N	Asn	Asparagine	13	132.33	1720.29	2.24
T	Thr	Threonine	34	119.17	4051.78	5.28
S	Ser	Serine	28	105.11	2943.08	3.83
E	Glu	Glutamic acid	59	147.18	8683.62	11.31
Q	Gln	Glutamine	20	146.02	2920.40	3.80
P	Pro	Proline	28	115.05	3221.40	4.20
G	Gly	Glycine	16	75.16	1202.56	1.57
A	Ala	Alanine	46	89.03	4095.38	5.33
V	Val	Valine	36	117.23	4220.28	5.50
I	Ile	Isoleucine	14	131.14	1835.96	2.39
L	Leu	Leucine	61	131.21	8003.81	10.43
Y	Tyr	Tyrosine	19	181.25	3443.75	4.49
F	Phe	Phenylalanine	27	165.28	4462.56	5.81
K	Lys	Lysine	59	146.17	8624.03	11.23
H	His	Histidine	17	155.23	2638.91	3.44
R	Arg	Arginine	23	174.18	4006.14	5.22
C	Cys	Cysteine	35	121.20	4242.00	5.53
M	Met	Methionine	4	148.90	595.60	0.78
W	Trp	Tryptophan	2	203.58	407.16	0.53
		TOT	582		76773.76	100.

Table 4. Effect of microwave hydrolysis time on recovery of amino acids from Bovine Serum Albumin (BSA). Constant pressure 100 psi (hydrolysis program 1)

BSA Amino acid	Theoric	Hydrolysis time		
		10 min	20 min	30 min
Asp + Asn	100	110.11 ± 2.57	115.12 ± 2.80	110.41 ± 4.16
Thr	100	77.11 ± 5.49	67.77 ± 1.07	70.69 ± 2.95
Ser	100	86.55 ± 0.55	70.59 ± 1.48	69.99 ± 2.77
Glu + Gln	100	130.00 ± 3.41	132.08 ± 3.27	130.18 ± 2.20
Pro	100	104.50 ± 2.00	109.68 ± 2.86	106.62 ± 0.17
Gly	100	102.39 ± 1.36	97.58 ± 3.63	103.17 ± 2.72
Ala	100	102.43 ± 2.91	102.32 ± 4.24	111.40 ± 3.31
Val	100	109.64 ± 2.06	116.39 ± 4.51	107.45 ± 6.83
Ile	100	93.88 ± 1.48	94.13 ± 2.96	98.41 ± 8.88
Leu	100	110.95 ± 0.48	111.56 ± 5.84	111.81 ± 1.43
Tyr	100	94.16 ± 1.89	90.14 ± 0.16	94.21 ± 0.16
Phe	100	94.63 ± 1.46	96.10 ± 2.56	95.51 ± 0.97
Lys	100	112.35 ± 1.51	116.94 ± 2.64	116.19 ± 0.19
His	100	86.66 ± 0.62	89.99 ± 1.44	90.99 ± 1.23
Arg	100	84.35 ± 0.68	89.55 ± 2.30	82.94 ± 0.41

Table 5. Effect of microwave hydrolysis time on recovery of amino acids from Bovine Serum Albumin (BSA). Constant pressure 80 psi (hydrolysis program 2)

BSA Amino acid	Theoric	Hydrolysis time		
		5 min	10 min	15 min
Asp + Asn	100	102.00 ± 6.96	113.52 ± 6.73	112.36 ± 4.24
Thr	100	88.19 ± 2.54	86.66 ± 1.47	81.54 ± 2.14
Ser	100	99.55 ± 8.12	89.15 ± 5.91	87.95 ± 0.74
Glu + Gln	100	100.60 ± 1.68	110.17 ± 2.15	111.39 ± 6.27
Pro	100	117.94 ± 1.01	121.19 ± 2.48	111.16 ± 1.85
Gly	100	114.08 ± 1.36	112.07 ± 4.08	109.65 ± 4.99
Ala	100	103.81 ± 1.46	107.15 ± 2.91	100.54 ± 6.09
Val	100	86.23 ± 2.45	88.89 ± 5.54	98.07 ± 2.45
Ile	100	92.21 ± 8.88	96.30 ± 0.30	94.03 ± 2.07
Leu	100	100.51 ± 0.14	104.40 ± 0.54	108.29 ± 2.71
Tyr	100	107.30 ± 5.37	101.70 ± 4.74	99.62 ± 5.68
Phe	100	99.36 ± 3.16	93.82 ± 2.92	94.31 ± 1.83
Lys	100	97.39 ± 0.19	106.93 ± 4.41	121.05 ± 4.85
His	100	98.79 ± 3.91	75.71 ± 5.87	76.04 ± 3.08
Arg	100	91.44 ± 0.95	92.43 ± 4.35	93.97 ± 4.63

protein hydrolysis due to insufficient temperature is evident, while good results were obtained from an 80 psi hydrolysis (Program 2). At this pressure, as can be seen in Table 5, an heating time of 5 minutes assures the best recovery of serine, threonine and tyrosine. Microwave hydrolysis of BSA for 5, 10 and 15 minutes

yields nearly constant values for isoleucine; leucine and valine increase with extending hydrolysis time while, as expected, heat sensitive amino acids follow an opposite trend. Data for the other amino acids show good agreement between the theoric and the experimental amount. Moreover the relevant chromatograms (not shown) suggest the presence of half-cystine and methionine that usually require different hydrolysis conditions. The experimental determined content of these amino acids is quite close to the expected theoretical values for the examined protein (75% for cystein and 100% for methionine).

Finally in Fig. 1 the results of a comparison between the chosen microwave protocols and the traditional hydrolysis method are reported.

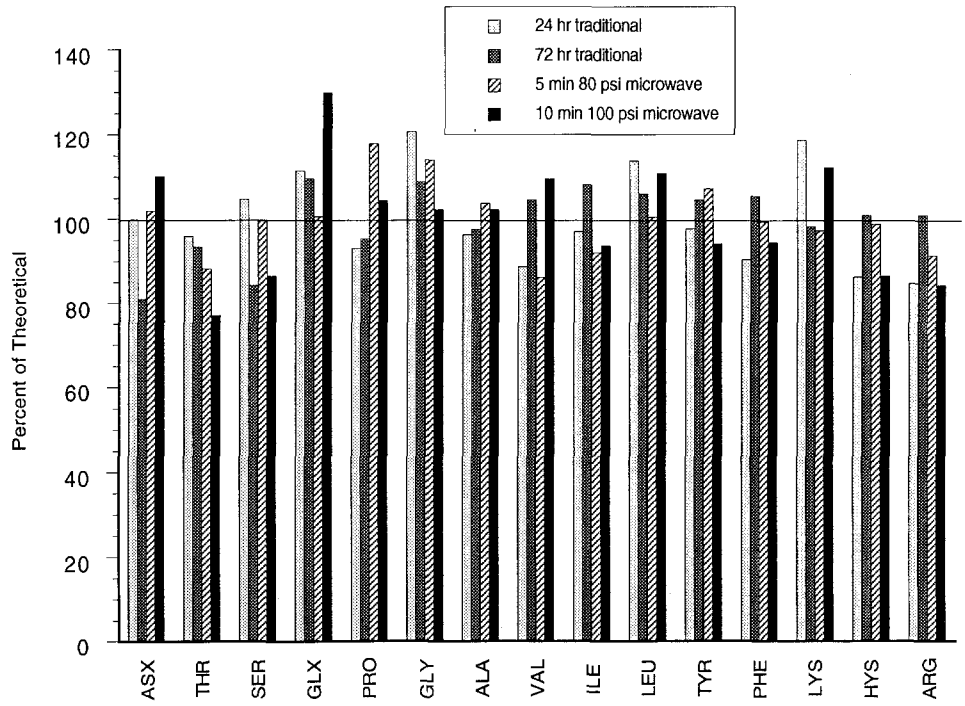

Fig. 1. Results of the comparative study between traditional and microwave hydrolysis of BSA

A mild microwave hydrolysis procedure (80 psi 5 min) gives satisfactory results for most of amino acids, whereas valine, leucine and isoleucine are more completly hydrolysed using the microwave heating at 100 psi for 10 min. Therefore it seems useful to combine both the microwave methods following the same procedure adopted to combine the results of traditional hydrolysis at 24 and 72 hours.

This procedure has been applied to some food samples (semolina and Pecorino romano) hydrolysed by both the traditional method and the proposed new microwave methods. The results of this comparative study given in Table 6 show a perfect correlation between the methods ($r = 0.99$). In addition the standard deviations of the microwave hydrolysis are comparable with the corresponding values of the traditional method.

Table 6. Samples of durum wheat semolina and Pecorino romano (Italian cheese) Comparative study between traditional and microwave hydrolysis

	Pecorino romano (a)		Pecorino romano (b)		Semolina	
Amino acid	Traditional aa/100 g Protein	Microwave	Traditional aa/100 g Protein	Microwave	Traditional aa/100 g Protein	Microwave
Asp + Asn	6.93 ± 0.08	7.29 ± 0.17	7.04 ± 0.17	7.00 ± 0.33	4.30 ± 0.16	4.55 ± 0.19
Thr	3.51 ± 0.12	3.30 ± 0.51	3.50 ± 0.07	3.58 ± 0.32	2.69 ± 0.20	2.73 ± 0.22
Ser	4.52 ± 0.66	4.47 ± 0.48	4.32 ± 0.77	4.38 ± 0.68	4.77 ± 0.38	4.93 ± 0.30
Glu + Gln	20.86 ± 0.07	20.75 ± 0.22	20.77 ± 0.20	20.32 ± 0.16	37.52 ± 0.48	36.00 ± 0.49
Pro	12.05 ± 1.15	11.98 ± 1.01	12.12 ± 0.91	12.24 ± 0.30	12.39 ± 0.87	12.68 ± 0.82
Gly	1.76 ± 0.04	1.71 ± 0.14	1.72 ± 0.05	1.76 ± 0.03	3.15 ± 0.12	3.32 ± 0.08
Ala	3.01 ± 0.09	3.18 ± 0.06	3.17 ± 0.30	2.98 ± 0.10	2.92 ± 0.09	3.15 ± 0.03
Val	6.78 ± 0.27	7.04 ± 0.70	6.94 ± 0.52	6.83 ± 0.06	4.79 ± 0.38	4.55 ± 0.59
Met	2.80 ± 0.03	2.85 ± 0.11	2.93 ± 0.18	2.69 ± 0.44	1.68 ± 0.06	1.76 ± 0.05
Ile	5.29 ± 0.25	4.98 ± 0.09	5.21 ± 0.31	4.95 ± 0.02	4.03 ± 0.15	3.80 ± 0.27
Leu	10.10 ± 0.04	10.23 ± 0.02	10.04 ± 0.13	10.13 ± 0.19	6.94 ± 0.26	7.07 ± 0.19
Tyr	4.97 ± 0.43	5.26 ± 0.27	4.86 ± 0.60	5.23 ± 0.07	2.61 ± 0.20	2.39 ± 0.19
Phe	4.94 ± 0.16	4.82 ± 0.13	4.88 ± 0.15	5.19 ± 0.26	4.26 ± 0.10	4.39 ± 0.09
Lys	7.84 ± 0.04	7.81 ± 0.07	7.86 ± 0.15	7.96 ± 0.10	2.14 ± 0.08	2.35 ± 0.05
His	2.38 ± 0.20	1.99 ± 0.38	2.30 ± 0.21	2.17 ± 0.21	2.46 ± 0.09	2.48 ± 0.08
Arg	2.27 ± 0.20	2.34 ± 0.31	2.37 ± 0.18	2.58 ± 0.30	3.34 ± 0.53	3.85 ± 0.60

In summary, this study confirms that liquid phase microwave hydrolysis of protein is an important and useful tool in food research. It is a great labor saving device, easy to operate it minimizes operator error and reduces the possibility of contamination from sample handling. The vacuum and nitrogen system eliminates any oxygen permeation during hydrolysis and the liquid phase allows the use of protectants (phenol, mercaptoethanol etc). The obtained results are consistent and reproducible. Moreover it is noteworthy that the amino acid chromatograms are generally clean with no ambiguos peaks, which is indicative of the specific cleavage of peptide bonds by microwave irradiation.

Further studies will be carried out to assess if complete amino acid recoveries can be obtained by a single program microwave hydrolysis and to investigate the fate of cysteine and methionine during microwave treatments.

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