Quantitative Analyses of Glutamine in Peptides and Proteins

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An easy and rapid procedure for the determination of glutamine in synthetic peptides and isolated proteins is developed. It involves a prehydrolysis reaction of glutamine residues with bis(1,1-trifluoroacetoxy)iodobenzene (BTI), protein hydrolysis using a microwave technique, and high-performance liquid chromatography (HPLC) after precolumn derivatization of amino acids with dansyl chloride. Optimum conditions for the BTI-mediated conversion of glutamine to the corresponding diaminobutyric acid (DABA) were established. DABA and the proteic amino acids could be simultaneously measured with high sensitivity (2.0 pmol/injection; S/N = 3:1) and good reproducibility (CV 2.8%). The linearity between DABA formation and glutamine concentration was excellent (r = 0.9996). Recovery of DABA (mean recovery 93 ± 4%) from the selected test peptides and proteins (cathepsin G peptide, whey proteins, ovalbumin) was in good agreement with data derived from sequence analyses.

Keywords: *Glutamine; content in protein/peptides; analysis; BTI; microwave hydrolysis; HPLC*

INTRODUCTION

Glutamine is considered to be a conditionally essential amino acid during episodes of catabolic stress and malnutrition (Lacey and Wilmore, 1990; Swails et al., 1992; Ziegler et al., 1993). Knowledge of glutamine contents of natural peptides and proteins is thus of utmost importance. Quantitative assessment of protein-bound glutamine is, however, hampered by glutamic acid formation during acid hydrolysis, invalidating subsequent distinction between glutamine and glutamic acid residues.

Reliable assessment of the "true" glutamine content might be acquired using laborious and cost intensive biotechnological methods (cDNA technology) (Lacey and Wilmore, 1990) or can be obtained from sequence analyses of purified protein/peptide fragments. Analyses of naturally occurring, complex peptide mixtures are, however, not feasible with these methods. Earlier attempts also include the estimation of ammonium liberation during acid hydrolysis (Wilcox, 1967), esterification−reduction of free carboxylic acid groups (Wilcox, 1967), carbodiimide modification of free carboxylic acid groups (Carraway and Koshland, 1972), and protease/peptidase-catalyzed polypeptide hydrolysis (Towers, 1967). Indeed, all of these methods are mainly focused upon measurement of the amide group in the polypeptide chain. None of these complex techniques, however, has been shown to be satisfactory due to the occurrence of side reactions, the instability of the liberated products, and the difficulty of differentiating between asparagine and glutamine amide groups.

The treatment of polypeptides with [bis(trifluoroacetoxy)iodo]benzene (BTI) prior to acid hydrolysis facilitates carboxyl-terminal sequential peptide amide degradation (Parham and Loudon, 1978). Accordingly, in the presence of BTI, N-protected glutamine residues are converted to acid stable L-2,4-diaminobutyric acid (DABA) (Radhakrishna et al., 1979; Loudon et al., 1984; Boutin and Loudon, 1984), whereas asparagine yields L-2,3-diaminopropionic acid (DAPA). Yet recent trials to assess protein bound glutamine content with this reagent have failed. Fouques and Landry found the conversion incomplete and highly variable (Fouques and Landry, 1991), whereas Soby and Johnson were unable to measure the conversion because of lacking methodology to directly assess DABA (Soby and Johnson, 1981).

In the present study we report for the first time a reliable procedure of glutamine determination in peptides and proteins. We carefully controlled the effects of temperature, duration, and BTI/sample ratio on the conversion of bound glutamine to DABA.

MATERIALS AND METHODS

Chemicals. Alanylglutamine was a gift from Degussa (Hanau, Germany). Cathepsin G peptide was purchased from Bachem (Heidelberg, Germany). Amino acid standards and test proteins (α-lactalbumin, β-lactoglobulin A, gliadin, and ovalbumin) were from Sigma (Deisenhofen, Germany), and BTI was from Aldrich (Steinheim, Germany). Solvents for HPLC analysis (acetonitril Roti-Solv HPLC, tetrahydrofuran Roti-Solv HPLC) were obtained from Carl Roth (Karlsruhe, Germany). All other chemicals were from E. Merck (Darmstadt, Germany).

Analytical. *Conversion of Bound Glutamine to Acid Stable DABA−BTI Treatment.* Two hundred microliters of a standard aqueous peptide or protein solution (5 μmol/mL or 2.5 mg/mL, respectively) was mixed in an Eppendorf reaction tube with 200 μL of a freshly prepared solution of excess BTI (2.5−10 mg/mL) in dimethylformamide (DMF) and with 50 μL of an aqueous pyridine solution (50 μmol/mL). The mixture was allowed to react for 4 h at a temperature of 50 °C in a dry-block thermostat. Subsequently, the samples were evaporated until complete removal of water/DMF in a Speed-Vac concentrator (room temperature). Depending on the number of samples, this procedure lasted for 3−4 h. The precipitates were redissolved in water containing norleucine (2.5 μmol/mL) as an internal standard.

Microwave Hydrolysis. The samples were hydrolyzed by using a novel microwave technology with an MLS 1200 mega-high-performance microwave digestion unit (Chiou and Wang, 1989; Engelhardt et al., 1990; Engelhart, 1990). Briefly, 100

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Table 1. Elution Program Used for Dansylamino Acid Separation

time (min)	% A[a]	% B[b]	time (min)	% A[a]	% B[b]
0	85	15	35	40	60
15	70	30	40	0	100
18	70	30	43	0	100
25	55	45	45	85	15
30	45	55			

[a] 3% tetrahydrofuran, 97% sodium phosphate buffer, pH 7.2. [b] 60% acetonitrile, 40% sodium phosphate buffer, pH 7.2.

μL of the aqueous BTI-treated sample was transferred to a conical glass vial and evaporated to dryness. The high-pressure rotor (volume 1 L) enabled the simultaneous analysis of up to 56 samples; the hydrolysis procedure with this rotor required 40 mL of constant boiling hydrochloric acid. Hydrolysis was performed under vacuum employing the following program: 250 W, 5 min; 0 W, 1 min; 600 W, 8 min; ventilation, 5 min.

Amino Acid Analysis. Amino acid composition was determined by RP-HPLC using precolumn derivatization with 5-(dimethylamino)naphthalene-1-sulfonyl chloride (dansyl chloride). Chromatography was carried out using an LKB HPLC system consisting of two LKB 2150 gradient pumps, an LKB 2152 HPLC controller (LKB, Bromma, Sweden), a Jasco 851 AS intelligent sampler, a Shimadzu RF 535 fluorescence spectromonitor, and a Shimadzu Chromatopac C-R6A data processor. The dansylamino acids were separated on a Hypersil ODS-2 column (3 μm, 150 × 4.6 mm, T = 25 °C, flow 0.9 mL/min). The elution program is given in Table 1. A Sigma AAS 18 amino acid standard, to which equimolar amounts of DABA and norleucine were added, was used as a reference sample. A typical chromatogram of an amino acid standard solution is shown in Figure 1A. Subsequent fluorescence detection was carried out at 330 nm excitation/520 nm emission (Fürst et al., 1990).

Studies with Alanylglutamine and Alanylasparagine. To study the influence of temperature, reaction time, and BTI to sample ratio on the BTI-mediated conversion of glutamine, aqueous solutions (5 μmol/mL) of the standard dipeptide L-alanyl-L-glutamine (Ala-Gln) were treated with BTI according to the procedure described above. The reaction was carried out at three temperatures (40−60 °C), at eight occasions (1−24 h), and with seven different BTI to peptide ratios (1:1 to 10:1), while all other reaction conditions were kept constant. Each incubation was performed at least in duplicate.

To evaluate the conversion of asparagine to DAPA, aqueous solutions of the dipeptide L-alanyl-L-asparagine were treated with BTI. The same reaction conditions were used as employed for glutamine (4 h, 50 °C, molar ratio of BTI to Asn = 3:1).

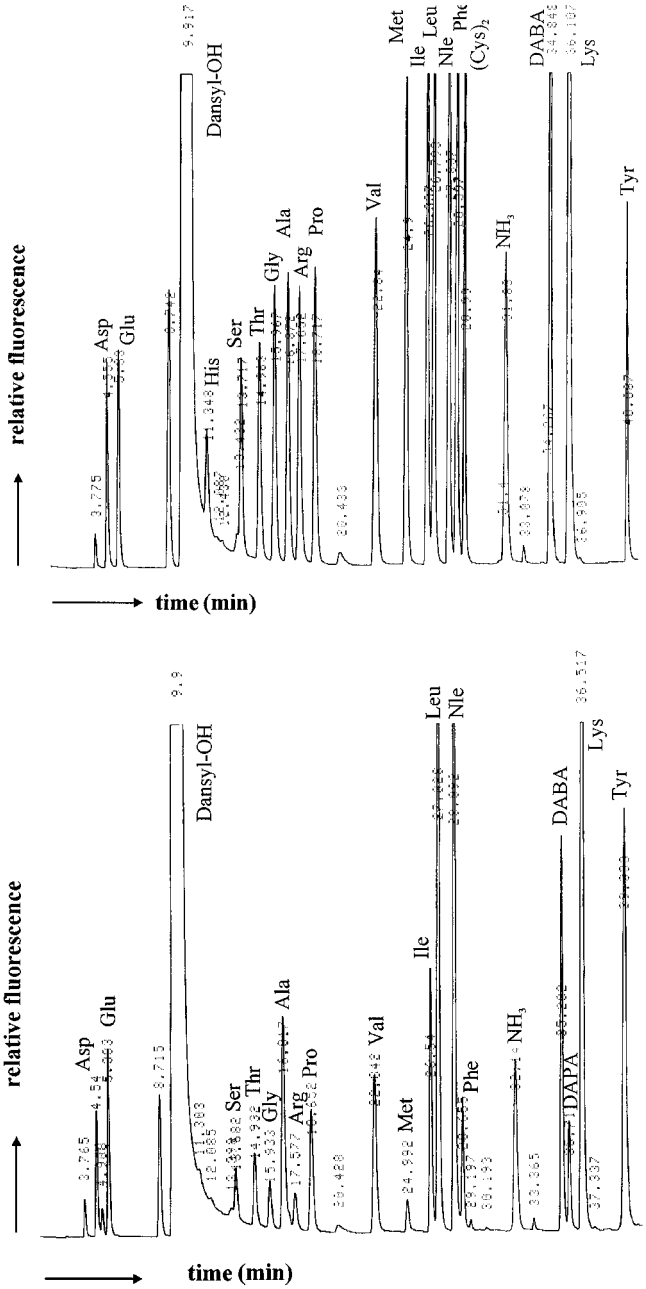

Figure 1. (A, top) HPLC analysis of a standard solution (17 proteic amino acids + DABA + norleucine as an internal standard, 2.0 nmol/mL each); (B, bottom) HPLC analysis of a BTI-treated protein hydrolysate (β-lactoglobulin A).

RESULTS

HPLC Analysis of DABA. The direct determination of the DABA and DAPA content in the hydrolysates was combined with simultaneous measurement of 17 proteic amino acids in a single run. In Figure 1B, the HPLC analysis of a BTI-treated hydrolysate from β-lactoglobulin A is depicted. The lower limit of sensitivity at a signal to noise ratio of 3:1 was 2.0 pmol per injection.

Studies with Alanylglutamine. Temperature and reaction time had little impact on conversion of glutamine residues to DABA. Nevertheless, the most suitable conditions appeared to be achieved after 4 h at 50 °C.

The amount of reagent strongly affected DABA recovery (Figure 2). Optimum results were obtained by using 3−5-fold BTI excess relative to the carboxamide concentration in the sample. The occurrence of glutamic acid in the hydrolysates at lower molar ratios suggested that an incomplete conversion of glutamine was associated with lower amounts of BTI in the reaction mixture. However, further increase in the reagent resulted in a diminished DABA recovery.

Studies with Alanylasparagine. BTI treatment of the dipeptide alanylasparagine (n = 23) resulted in a poor recovery of DAPA. Mean ± S_M was 13.2 ± 7.8% of theoretical.

Evaluation of Glutamine Content. Recoveries of DABA from Ala-Gln, the heptapeptide cathepsin G, and the two whey proteins were in good agreement with theoretical values in synthetic peptides and with data acquired from available sequence analyses (Walstra and Jeness, 1984; van Boekel and Ribadeau-Dumas, 1987; Lacey and Wilmore, 1990), respectively. Results are summarized in Table 2.

Statistical Evaluation. In 24 successive analyses of an Ala-Gln sample (5 μmol/mL) the *precision* of the BTI method, including all analytical procedures was 0.13 (S_M) and the percentage error was 2.8% [($S_M/\bar{x}$) ×

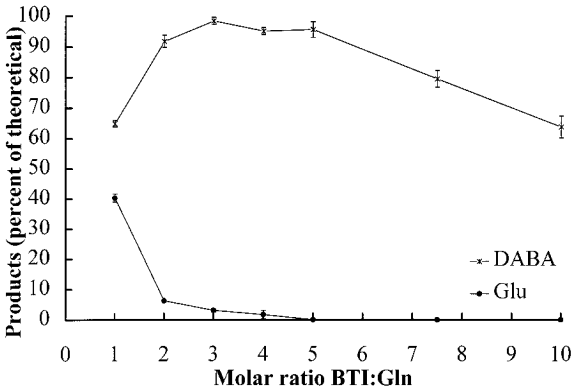

Figure 2. Influence of the amount of reagent on conversion rates of Gln to DABA (mean ± S_M, $n = 3$), $T = 50$ °C, 4 h reaction time.

Table 2. Recovery of DABA-Converted Glutamine (T = 50 °C, 4 h Reaction Time, BTI:Sample Ratio = 3:1 to 5:1) from the Dipeptide Ala-Gln and Selected Proteins Expressed in Grams per 16 g of Nitrogen ($n = 2-24$, Mean ± S_M) and Comparison of Data with Theoretical Values/Sequence Analyses (SA)

	% Gln BTI method	% Gln SA	recovery in % of SA
Ala-Gln[a] (24)	55.8 ± 1.6	59.0	95
cathepsin G[a] (2)	23.5/26.1	27.3	91
α-lactalbumin[b] (8)	5.3 ± 0.1	5.5	96
β-lactoglobulin[b] (8)	5.9 ± 0.1	6.3	94
ovalbumin[c] (5)	3.3 ± 0.1	3.8	87

[a] Theoretical values. [b] Walstra and Jenness (1984); van Boekel and Ribadeau-Dumas (1987). [c] Lacey and Wilmore (1990).

100]. By employing the one-sample t test [$t = (\bar{x} - \zeta)/S_{\bar{x}}$] (Marthaler, 1974), we assessed the *accuracy* of the method. The mean value was 4.73 μmol/mL, and the standard error of the mean was $S_{\bar{x}} = 0.03$ ($p < 0.0001$).

The relationship between amount of peptide (glutamine) and DABA formation was derived from triplicate analyses at seven Ala-Gln concentrations (1−10 μmol/mL). The regression line obtained was highly significant ($y = 1.00x - 0.40$; $r = 0.9996$).

DISCUSSION

Prerequisite for the use of the BTI method is a reliable, reproducible, and routinely manageable technique allowing assessment of the DABA content in acid hydrolysates. In the present investigation, we successfully employed precolumn derivatization with dansyl chloride and RP-HPLC for the simultaneous analysis of DABA, DAPA, and the 17 proteic amino acids in a single run (Figure 1). DAPA was not included to the standard solution, as results obtained from the BTI treatment showed that the conversion of Asn to DAPA is incomplete and not reproducible. It appears that the BTI method is not suitable for the simultaneous determination of asparagine and glutamine. Pertinent clinical questions in the frame of current nutritional studies are generally related to the glutamine content, only.

The knowledge of the course of BTI-mediated conversion of bound glutamine to DABA is the first prerequisite to establish a reliable method. This task was approached with the use of the synthetic dipeptide Ala-Gln revealing sufficiently high purity (>99.5%) and, in contrast to free glutamine, high solubility (>500 g/L). In the dipeptide molecule the α-amino group of the glutamine residue is "protected" by a peptide bond. According to early observations, N-terminal glutamine residues in oligopeptides or free glutamine are not converted to DABA (Loudon et al., 1984). In agreement with these results we found only minute conversion following BTI treatment of free glutamine or of the dipeptide L-glutaminylglycine (Gln-Gly). This would mean that glutamine with an unprotected α-amino group might undergo oxidative decarboxylation combined with deamidation (Loudon et al., 1984). Therefore, one may contemplate a slight underestimation of the glutamine content. On the other hand, N-terminal glutamine is as instable as free glutamine (Meister, 1956) and might be promptly degraded to yield pyroglutamic acid during processing and storage. Thus, the BTI method provides information about the amount of stable glutamine, which knowledge is pertinent in clinical nutrition. Currently, there is great interest devoted to the analysis of the content of glutamine in nutritional preparations. In numerous studies it is demonstrated that glutamine is an essential nutrient during catabolic stress (Fürst, 1994).

Table 3. Concentration of BTI Reagent According to the Estimated Carboxamide Content (in Percent of Weight) in the Protein/Peptide Sample ($C_{protein/peptide}$ = 2.5 mg/mL)

BTI concn (mg/mL)	carboxamide content (% of wt)	BTI concn (mg/mL)	carboxamide content (% of wt)
1	<5	6	14−26
2	5−9	8.5	19−34
3	7−13	10	>35
4.5	10−20		

Obviously, alterations in temperature between 40 and 60 °C have little influence on DABA recovery. We employed three reaction temperatures (40, 50, and 60 °C) and found that the fastest quantitative conversion was achieved at 50 °C. This temperature is in agreement with previous studies recommending 60 °C reaction temperature (Soby and Johnson, 1981; Fouques and Landry, 1991). Conversion of glutamine to DABA is virtually complete after 2 h and reaches 93−95% recovery after 4 h of reaction time. A further prolongation of reaction time to 5, 6, or 8 h does not increase the recovery of DABA; after 24 h of reaction time, a slight decrease can be observed.

In the original BTI method, the necessity of considerable excess of reagent (>200) is claimed to facilitate carboxamide degradation (Soby and Johnson, 1981). The reasons for this proposal are not explained, yet an obvious disadvantage associated with the method is the laborious removal of excess BTI before hydrolysis. Indeed, the observation of the present study (Figure 2) strongly indicates that this high excess of BTI might be one of the explanations for controversial results obtained with this method (Soby and Johnson, 1981; Vendrell and Aviles, 1986; Fouques and Landry, 1991). Since, with high excess of BTI, no glutamate could be detected in the acid hydrolysates, it is assumed that the glutamine residues were quantitatively converted to DABA. This would indicate that the decreased recovery of DABA is the result of a secondary decomposition, possibly mediated by untreated BTI in the incubation mixture. On the basis of this reasoning we recommend three to five parts of BTI per part of carboxamide to facilitate complete conversion of glutamine and to minimize DABA decomposition (Figure 2). A guideline to estimate the necessary excess of BTI in relation to the carboxamide content in unknown samples is given in Table 3.

The fact that DABA recovery remained consistently below 100% suggests that it might not be practical to

completely suppress undesirable secondary reactions. Calculations of the glutamine content in various proteins on the basis of DABA formation are in fair agreement with available sequence analysis data, resulting in a recovery of 93 ± 4% (Table 2). The results of the present study are in contrast to a previous observation with BTI (Fouques and Landry, 1991) reporting a maximum recovery for DABA of 75% in various proteins investigated. The explanation for the lower recovery in this study might be due to the high amount of BTI, as well as the acidic reaction conditions (0.01 M trifluoroacetic acid as a solvent for proteins, no addition of pyridine). In addition, excess BTI might interfere with the formation of the PITC derivatives, thereby disturbing UV detection of DABA subsequent to RP-HPLC.

In model experiments with the synthetic dipeptide Ala-Gln the overall reduction of the rate of conversion is apparently independent of glutamine concentration. While the uniform reduction of DABA recovery is virtually constant (0.4 μmol/mL) for each glutamine concentration, this systematic reduction does not appreciably influence the results.

Indeed, the method as presented combines several methods including BTI reaction, hydrolysis, and HPLC. We are attempting to simplify these methods, thereby facilitating a more user friendly system. Yet the modified BTI method shows good reproducibility and provides a reliable, routinely available method for measurement of the glutamine content in oligopeptides and proteins.

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