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Optimum conditions of autoclaving for hydrolysis of proteins and urinary peptides of prolyl and hydroxyprolyl residues and HPLC analysis

Mallikarjun Badadani, S.V. SureshBabu, K. Taranath Shetty *

Department of Neurochemistry, National Institute of Mental Health and Neurosciences, Hosur Road, Bangalore 560029, India

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Abstract

A method for urinary peptide(s) and protein hydrolysis, involving autoclaving at 15 psi (121 °C) for 60 min, is described. Using three candidate proteins (bovine serum albumin, casein and gelatin) and urine specimens, the effect of autoclaving with respect to the optimum time required for hydrolysis under both acidic (6 N HCl) and alkaline (6 N KOH) conditions was studied. Recoveries of total amino acids from proteins and urine hydrolysate(s) suggest that complete hydrolysis of proteins and urinary peptides could be achieved by autoclaving for 30–60 min instead of 16 h of incubation at 110 °C. Further, stability of some of the individual amino acids was also studied. The observed differential stability of amino acids under acidic and alkaline conditions, as demonstrated in this study by HPLC analysis, makes it imperative to choose the appropriate hydrolytic condition while studying the composition of any given amino acids in urinary peptide(s)/protein hydrolysates. Further, the finding that both Pro and Hyp were stable under alkaline conditions of hydrolysis by autoclaving renders this method suitable for assaying these two amino acids from urine hydrolysates, hence its utility in the study of urinary peptide derived Hyp and Pro in bone/cartilage disorders.

Keywords: Autoclave; Proteins; Urinary peptides; Prolyl residues; Hydrolysis; Bone disorders

1. Introduction

Amino acids composition of urine, including the peptide derived, offers a means of diagnosis of a variety of diseases [1,2]. In particular, the assay of hydroxy proline (Hyp), including the urinary peptide derived, has a diagnostic significance with reference to osteoporosis associated with a variety of diseases such as primary hyperparathyroidism, Paget's disease, post menopausal osteoporosis and other metabolic diseases of the bone as an index of collagen catabolism [3–5]. Analysis of urinary peptide(s) derived amino acids involve prior hydrolysis of the specimens; hence, the method employed to hydrolyze the urine attains significance. The hydrolysis of protein(s) and urinary peptide(s) based on chemical methods, involving overnight incubation in high acidic condition, continues to be the first step to analyze the amino acid(s) composition of unknown specimens [6–10]. However, the inherent problem of degradation of acid labile amino acids and the conversion of Asn and Gln to Asp and Glu is often a matter of concern while quantifying the amino acids from acid hydrolysate(s). The inclusion of additives during hydrolysis, such as dodecanethiol [11] and thioglycolic acid [12], to prevent degradation of acid labile amino acids and bis (1,1-trifluroacetoxy) iodobenzene[13] to convert Asn and Gln to diaminopropionic and diaminobutyric acid, respectively [14], to avoid over estimation of respective dicarboxylic amino acids has been recommended. Further, though the attention is paid to minimize the duration required for hydrolysis of proteins, the recommended method(s) often involving a mixture of strong acids with heat [15] and microwave irradiation [16] continues to suffer from the inherent degradation of certain amino acids.

Present study describes a method based on autoclaving for relatively shorter period as an alternate step for extended overnight incubation at 110 °C for chemical hydrolysis of proteins as well as urine specimens. Further the method also evaluates the relative stability of some of the candidate amino

Abbreviations: BSA, bovine serum albumin; DNFB, 2,4-dinitro fluoro benzene; DNP, dinitrophenyl; Pro, proline; Hyp, hydroxyproline; OPA, *O*-pthalaldehyde

* Corresponding author. Tel.: +91 80 26995158; fax: +91 80 26564830/26562121.
E-mail addresses: tara@nimhans.kar.nic.in, taranathshetty@gmail.com (K.T. Shetty).

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acids, including proline (Pro) and hydroxyproline (Hyp), under defined conditions of autoclaving recommended to achieve complete hydrolysis of urinary peptides with an emphasis on the analysis of urinary peptide derived Pro and Hyp.

2. Experimental

2.1. Chemicals

Bovine serum albumin (BSA; Sigma Cat No.: A-7906), casein (Sigma Cat No.: C-5890), gelatin (Sigma Cat No.: G-2500), dinitrofluorobenzene (DNFB), *O*-pthalaldehyde (OPA), β-mercaptoethanol and (β-ME) were purchased from Sigma Chem. Co., USA. Other chemicals and solvents, including acid (HCl) and alkali (KOH), used were of analytical grade. Methanol (HPLC grade) was purchased from E-Merck.

2.2. Reagents and solutions

Stock DNFB reagent was prepared by diluting 625 μl of DNFB to 25 ml with acetone and stored in an amber colored bottle at 4 °C. Stock solutions (5 mg/ml) of candidate proteins (BSA, casein and gelatin) sodium borate solution (155 mM/l) were prepared in distilled water. DNFB working solution was prepared fresh, just before use, by diluting 1 ml of stock DNFB reagent to make up to 10 ml with sodium borate solution. Sodium borate buffer (50 mM, pH 6.8) prepared in double distilled water was filtered through a 0.45 μm pore size filter (Sartorius, Germany) prior to use in HPLC analysis.

2.3. Proteins and urine hydrolysis

Urine specimens and individual solutions (1 ml each) of BSA, casein, gelatin (5 mg/ml) and urine specimens were mixed with an equal volume of 12 N HCl or KOH solutions in teflon coated glass tube (Chromsystems, Germany) and capped tightly. Hydrolysis was carried out by autoclaving at 15 psi (121 °C) for different time (10–90 min) intervals at 15 psi (121 °C) or by incubating at 110 °C for 16 h as described in earlier works [9,17]. Hydrolysates, after adjusting the pH to ∼7.0 + 0.1, were made up to a final volume of 10 ml each.

2.4. Dinitrophenyl derivatisation and assay of total amino acids

Urine and protein hydrolysates were mixed with methanol (1:4, v/v) and allowed to stand for 10 min in ice bucket and centrifuged (5000 × *g*/10 min). The supernatant was used for the assay of total amino acids by dinitrophenyl (DNP) derivatisation method [18,19]. In brief, 100 μl of supernatant was made up to 250 μl with 80% methanol. An equal volume of sodium borate solution (155 mM/l) was added followed by 500 μl of working DNFB reagent, and the tubes were transferred to water bath maintained at 45 °C. After 30 min of incubation, the tubes were allowed to attain ambient temperature, and the reaction mixture was acidified by adding 1 ml of 2% (v/v) HCl. Absorbance was measured at λ_{max} 380 nm and the amino acids content was calculated by referring to a standard curve developed by using a mixture of an equimolar concentration of Glu and Gly.

2.5. OPA derivatisation and HPLC analysis of amino acids

Amino acids profile, except for Pro and Hyp, of hydrolysate(s) were analyzed by reverse phase HPLC analysis based on pre-column derivatisation with *O*-pthalaldehyde as described earlier [20]. In brief, OPA derivatisation was achieved by mixing methanol extract of urine/protein hydrolysates (100 μl ≡ ∼15 ± 100 nmol) with 50 μl sodium tetra borate buffer (500 mM/l, pH 10.5), 5 μl of β-mercapto ethanol and 25 μl of freshly prepared OPA solution (1 mg/ml) in water and allowed to stand in dark for 3 min. Derivatized solution was made up to 1 ml with start eluent [mixture of sodium acetate buffer (50 mM/l, pH 6.8) and methanol (4:1, v/v)].

The individual amino acids were resolved and analyzed by injecting to HPLC system equipped with 20 μl injection loop and C18 resolution (Supelcosil 25 cm × 4.6 mm, 5 μm) and guard (Supelcosil 1 cm × 4.6 mm, 5 μm) columns housed in an incubator set at 40 °C constant temperature. Reverse phase binary gradient system (methanol: sodium acetate buffer (50 mM/l, pH 6.8), with flow rate of 1.5 ml/min. Fluorescence detector set at 330 nm and 450 nm for excitation and emission, respectively, was used to monitor the resolution of OPA derivatives.

2.6. HPLC analysis of DNP derivatives of proline and hydroxyproline

An aliquot of DNP derivatives of hydrolysates, as described for the assay of total amino acids, were also used for HPLC analysis of Pro and Hyp by reverse phase separation, experimental conditions being same as described for OPA derivatives except for monitoring spectrophotmetrically at 380 nm.

2.7. Statistical analysis

Data were analyzed using SPSS program version 10.0 of Windows 2000. Statistical analysis with respect to mean ± SD was determined with the Boeneferroni '*t*'-test.

3. Results

3.1. Hydrolysis of proteins and urine specimens

Using three proteins, viz., bovine serum albumin, casein and gelatin; the optimum time required to bring about complete hydrolysis was established (Fig. 1, Panel A). Similar findings were also observed with urine specimens subjected to hydrolysis (Fig. 1 Panel B). Thus, depending on the proteins, a total duration of 30–60 min of autoclaving resulted in maximum recovery of total amino acids (Fig. 1) from proteins; the recovery of total amino acids were comparable with that of hydrolysis by incubation for 16 h at 110 °C (Table 1A). Similar studies on urine hydrolysis by autoclaving also demonstrated complete hydrolysis of urinary peptides by 60 min of autoclaving (Table 1B). When compared, the recoveries of total amino acids of a given

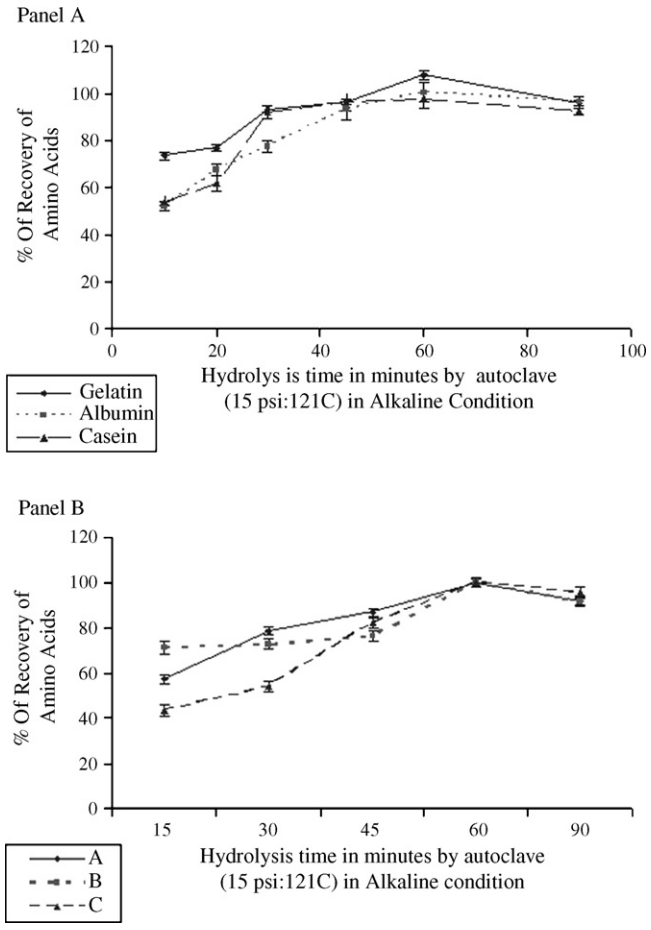

Fig. 1. Optimum time requirement for complete hydrolysis, based on the recovery of total amino acids content of protein hydrolysate(s) Panel A and urine specimens Panel B, by autoclaving. Panel A: The observed percent recovery is related to amino acids content (considered as 100%) of respective protein hydrolysate(s) at 110 °C incubation for 16 h. Panel B: Three separate urine specimens were autoclaved same as for proteins, and the maximum amino acids recovered from each of the specimen is considered as 100%. The values represent the mean ± SD of three independent experiments. Note the differential optimum time ranging from 30 to 60 min, for complete hydrolysis as evidenced from the maximum recovery followed by the decrease in the recovery afterwards.

Table 1A
Recovery of the total amino acids (values expressed as mean ± SD) by autoclaving at 15 psi (121 °C) in acidic and alkaline conditions

Proteins	Total amino acids (μmol/mg protein)	
	Acidic condition	Alkaline condition
Bovine serum albumin	7.62 ± 0.0.35 (45′) 8.45 ± 0.14 (60′)	7.48 ± .0.21(45′) 7.78 ± 0.21 (60′)
Casein	7.62 ± 0.35 (45′) 7.68 ± 0.34 (60′)	7.33 ± 0.64 (45′) 6.48 ± 0.64 (60′)
Gelatin	10.50 ± 0. 35 (45′) 11.73 ± 0.40 (60′)	10.54 ± 0.28 (45′) 11.40 ± 0.11 (60′)

Note the difference with respect to the optimum time (in minutes indicated within the parenthesis) required for better requirement of amino acids from different proteins.

protein were found to vary under different experimental conditions. Thus, nearly 100% recovery of total amino acids, as compared to hydrolysis by incubation at 110 °C, from all the three candidate proteins and urine specimens could be achieved by autoclaving for 30–60 min under both acidic and alkaline conditions. Any further extending of autoclaving invariably resulted in the loss of recovery of amino acids (Fig. 1). Thus, nearly 96% of total amino acids, as compared to hydrolysis by incubation at 110 °C, could be recovered from gelatin by autoclaving for 30 min in both acidic and alkaline conditions, whereas casein and BSA were found to require 45 min of autoclaving. In general, hydrolysis by autoclaving in acidic and alkaline conditions, apart from shorter duration, also suggested differential optimum time for recovery of total amino acids for the candidate proteins viz., BSA, casein and gelatin (Table 1A). On the other hand, findings on total amino acids from urine hydrolysates suggested better recovery under alkaline conditions than in acidic conditions (Table 1B). Further, studies on candidate amino acids suggested a relatively better stability for Gly, Tyr, Lys, Pro and Hyp in alkaline as compared with acidic conditions, whereas Glu and His were relatively more stable in acidic conditions (Table 4).

HPLC profile of amino acids from protein hydrolysates suggested differential stability of different amino acids under different conditions of hydrolysis (Tables 3A–3C). Most of the

Table 1B
Recovery of total amino acids from hydrolysate(s) of three different urine specimens (A, B and C) by autoclaving under acidic and alkaline conditions

Amino acids content (mmol/mol creatinine)				
Urine specimens	Free amino acids content	Hydrolysis condition	Total amino acids content (free + peptide derived)	
			Acid hydrolysate	Alkaline hydrolysate
A	0.679 ± 0.10	Autoclave 1 h at 15 psi (121 °C) Incubation (16 h) at 110 °C	2.66 ± 0.15 2.04 ± 0.12	5.26 ± 0.12 (198%) 5.49 ± 0.15 (269%)
B	1.225 ± 0.11	Autoclave 1 h at 15 psi (121 °C) Incubation (16 h) at 110 °C	3.14 ± 0.17 3.40 ± 0.14	5.93 ± 0.18 (189%) 6.00 ± 0.20 (176%)
C	0.966 ± 0.12	Autoclave 1 h at 15 psi (121 °C) Incubation (16 h) at 110 C	2.35 ± 0.18 2.99 ± 0.20	4.49 ± 0.14 (191%) 5.00 ± 0.16 (168%)

The values represent the mean ± SD of three independent experiments. Numbers in parenthesis represent the percent of recovery as compared to respective acid hydrolysate(s). *Note*: Better recovery (168–269%) of total amino acids in alkaline hydrolysates as compared with acidic hydrolysis.

Table 2
Stability of candidate amino acids under defined conditions of hydrolysis by autoclaving (15 psi/1 h/121 °C) in acidic (6 N HCl) and alkaline (6 N KOH) conditions

S. no.	Amino acid	Absorbance (%recovery)		
		Control* (without hydrolysis)	Hydrolysis by autoclaving (15 psi/1 h/121 °C)	
			Acidic condition	Alkaline condition
1	Gly	0.070 (100)	0.04 1(58.50)	0.053 (76.50)
2	Glu	0.170 (100)	0.100 (58.82)*	0.082 (48.30)
3	Tyr	0.170 (100)	0.140 (82.35)	0.137 (81.00)
4	Lys	0.110 (100)	0.060 (54.54)	0.081 (73.60)
5	His	0.150 (100)	0.097 (64.66)*	0.060 (40.00)
6	Pro	0.190 (100)	0.134 (70.6)	0.200 (105.5)
7	Hyp	0.090 (100)	0.080 (88.88)	0.090 (100)

Note the stability of Pro and Hyp in alkaline as compared with acid hydrolysate. Also, note the differential stability of other amino acids in acidic and alkaline conditions.
* Respective amino acids of equimolar concentrations without undergoing autoclave constitute the controls.

amino acids were unstable either in acidic or alkaline conditions of hydrolysis. In general, basic amino acids such as Asn, Gln and His tend to get destroyed to a great extent during hydrolysis under both acidic and alkaline conditions (Table 4). On the other hand, both Pro and Hyp were found to be stable in alkaline condition (Table 2). Thus, triplicate analysis of a random urine specimen (Table 4) demonstrated a better recovery of Pro and Hyp in alkaline hydrolysates by autoclaving as compared with acidic hydrolysates. Total content of both Hyp (27.5 ± 0.32 μmol/mmol creatinine) and Pro (66.0 ± 0.7 μmol/mmol creatinine) of urinary alkaline hydrolysate was found to be significantly high as compared with the corresponding value of 10.60 ± 0.13 μmol/mmol creatinine and 30.4 ± 0.35 μmol/mmol creatinine from acidic hydrolysate. Similar findings on the stability of Pro and Hyp were also observed from studies on hydrolysate(s) of candidate proteins (Tables 3A–3C). The inter-assay studies, involving hydrolysis of same specimens, carried out on different days spanning over 3 months confirmed the good reproducibility (Tables 3A–3C). Further, the amino acids content of protein and urine hydrolysates, after adjusting the pH to near neutral (7.0 ± 0.1) and stored at −20 °C, were found to be stable at least for 6 months.

4. Discussion

Instability of the amino acids under extreme conditions of pH and temperature and the lengthy duration of hydrolysis are the main constrains in the analysis of amino acids composition of proteins and peptides. Several investigators have employed different strategies to overcome these limitations of chemical hydrolysis [11–16], with little success. In the absence of an ideal method for protein hydrolysis to guarantee the stability of all the amino acids, it is wide open for the investigators to establish hydrolytic conditions suitable for the stability of given amino acids under study. Though the available literature on hydrolysis and the analysis of amino acid composition of protein [16,19] are suggestive of differential recovery for the amino acids in general, very little is known about the stability and recovery of Pro and Hyp under extreme conditions of pH and temperature.

The observed difference in recovery of total amino acids in acidic and alkaline conditions of autoclaving, as seen in the present study (Tables 1A and 3B), appears to be due to the combined effect of differential susceptibility of individual amino acid(s). As one would expect the amino acids profiles, based on HPLC analysis, were found to differ in acidic and alkaline hydrolysate(s) of candidate proteins (Table 3) and urine specimen (Table 4). This observation is substantiated from findings on the differential stability of certain candidate amino acids under alkaline and acidic conditions of autoclaving (Table 2). Thus, as could be seen from the amino acids composition of protein and urine hydrolysate(s), amino acids such as Asn and Gln were found to be highly degradable under acidic conditions (Tables 3 and 4), an observation in conformity with earlier reports [14,15]. The observed poor recoveries of both serine and threonine from alkaline hydrolysate(s) is in agreement with earlier report [21] and were found to be relatively stable in acidic compared with alkaline hydrolysis (Table 3). In addition to ser and Thr, both Glu and His are also unstable in alkaline conditions, where as other amino acids were found to be relatively stable in alkaline as compared to acidic hydrolysis. The relatively better stability of Lys observed in alkaline condition of autoclaving (Table 3) is substantiated by a comparable increase in recovery of this amino acid in alkaline hydrolysates of proteins (Table 3) and urine specimen (Table 4) as compared with acidic hydrolysates. Thus, the inherent instability of both Asn and Gln under extreme conditions of pH and temperature, giving rise to respective dicarboxylic acids, is likely to contribute to the overestimation of Asp and Glu. Hence, it is likely that the values of Asp and Glu based on hydrolysis under extreme conditions of pH and temperature, as depicted in Table(s) 3 and 4, may represent the summation effect of contribution from respective amides and partial degradation of individual dicarboxylic amino acids under the given conditions of hydrolysis.

As shown in Fig. 2, OPA derivatisation followed by reverse phase HPLC analysis of gelatin and urine hydrolysate(s) demonstrated the presence of most of the amino acids except for Pro and Hyp. On the other hand, amino acids profiles of the same hydrolysates based on DNP derivatisation followed by HPLC analysis (Fig. 3), is conspicuous with the presence of both Pro

Table 3A
Amino acids profile of bovine serum albumin hydrolysate(s) by autoclaving (15 psi/1 h) compared with that of incubation at 110 °C for 16 h at acidic and alkaline conditions

Amino acids	mmol of amino acid/G protein (calculated value based on HPLC analysis of protein hydrolysate: mean ± SD)															
	Intra assay within a day on single plate								Inter assay on different days of total 30 days							
	Alkali hydrolysis				Acid hydrolysis				Alkali hydrolysis				Acid hydrolysis			
	1 h autoclave at 15 psi (121 °C)		16 h incubation at 110 °C		1 h autoclave at 15 psi (121 °C)		16 h incubation at 110 °C		1 h autoclave at 15 psi (121 °C)		16 h incubation at 110 °C		1 h autoclave at 15 psi (121 °C)		16 h incubation at 110 °C	
		CV%		CV%		CV%		CV%		CV%		CV%		CV%		CV%
asp	0.691 ± 0.007	1.013	0.656 ± 0.007	1.067	0.750 ± 0.008	1.066	0.765 ± 0.008	1.045	0.646 ± 0.007	1.083	0.626 ± 0.007	1.118	0.720 ± 0.008	1.111	0.708 ± 0.008	1.129
asn	ND	–	ND	–	ND	–	ND	–	ND	–	ND	–	ND	–	ND	–
glu	0.831 ± 0.009	1.083	0.837 ± 0.009	1.075	0.911 ± 0.010	1.106	0.904 ± 0.010	1.106	0.813 ± 0.009	1.107	0.837 ± 0.009	1.075	0.911 ± 0.010	1.097	0.904 ± 0.010	1.106
gln	ND	–	ND	–	ND	–	ND	–	ND	–	ND	–	ND	–	ND	–
his	0.031 ± 0.001	3.22	ND	–	0.070 ± 0.001	1.428	0.100 ± 0.001	1.000	0.029 ± 0.001	3.448	ND	–	0.061 ± 0.001	1.639	0.092 ± 0.001	1.086
ser	0.283 ± 0.003	1.06	0.301 ± 0.004	1.328	0.682 ± 0.007	1.026	0.631 ± 0.007	1.105	0.268 ± 0.003	1.119	0.316 ± 0.004	1.265	0.698 ± 0.007	1.002	0.617 ± 0.007	1.134
thr	0.023 ± 0.001	4.347	0.020 ± 0.001	5.000	0.455 ± 0.005	1.098	0.455 ± 0.005	1.098	0.023 ± 0.001	4.347	0.020 ± 0.001	5.000	0.470 ± 0.005	1.063	0.476 ± 0.005	1.050
gly	0.447 ± 0.005	1.118	0.494 ± 0.005	1.010	0.786 ± 0.008	1.078	0.711 ± 0.008	1.125	0.470 ± 0.005	1.063	0.480 ± 0.005	1.041	0.756 ± 0.008	1.058	0.679 ± 0.007	1.030
tyr	0.155 ± 0.002	1.290	0.156 ± 0.002	1.282	0.167 ± 0.002	1.197	0.156 ± 0.002	1.282	0.150 ± 0.002	1.333	0.152 ± 0.002	1.315	0.162 ± 0.002	1.234	0.147 ± 0.002	1.360
phe	0.323 ± 0.004	1.238	0.298 ± 0.003	1.006	0.337 ± 0.004	1.186	0.313 ± 0.004	1.277	0.319 ± 0.004	1.253	0.289 ± 0.003	1.038	0.325 ± 0.004	1.215	0.301 ± 0.004	1.328
ala	0.501 ± 0.006	1.197	0.470 ± 0.005	1.063	0.408 ± 0.005	1.225	0.394 ± 0.004	1.015	0.526 ± 0.006	1.140	0.459 ± 0.005	1.089	0.389 ± 0.004	1.028	0.385 ± 0.004	1.038
lys	0.694 ± 0.007	1.008	0.768 ± 0.008	1.04	0.971 ± 0.010	1.029	0.858 ± 0.009	1.052	0.674 ± 0.007	1.038	0.735 ± 0.008	1.088	0.952 ± 0.010	1.05	0.838 ± 0.009	1.07
arg	0.022 ± 0.001	4.545	0.022 ± 0.001	4.545	0.468 ± 0.005	1.068	0.470 ± 0.005	1.063	0.021 ± 0.001	4.761	0.021 ± 0.001	4.761	0.444 ± 0.005	1.126	0.450 ± 0.005	1.111
met	0.050 ± 0.001	2.000	0.038 ± 0.001	2.631	0.079 ± 0.001	1.265	0.019 ± 0.001	5.263	0.047 ± 0.001	2.127	0.034 ± 0.001	2.964	0.079 ± 0.001	1.265	0.019 ± 0.001	5.263
ile	0.114 ± 0.002	1.754	0.125 ± 0.002	1.60	0.182 ± 0.002	1.098	0.183 ± 0.002	1.092	0.117 ± 0.002	1.709	0.126 ± 0.002	1.587	0.177 ± 0.002	1.129	0.174 ± 0.002	1.149
leu	0.622 ± 0.007	1.125	0.671 ± 0.007	1.043	0.705 ± 0.008	1.134	0.732 ± 0.008	1.092	0.604 ± 0.007	1.158	0.649 ± 0.007	1.078	0.705 ± 0.008	1.134	0.708 ± 0.008	1.129
val	0.240 ± 0.003	1.25	0.279 ± 0.003	1.075	0.705 ± 0.008	1.134	0.732 ± 0.008	1.092	0.229 ± 0.003	1.310	0.259 ± 0.003	1.158	0.392 ± 0.004	1.020	0.398 ± 0.004	1.005
hyp*	ND	–	ND	–	ND	–	ND	–	ND	–	ND	–	ND	–	ND	–
pro*	0.443 ± 0.005	1.128	0.356 ± 0.004	1.123	0.326 ± 0.004	1.226	0.183 ± 0.002	1.092	0.425 ± 0.005	1.176	0.340 ± 0.004	1.176	0.310 ± 0.004	1.290	0.176 ± 0.002	1.136

Calculated amino acids profile of protein is based on HPLC analysis of protein hydrolysates by pre-column OPA or DNP derivatisation (*Pro and Hyp only). Values expressed as mean ± SD of nine individual experiments. ND: Not detectable.

Table 3B
Amino acids profile of α-casein hydrolysate(s) by autoclaving (15 psi/1 h) compared with that of incubation at 110 °C for 16 h at acidic and alkaline conditions

Amino acids	mmol of amino acid/G protein (calculated value based on HPLC analysis of protein hydrolysate: mean ± SD)															
	Intra assay within a day on single plate								Inter assay on different days of total 30 days							
	Alkali hydrolysis				Acid hydrolysis				Alkali hydrolysis				Acid hydrolysis			
	1 h autoclave at 15 psi (121 °C)		16 h incubation at 110 °C		1 h autoclave at 15 psi (121 °C)		16 h incubation at 110 °C		1 h autoclave at 15 psi (121 °C)		16 h incubation at 110 °C		1 h autoclave at 15 psi (121 °C)		16 h incubation at 110 °C	
		CV%		CV%		CV%		CV%		CV%		CV%		CV%		CV%
asp	0.340 ± 0.004	1.176	0.444 ± 0.005	1.126	0.455 ± 0.005	1.098	0.400 ± 0.005	1.25	0.337 ± 0.004	1.186	0.437 ± 0.005	1.101	0.416 ± 0.005	1.201	0.361 ± 0.004	1.108
asn	ND	–	ND	–	ND	–	ND	—	ND	–	ND	–	ND	–	ND	–
glu	0.785 ± 0.008	1.019	0.996 ± 0.010	1.004	0.892 ± 0.009	1.008	0.822 ± 0.009	1.094	0.770 ± 0.008	1.038	0.929 ± 0.010	1.076	0.870 ± 0.009	1.034	0.822 ± 0.009	1.094
gln	ND	–	ND	–	ND	–	ND	–	ND	–	ND	–	ND	–	ND	–
his	ND	–	ND	–	0.111 ± 0.002	1.80	0.096 ± 0.001	1.041	ND	–	ND	–	0.114 ± 0.002	1.754	0.100 ± 0.002	2.000
ser	0.144 ± 0.002	1.380	0.333 ± 0.004	1.201	0.588 ± 0.006	1.020	0.425 ± 0.005	1.176	0.140 ± 0.002	1.428	0.327 ± 0.004	1.223	0.548 ± 0.006	1.094	0.400 ± 0.005	1.25
thr	ND	–	0.055 ± 0.001	1.818	0.272 ± 0.003	1.102	0.222 ± 0.003	1.351	ND	–	0.052 ± 0.001	1.923	0.267 ± 0.003	1.123	0.216 ± 0.003	1.388
gly	0.274 ± 0.003	1.094	0.201 ± 0.003	1.492	0.374 ± 0.004	1.069	0.448 ± 0.005	1.116	0.271 ± 0.003	1.107	0.192 ± 0.002	1.041	0.351 ± 0.004	1.139	0.429 ± 0.005	1.165
tyr	0.125 ± 0.002	1.6	0.157 ± 0.002	1.273	0.079 ± 0.001	1.265	0.120 ± 0.002	1.666	0.120 ± 0.002	1.66	0.152 ± 0.002	1.315	0.076 ± 0.001	1.315	0.111 ± 0.002	1.80
phe	0.168 ± 0.002	1.190	0.216 ± 0.003	1.388	0.203 ± 0.003	1.298	0.170 ± 0.002	1.176	0.159 ± 0.002	1.257	0.198 ± 0.002	1.010	0.194 ± 0.002	1.030	0.157 ± 0.002	1.273
ala	0.222 ± 0.003	1.351	0.225 ± 0.003	1.335	0.185 ± 0.002	1.081	0.153 ± 0.002	1.324	0.212 ± 0.003	1.415	0.220 ± 0.003	1.357	0.179 ± 0.002	1.117	0.144 ± 0.002	1.388
lys	0.437 ± 0.005	1.144	0.351 ± 0.004	1.139	0.474 ± 0.005	1.054	0.377 ± 0.004	1.061	0.475 ± 0.054	1.13	0.342 ± 0.004	1.169	0.130 ± 0.002	1.53	0.260 ± 0.003	1.153
arg	ND	–	ND	–	0.181 ± 0.002	1.104	0.166 ± 0.002	1.204	ND	–	ND	–	0.175 ± 0.002	1.142	0.161 ± 0.002	1.242
met	0.118 ± 0.002	1.694	0.146 ± 0.002	1.369	0.114 ± 0.002	1.754	0.094 ± 0.001	1.063	0.114 ± 0.002	1.754	0.129 ± 0.002	1.55	0.103 ± 0.002	1.456	0.083 ± 0.001	1.204
ile	0.111 ± 0.002	1.80	0.151 ± 0.002	1.324	0.214 ± 0.003	1.401	0.148 ± 0.002	1.351	0.105 ± 0.002	1.904	0.146 ± 0.002	1.369	0.222 ± 0.003	1.351	0.142 ± 0.002	1.408
leu	0.381 ± 0.004	1.049	0.488 ± 0.005	1.024	0.470 ± 0.005	1.063	0.400 ± 0.005	1.25	0.385 ± 0.004	1.038	0.477 ± 0.005	1.048	0.459 ± 0.005	1.089	0.038 ± 0.004	1.044
val	0.114 ± 0.002	1.754	0.156 ± 0.002	1.282	0.296 ± 0.003	1.013	0.216 ± 0.003	1.388	0.112 ± 0.002	1.785	0.153 ± 0.002	1.307	0.288 ± 0.003	1.041	0.205 ± 0.003	1.463
hyp*	ND	–	ND	–	ND	–	ND	–	ND	–	ND	–	ND	–	ND	–
pro*	0.462 ± 0.005	1.082	0.437 ± 0.005	1.144	0.448 ± 0.005	1.116	0.129 ± 0.002	1.550	0.466 ± 0.005	1.072	0.425 ± 0.005	1.176	0.437 ± 0.005	1.144	0.120 ± 0.002	1.66

Calculated amino acids profiles of casein hydrolysate are based on HPLC analysis of protein hydrolysates by pre-column OPA or DNP derivatisation (*Pro and Hyp only). Values expressed as mean ± SD of nine individual experiments. ND: Not detectable.

Table 3C
Amino acids profile of gelatin hydrolysate(s) by autoclaving (15 psi/1 h) compared with that of incubation at 110 °C for 16 h at acidic and alkaline conditions

Amino acids	mmol of amino acid/G protein (calculated value based on HPLC analysis of protein hydrolysate: mean ± SD)															
	Intra assay within a day on single plate								Inter assay on different days of total 30 days							
	Alkali hydrolysis				Acid hydrolysis				Alkali hydrolysis				Acid hydrolysis			
	1 h autoclave at 15 psi (121 °C)		16 h incubation at 110 °C		1 h autoclave at 15 psi (121 °C)		16 h incubation at 110 °C		1 h autoclave at 15 psi (121 °C)		16 h incubation at 110 °C		1 h autoclave at 15 psi (121 °C)		16 h incubationat 110 °C	
		CV%		CV%		CV%		CV%		CV%		CV%		CV%		CV%
asp	0.234 ± 0.003	1.282	0.253 ± 0.003	1.185	0.327 ± 0.004	1.22	0.295 ± 0.003	1.016	0.227 ± 0.003	1.32	0.231 ± 0.003	1.29	0.320 ± 0.004	1.25	0.309 ± 0.004	1.294
asn	ND	–	ND	–	ND	–	ND	–	ND	–	ND	–	ND	–	ND	–
glu	0.342 ± 0.004	1.169	0.386 ± 0.004	1.036	0.428 ± 0.005	1.168	0.423 ± 0.005	1.182	0.328 ± 0.004	1.219	0.372 ± 0.004	1.075	0.395 ± 0.004	1.012	0.387 ± 0.004	1.033
gln	ND	–	ND	–	ND	–	ND	–	ND	–	ND	–	ND	–	ND	–
his	0.082 ± 0.001	1.21	0.034 ± 0.001	2.94	0.050 ± 0.001	2	0.074 ± 0.001	1.351	0.080 ± 0.001	1.25	0.036 ± 0.001	2.77	0.048 ± 0.001	2.08	0.072 ± 0.001	1.388
ser	0.318 ± 0.004	1.257	0.330 ± 0.004	1.21	0.050 ± 0.001	2.00	0.074 ± 0.001	1.351	0.303 ± 0.004	1.320	0.305 ± 0.004	1.311	0.393 ± 0.004	1.017	0.398 ± 0.005	1.256
thr	0.057 ± 0.001	1.754	0.064 ± 0.001	1.562	0.139 ± 0.002	1.438	0.136 ± 0.002	1.470	0.054 ± 0.001	1.85	0.060 ± 0.001	1.666	0.133 ± 0.002	1.503	0.128 ± 0.002	1.562
gly	1.216 ± 0.020	1.644	1.878 ± 0.020	1.064	1.835 ± 0.020	1.089	1.842 ± 0.020	1.085	1.223 ± 0.020	1.63	1.857 ± 0.020	1.077	1.814 ± 0.020	1.102	1.828 ± 0.020	1.094
tyr	0.019 ± 0.001	5.263	0.019 ± 0.001	5.263	0.022 ± 0.001	4.545	0.022 ± 0.001	4.545	0.018 ± 0.001	5.555	0.018 ± 0.001	5.555	0.021 ± 0.001	4.761	0.021 ± 0.001	4.761
phe	0.111 ± 0.002	1.80	0.109 ± 0.002	1.834	0.118 ± 0.002	1.694	0.114 ± 0.002	1.754	0.102 ± 0.002	1.96	0.095 ± 0.001	1.05	0.103 ± 0.002	1.941	0.114 ± 0.002	1.75
ala	0.457 ± 0.005	1.09	0.424 ± 0.005	1.179	0.379 ± 0.004	1.055	0.462 ± 0.005	1.082	0.442 ± 0.005	1.131	0.403 ± 0.005	1.24	0.363 ± 0.004	1.10	0.439 ± 0.005	1.138
lys	0.266 ± 0.003	1.127	0.282 ± 0.003	1.063	0.309 ± 0.004	1.294	0.298 ± 0.003	1.006	0.253 ± 0.003	1.185	0.282 ± 0.003	1.063	0.295 ± 0.003	1.016	0.285 ± 0.003	1.052
arg	0.040 ± 0.001	2.5	0.027 ± 0.001	3.701	0.432 ± 0.005	1.157	0.430 ± 0.005	1.162	0.035 ± 0.001	2.85	0.024 ± 0.001	4.16	0.418 ± 0.005	1.196	0.408 ± 0.005	1.225
met	0.044 ± 0.001	2.272	0.040	2.5	0.016	6.25	0.027	3.703	0.043 ± 0.001	2.325	0.040 ± 0.001	2.50	0.016	6.25	0.027 ± 0.001	3.70
ile	0.064 ± 0.001	1.56	0.146 ± 0.002	1.369	0.085 ± 0.001	1.176	0.075 ± 0.001	1.333	0.070 ± 0.001	1.428	0.138 ± 0.002	1.449	0.075 ± 0.001	1.333	0.067 ± 0.001	1.492
leu	0.272 ± 0.003	1.102	0.223 ± 0.003	1.345	0.208 ± 0.003	1.442	0.269 ± 0.003	1.115	0.262 ± 0.003	1.145	0.217 ± 0.003	1.382	0.189 ± 0.002	1.058	0.254 ± 0.003	1.181
val	0.123 ± 0.002	1.626	0.136 ± 0.002	1.47	0.177 ± 0.002	1.129	0.161 ± 0.002	1.242	0.117 ± 0.002	1.709	0.138 ± 0.002	1.449	0.154 ± 0.002	1.29	0.147 ± 0.002	1.36
hyp*	0.542 ± 0.006	1.107	0.303 ± 0.004	1.320	0.475 ± 0.005	1.052	0.331 ± 0.004	1.208	0.527 ± 0.006	1.13	0.288 ± 0.003	1.04	0.453 ± 0.005	1.103	0.317 ± 0.004	1.261
pro*	0.697 ± 0.007	1.004	0.637 ± 0.007	1.098	0.633 ± 0.007	1.105	0.651 ± 0.007	1.075	0.680 ± 0.007	1.03	0.614 ± 0.007	1.14	0.619 ± 0.007	1.130	0.637 ± 0.007	1.098

Calculated amino acids profile gelatin hydrolysate is based on HPLC analysis of protein hydrolysates by pre-column OPA or DNP derivatisation (*Pro and Hyp only). Values expressed as mean ± SD of nine individual experiments. ND: Not detectable.

Table 4
Typical amino acid(s) profile of urine without hydrolysis (free amino acids) and after hydrolysis (total amino acids) by autoclaving (15 psi, 121 °C) or by incubation at 110 C for 16 h (total: free + peptide(s) derived)

S.no.	Amino Acid	Free amino acids	Alkali hydrolysate (μmol/mmol creatinine: mean ± SD)				Acid hydrolysate (μmol/mmol creatinine: mean ± SD)			
			1 h autoclave at 15 psi (121 °C)	Peptide derived	16 h incubation at 110 °C	Peptide derived	1 h autoclave at 15 psi (121 °C)	Peptide derived	16 h incubation at 110 °C	Peptide derived
1	asp	1.84 ± 0.02	74.6 ± 0.78	72.8 ± 0.75	83.8 ± 0.86	81.9 ± 0.84	95.3 ± 1.01	93.5 ± 0.97	169 ± 1.75	167 ± 1.70
2	glu	11.0 ± 0.12	136 ± 1.40	125 ± 1.30	123 ± 1.30	112 ± 1.15	156 ± 1.70	145 ± 1.50	187 ± 1.92	176 ± 1.80
3	asn	2.75 ± 0.03	0.00 ± 0.00	0.00 ± 0.00	5.40 ± 0.06	2.65 ± 0.03	0.00 ± 0.00	0.00 ± 0.00	3.80 ± 0.04	1.05 ± 0.01
4	ser	38.4 ± 0.40	77.2 ± 0.80	38.8 ± 0.41	67.2 ± 0.72	28.8 ± 0.30	74.3 ± 0.80	35.9 ± 0.40	106 ± 1.10	37.6 ± 0.40
5	gln	2.35 ± 0.03	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	19.2 ± 0.21	16.8 ± 0.19	21.9 ± 2.3	19.5 ± 0.25
6	his	96.5 ± 0.98	60.1 ± 0.64	0.00 ± 0.00	82.3 ± 0.86	0.00 ± 0.00	118 ± 1.25	21.5 ± 0.25	119 ± 1.25	22.5 ± 0.25
7	gly	111 ± 1.13	281 ± 2.90	170 ± 1.80	184.3 ± 0.62	73.3 ± 0.78	315 ± 3.12	204 ± 2.15	343 ± 3.50	232 ± 2.40
8	thr	10.6 ± 0.12	12.7 ± 0.15	2.10 ± 0.025	15.7 ± 0.18	5.10 ± 0.06	32.8 ± 0.34	22.2 ± 2.25	41.3 ± 0.45	30.7 ± 0.34
9	arg	10.0 ± 0.12	12.8 ± 0.15	2.80 ± 0.03	11.6 ± 0.13	1.60 ± 0.02	7.40 ± 0.08	0.00 ± 0.00	10.6 ± 0.14	0.60 ± 0.65
10	ala	28.5 ± 0.30	44.4 ± 0.48	15.9 ± 0.17	43.9 ± 0.47	15.4 ± 0.17	54.2 ± 0.60	25.7 ± 0.30	55.5 ± 0.62	27.0 ± 0.30
11	tyr	5.40 ± 0.06	16.7 ± 0.19	11.3 ± 0.13	12.8 ± 0.15	7.40 ± 0.09	17.7 ± 0.20	12.3 ± 0.15	21.5 ± 0.25	16.1 ± 0.20
12	met	1.80 ± 0.02	13.9 ± 0.16	12.1 ± 0.14	4.70 ± 0.05	2.90 ± 0.03	13.8 ± 0.16	12.0 ± 0.18	6.05 ± 0.08	4.25 ± 0.05
13	val	4.35 ± 0.05	43.3 ± 0.46	39.0 ± 0.42	14.4 ± 0.16	10.0 ± 0.12	17.3 ± 0.19	12.9 ± 0.15	18.4 ± 0.25	14.0 ± 0.18
14	phe	2.60 ± 0.03	46.0 ± 0.50	43.4 ± 0.45	134 ± 1.40	131 ± 1.38	19.5 ± 0.22	16.9 ± 0.18	11.4 ± 0.14	8.80 ± 0.10
15	ile	0.80 ± 0.01	9.20 ± 0.10	8.40 ± 0.10	8.25 ± 0.09	7.45 ± 0.08	6.85 ± 0.08	6.05 ± 0.08	8.55 ± 0.10	7.70 ± 0.09
16	leu	2.15 ± 0.025	13.5 ± 0.15	11.3 ± 0.13	30.9 ± 0.32	28.7 ± 0.30	17.4 ± 0.20	15.2 ± 0.18	25.1 ± 0.21	22.9 ± 0.25
17	lys	15.1 ± 0.17	38.1 ± 0.40	23.0 ± 0.28	36.1 ± 0.40	21.0 ± 0.27	40.1 ± 0.38	25.0 ± 0.33	32.2 ± 0.35	17.1 ± 0.20
18	hyp*	1.25 ± 0.02	27.5 ± 0.32	26.25 ± 0.30	19.5 ± 0.22	18.25 ± 0.2	10.60 ± 0.13	9.35 ± 0.11	11.5 ± 0.15	10.25 ± 0.1
19	pro*	16.1 ± 0.18	66.0 ± 0.70	49.9 ± 0.54	53.4 ± 0.58	37.3 ± 0.40	30.4 ± 0.35	14.3 ± 0.18	32.6 ± 0.38	16.5 ± 0.20

Urine specimens were subjected to hydrolysis by autoclaving at both acidic and alkaline conditions as described in text for protein solutions. Amino acids profiles are based on HPLC analysis of urine hydrolysate by pre-column OPA or DNP derivatisation (*Pro and Hyp only). Values expressed as mean ± SD of nine individual experiments.

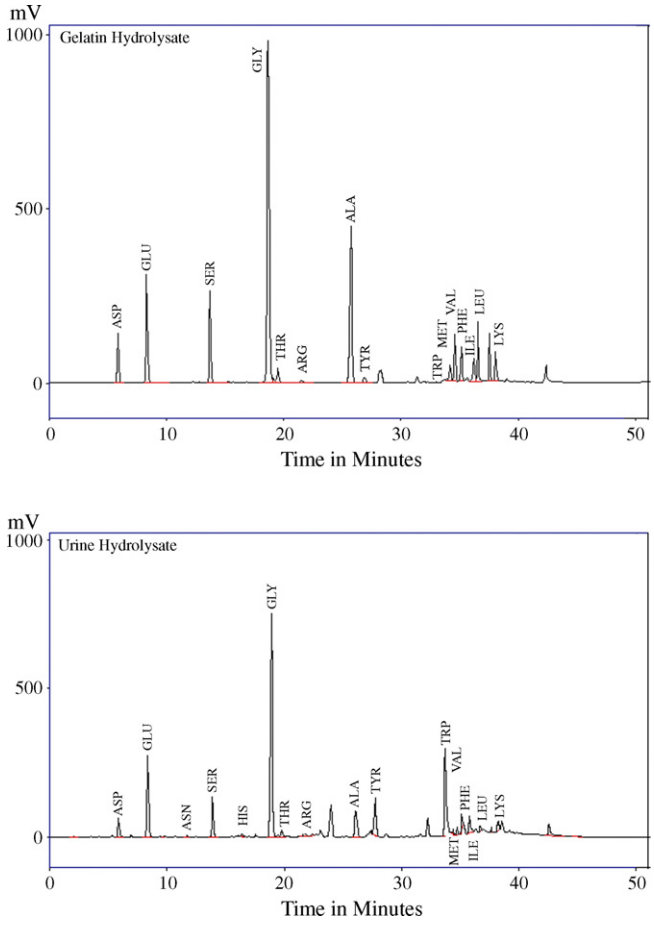

Fig. 2. Typical profiles of OPA derivatives of amino acids from gelatin (top panel) and urine (lower panel) hydrolysates under alkaline conditions of autoclaving. Note the absence of Hyp and Pro in both the chromatograms.

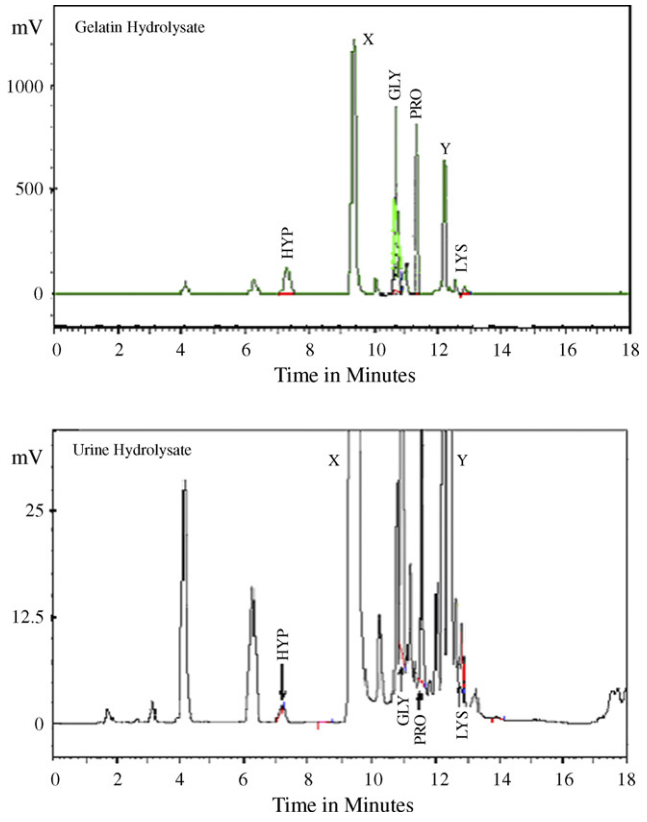

Fig. 3. Typical profiles of DNP derivatives of amino acids from gelatin (top panel) and urine (lower panel) hydrolysates under alkaline conditions of autoclaving. Note the presence of Hyp, Gly, Pro and Lys in both chromatograms. Also, note the difference with, respect to the unidentified peaks in both the chromatograms marked as 'X' and 'Y', which represent the degradation products of DNFB reagent in both the chromatograms.

and Hyp. The absence of Pro and Hyp peaks in OPA derivatisation method is attributed to the inability of OPA to react with secondary amino group of Pro and Hyp, which needs to be oxidized prior to derivatisation with primary amino group reagents such as OPA [20]. Thus, the ability of DNFB reagent to derivatize both primary and secondary amino groups and also the major components of collagen (Hyp, Hyl, Gly, Pro and Lys) render this method ideal for the study of collagen disorders. In addition to the respective amino acid(s) derivatives, two prominent additional peaks (labeled as 'X' and 'Y') of varied height, appeared in the chromatograms of protein and urine (Fig. 3) hydrolysate as well as in 'reagent blank' (not shown). The observation that the area under the curve of these unknown peaks is variable, depending on the total amino acids in the reaction mixture, implies that they represent the byproducts of unreacted DNFB reagent. In view of the fact that many of the amino acids other than Hyp and Pro are unstable in the hydrolytic conditions, the present method based on DNP derivatization is restricted for analyzing Hyp and Pro from the hydrolysates. Detectable range for Hyp, Gly, Pro and Lys was found to be within the range of 5–40 μM (0.5–4.0 nmol/100 μl injection). Thus, the present method has an advantage over the other similar methods based on HPLC and UV detection {38–760 μmol/l} [22] with a linearity of up to 10 mmol/l [23,24].

In contrast to the differential stability of most of the amino acids by alkaline and acidic hydrolysis, the studies on candidate amino acids suggest better stability of both Hyp and Pro, particularly in alkaline condition (Table 2). These observations were further substantiated from the composition of these amino acids from the hydrolysates of candidate proteins (Table 3) and urine hydrolysate (Tables 3 and 4). Thus it is a fact that the assay of both Pro and Hyp has important implications in the diagnosis of proline related disorders [3–5]. Further, the urinary peptide derived Hyp in particular is of diagnostic significance in diagnosis and monitoring of bone disorders such as [1,2,17], the observed stability of both Pro and Hyp during hydrolysis by autoclaving in alkaline conditions renders it suitable to quantify urinary peptide derived Pro and Hyp as an index of bone/collagen turn over.

The assay of Pro and Hyp attains diagnostic significance in inborn metabolic disorders such as prolinemia and diseases leading to osteoporosis [3–5]. Further, it is a fact that urinary excretion of collagen related peptides being a better marker for collagen degradation calls for urinary peptide derived Pro and Hyp as a biomarker of bone disorders. Hence, the observations made in the present study that both Pro and Hyp, unlike other amino acids, are stable under the hydrolytic conditions described and also the fact that DNP derivatives of these imino acids have

retention time less than 13 min that makes it ideal for the assay of protein and peptide derived prolyl and hydroxyprolyl residues.

5. Conclusion

To summarize, a chemical method of protein and urine hydrolysis based upon autoclaving requiring relatively shorter duration is described. The relative stability of some of the candidate amino acids, including Pro and Hyp, under the defined conditions of hydrolysis is defined. The differential stability and recovery of total and individual amino acids from protein and urine hydrolysate(s) were assessed. The observed stability of Pro and Hyp in alkaline autoclaving conditions establishes its utility to quantify the peptide derived Hyp and Pro in urine. Accordingly, a method based on DNP derivatisation followed by HPLC analysis of urinary peptide derived Pro and Hyp is described and its application in the study of bone/collagen disorders is underscored.

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