Analysis of Dabsyl-Cl Derivated Amino Acids by High Performance Liquid Chromatography and Tandem Mass Spectrometry

Yi-Hong CHEN, Ling-Ling SHIH, Su-Er LIOU and Chu-Chin CHEN

Food Industry Research and Development Institute, Hsinchu 30099, Taiwan, R.O.C.

Received December 13, 2002; Accepted April 4, 2003

A high performance liquid chromatograph coupled with a tandem mass (MS/MS) detector was used for the analysis of 4-dimethylaminoazobenzene-4-sulfonyl chloride (dabsyl-Cl) derivated amino acids. Apart from dabsyl-Cys and dabsyl-Lys, the other 18 common amino acid derivatives had good linear relationship between the responding peak area and concentrations between 10–250 μM , $R^2 > 0.99$. The responding peak area of dabsyl-Lys at a low concentration was suppressed by that of dabsyl-Gln in samples of the amino acid mixture, because signals of both amino acid derivatives were present in the same selected reaction monitoring (SRM) channel. A linear regression curve of dabsyl-Lys was therefore obtained only in the range of 250–1000 μM , $R^2 > 0.96$. Dabsyl-Cys was destructed to get a unique ion of m/z 290 before entering the MS/MS detector. The regression curve of the responding peak area and concentration of dabsyl-Cys was therefore obtained with the SRM of m/z 290 to its product ion m/z 225.1. A linear relationship was obtained between 1–10 mM, $R^2 > 0.98$. The established method was used in the analysis of amino acids in a koji liquid. The results showed better identification of the dabsyl-AA and the method is, therefore, more reliable in analyzing amino acid contents in complex food mediums.

Keywords: amino acid, dabsyl chloride, analysis, HPLC-MS/MS, mass spectrometry

Amino acids and proteins are essential food components and also play a crucial role in the life phenomenon as enzymes, hormones, etc. With the recent advances in biotechnology, research on the structure analyses and functions of amino acids and proteins have become important. Several methods have been developed for the analysis of amino acids which include separation on an ion exchange resin followed by postcolumn reaction with chromogenic reagents or precolumn derivatization of amino acids for reversed-phase chromatography with ultraviolet (UV) or fluorescent detectors (Duncan & Poljak, 1998). Postcolumn derivatization with ninhydrin has been well developed (Moore & Stein, 1951; Spackman *et al.*, 1958). Recently, precolumn derivatization methods have become popular because they not only offer better chromatographic reproducibility (Cohen & Strydom, 1988), but also can be performed ubiquitously on any high performance liquid chromatographic (HPLC) system. Several reagents offering different advantages and feasibilities have been in common used: 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) (Pawlowska *et al.*, 1993), 4-dimethylaminoazobenzene-4-sulfonyl chloride (Dabsyl-Cl) (Lin & Wang, 1980), dimethylaminonaphthalene-1-sulfonyl chloride (Dansyl-Cl) (Schmidt *et al.*, 1979), 9-fluorenylmethyl chloroformate (Fmoc-Cl) (Einarsson *et al.*, 1983), phenyl isothiocyanate (PITC) (Heinrikson & Meredith, 1984) and Ophthalaldehyde (OPA) (Herbert *et al.*, 2000). However, neither a precolumn nor a postcolumn method has been successful in analyzing the free amino acids in a protein hydrolysate because all of the derivatization reagents mentioned above also react with peptides and proteins in the hydrolysate, thus creating interference (Gartenmann & Kochhar,

1999). Although a better separation technique with capillary electrophoresis (CE) has been attempted (Terabe *et al.*, 1991), the selectivity with UV or fluorescent spectrometry still has its limitations. Mass spectrometry (MS) has been employed successfully with CE in the analysis of phenolic compounds (Lafont *et al.*, 1999), herbicides (Song & Budde, 1996), drugs and their metabolites (Lu *et al.*, 1996), and carboxylic acids (Johnson *et al.*, 1999) with the advantage of high selectivity and sensitivity. It was not until recently that CE-MS was claimed to be effective in the analysis of underivatized amino acids (Soga & Heiger, 2000). However, HPLC-MS has worked well with some of the derivatized amino acids. van Leuken *et al.* (1995) coupled OPA and N-acetyl-L-cysteine derivatives to determine the D- and L-amino acids in peptide solutions; Liu *et al.* (1995) analyzed AQC derivatized amino acids in foods and feeds with HPLC-MS. Fujii *et al.* (1998), on the other hand, used DLFDLA(1-fluoro-2,4-dinitrophenyl-5-D-leucinamide & -L-leucinamide) derivatives. Dabsyl-Cl derivatization coupled with HPLC resolution and UV (436 nm) detection has long been used in our laboratory for amino acid analysis (Chen *et al.*, 1993; Chien *et al.*, 1993). Since there has been no report dealing with HPLC-MS analysis of dabsyl-Cl derivatized amino acids (dabsyl-AA) thus far, it is attempted in this paper to establish a method for dabsyl-AA analysis by taking advantage of HPLC ubiquity and MS selectivity.

Materials and Methods

Materials Twenty common amino acids and 4-dimethylaminoazobenzene-4-sulfonyl chloride (dabsyl-Cl) were purchased from Sigma (St. Louis, MO). Ethanol, hydrochloric acid, sodium bicarbonate and sodium orthophosphate were products of Merck (Darmstadt, Germany). Formic acid was purchased

from Riedel-de Haen (Seelze, Germany) and acetonitrile (HPLC grade) was purchased from Tedia (Fairfield, OH). A koji liquid was obtained from the Product Processing and Chemistry laboratory of our Institute. It was a clear solution of koji after incubation of the raw materials, soy and wheat, with microorganisms for an appropriate period of time.

Methods

Preparation of dabsyl-Cl derivatized amino acids (dabsyl-AA)
Protocols of Chang and Liu (1988) were followed. Each single amino acid or amino acid mixtures was dissolved in 0.1 N HCl solution. Dabsyl-Cl (26 mg) was dissolved in 20 ml acetonitrile. A 0.1 M NaHCO_3 solution (pH 8.3) and a 0.05 M Na_2HPO_4 solution (pH 7.0) were separately mixed with an equal volume of

ethanol.

A 0.5 ml aliquot of amino acid solution or koji liquid was placed in a test tube and mixed well with 0.5 ml NaHCO_3 solution and 2 ml dabsyl-Cl solution. The test tube was incubated in a 70°C water bath for 15 min before 3 ml Na_2HPO_4 /ethanol solution was added to stop the reaction.

MS analysis of dabsyl-AA An HPLC-MS/MS system including a Waters 600E multi-solvent delivery pump, a Waters 2487 UV/VIS detector (Milford, MA) and a Quattro LC MS/MS detector (Wythenshawe, UK) with electrospray ionization (ESI) was used. Desolvation temperature was 350°C. Extraction cone voltage was 10 V.

Full mass of each dabsyl-AA sample was scanned between

Fig. 1. ESI mass spectrum of dabsyl-Gly.

Fig. 2. ESI mass spectrum of dabsyl-Cys.

m/z 70–500 by pumping 10 μ l of each dabsyl-AA solution into the MS/MS detector directly with H₂O/acetonitrile (50/50, containing 0.1% (v/v) formic acid). Flow rate was 0.1 ml/min. The concentration of each amino acid was 20 μ M. Product ions of each dabsyl-AA were also monitored in another injection by applying argon collision with 20 V energy under the same experimental condition.

Dabsyl-AA mixtures, on the other hand, were resolved with a LiChroCART 125-2 Superspher 100 RP-18 column (Merck) before entering the UV/VIS detector and then the MS/MS detector. Sample size was 10 μ l. Flow rate was 0.3 ml/min. Both solvent A (H₂O) and solvent B (acetonitrile) contained 0.1% (v/v) formic acid. The gradient was programmed as follows: 15% B for 3 min, linear gradient from 15% to 85% B for 60 min, and 85% B for 7 min. Relationship of responding peak area and concentration of each dabsyl-AA was calculated with Excel software (Microsoft Co., Seattle, WA).

Results and Discussion

Each dabsyl-AA was first full mass scanned between m/z 70–500. It was found for most of the dabsyl-AA that the major MS signal always appeared at the calculated m/z , which is the m/z of the corresponding amino acid plus 287. For example, the total ion chromatogram (TIC) of dabsyl-Gly (Fig. 1) shows only the calculated m/z 363. Other dabsyl-AA may be destructed by the high temperature during desolvation and the high voltage at the sample cone before entering the MS detector. Signals of the breakdown products at lower masses than the calculated ones were thus observed. The profiles and intensities of low mass ions were different for each dabsyl-AA. Nevertheless, significance of the calculated m/z for each dabsyl-AA was still observed (data not shown) except that of dabsyl-Cys. The TIC of dabsyl-Cys showed a highest ion at m/z 290 together with a very low signal at the calculated m/z 409. The ion at m/z 290 was the ionized

dabsyl group destructed from the dabsyl-Cys (Fig. 2). This ion at m/z 290 was not observed for any other dabsyl-AA or the blank dabsyl-Cl derivatized sample without amino acid, and was therefore used as the M+H ion for dabsyl-Cys in the following experiment.

Product ion scanning was further performed by choosing the calculated m/z of each dabsyl-AA as the precursor ions except that of dabsyl-Cys, where the product ions of m/z 290 were monitored. A pre-experiment with different argon collection energies was applied and 20 V was chosen. The product ion spectrum

Table 1. Calculated m/z values of amino acids, dabsyl-AA, and the selected product ions of dabsyl-AA for MS/MS analysis.

Amino acid (AA)	Mass of AA (M+H)	Mass of dabsyl-AA (M+H)	Mass of selected product ion of dabsyl-AA ^{a)}
Gly	76.0	363.1	214.0
Ala	90.1	377.1	183.0
Ser	106.0	393.1	224.1
Pro	116.1	403.1	224.1
Val	118.1	405.2	211.0
Thr	120.1	407.1	290.1
Cys	122.0	409.1	225.1 ^{b)}
Leu	132.1	419.2	224.1
Ile	132.1	419.2	224.1
Asn	133.1	420.1	224.1
Asp	134.0	421.1	224.1
Gln	147.1	434.1	224.1
Lys	147.1	434.2	224.1
Glu	148.1	435.1	224.1
Met	150.1	437.1	224.1
His	156.1	443.1	224.1
Phe	166.1	453.2	224.1
Arg	175.1	462.2	224.1
Tyr	182.1	469.1	224.1
Trp	205.1	492.2	224.1

^{a)} Argon collision energy: 20 V.

^{b)} Product ion of m/z 290.

Fig. 3. HPLC chromatograms of the mixture of 20 dabsyl-AA. A: absorbance at 436 nm, B: total ion chromatogram of 19 SRM channels. The concentration for each amino acid was 1 mM.

Fig. 4. Total ion chromatograms of the 19 SRM channels for 20 dabsyl-AA. The concentration of each dabsyl-AA was 1 mM, except 10 mM for dabsyl-Cys.

revealed the breakdown ions of the designated precursor ion for each dabsyl-AA, and a selected reaction monitoring (SRM) mode (precursor ion to product ion) for the MS/MS analysis could therefore be chosen (Table 1).

It was found that most of the dabsyl-AA had a major product ion of m/z 224.1. This is similar to the results of Cooper and Morris (2000) who found a common ion of m/z 171 for almost

all the AccQ-Tag (6-aminoquinolyl-N-hydroxysuccinimidyl carbamate) derivatized amino acids.

A mixture of 20 amino acids was then derivatized with dabsyl-Cl and applied to the HPLC-MS/MS. The product was resolved with a column as specified above and was monitored at UV 436 nm and then MS/MS of 19 SRM modes (One SRM each for 18 dabsyl-AA, and another SRM setting for dabsyl-Leu

(Fig. 4 continued)

and dabsyl-Ile because they have the same mass values for precursor and product ions). It was found that the mass chromatogram was neater than the UV chromatogram (Fig. 3). This was because only the mass signals of the 20 dabsyl-AA were monitored. For example, the underivatized dabsyl-Cl gave a high signal at 22.67 min in the UV chromatogram but it did not appear in the mass chromatogram. There was also about a 0.2 min lag between the UV signals and corresponding mass signals.

The mass chromatogram of Fig. 3 also suggests that the resolution with HPLC was not successful in separating all the 20 dabsyl-AA because fewer peaks were observed. However, this will not be a problem if each SRM channel is viewed separately,

where only the corresponding dabsyl-AA will be present (Fig. 4). Dabsyl-Leu and dabsyl-Ile are present in the same SRM 419.2>224.1 channel as a single peak at a retention time (RT) of 47.20. These two amino acid derivatives were not able to be differentiated by this method.

The chromatograms of Dabsyl-Gln and Dabsyl-Lys were identical. This is because the difference between the selected mass reactions of dabsyl-Gln (434.1>224.1) and dabsyl-Lys (434.2>224.1) was so small that both their peaks are present in SRM 434.1>224.1 and in SRM 434.2>224.1 channels. The RT of dabsyl-Gln and dabsyl-Lys were further identified by applying each one separately. It was found that the larger peak at 33.86

Table 2. Retention time, reproducibility and linearity of dabsyl-AA analyzed by the HPLC-MS/MS.

Dabsyl-AA	Retention time (min)	RSD ($n=8$) (%)		$R^{2a)}$
		Retention time	Peak area	
Gly	38.52	0.25	9.05	0.9983
Ala	40.13	0.19	6.93	0.9991
Ser	35.33	0.42	7.30	0.9972
Pro	44.15	0.16	6.79	0.9968
Val	44.43	0.16	7.25	0.9996
Thr	36.98	0.30	10.73	0.9989
Cys	27.17	0.44	10.41	0.9827 ^{b)}
Ile+Leu	47.20	0.15	6.90	0.9988
Asn	33.75	0.56	8.55	0.9991
Asp	36.31	0.35	9.69	0.9907
Gln	33.86	0.50	7.89	0.9993
Lys	42.82	1.06	11.27	0.9632 ^{c)}
Glu	36.24	0.32	8.45	0.9989
Met	44.08	0.16	7.84	0.9969
His	58.52	0.14	6.72	0.9910
Phe	47.62	0.15	7.50	0.9994
Arg	47.20	1.04	9.41	0.9949
Tyr	40.83	0.16	6.75	0.9953
Trp	45.76	0.15	7.50	0.9966

^{a)} Peak area was dependent on the concentration of dabsyl-AA. Curves were generated with the least square method by using points between 10–250 μM , unless specified.

^{b)} Calculated from points between 1–10 mM.

^{c)} Calculated from points between 250–1000 μM .

min was dabsyl-Gln and the smaller peak at 42.82 min was dabsyl-Lys. Other dabsyl-AA had clear RT as listed in Table 2.

It was perceived that reproducibilities of all dabsyl-AA were excellent ($\text{RSD}<10$) in retention time with all RSD values ($n=8$) lowered than 1.06%, and were excellent in peak areas for most of the dabsyl-AA and good ($10<\text{RSD}<15$) for dabsyl-Cys, dabsyl-Lys and dabsyl-Thr (Table 2).

Eight 20 dabsyl-AA mixtures at 10, 25, 50, 100, 250, 500, 750 and 1000 μM for each dabsyl-AA were used to determine the relationship between response peak area and dabsyl-AA concentration by HPLC-MS/MS analysis. For 18 out of 20 dabsyl-AA, the regression curve between response peak area and dabsyl-AA concentration showed good linearity between 10 and 250 μM with R^2 over 0.99 (Table 2). However, peak signal of dabsyl-Cys was only observed and the area integrated at 1000 μM . Five dabsyl-Cys samples with concentrations ranging from 1 to 10 mM were performed and a regression curve with $R^2=0.9827$ was obtained. The peak signal of dabsyl-Lys, on the other hand, was suppressed by that of dabsyl-Gln and it was not observed until 250 μM . The regression curve for dabsyl-Lys was calculated by using the points between 250 and 1000 μM and an equation with $R^2=0.9632$ was obtained. The curve obviously lost its linearity at a high concentration range, although it was still acceptable at a 95% confidence level. In a separate experiment, dabsyl-Lys samples in the absence of dabsyl-Gln were prepared and analyzed, and a regression curve with good linearity ($R^2>0.99$) was obtained between 10 and 250 μM (data not shown). However, this situation is not likely to occur in any enzymatic protein hydrolysate.

The established method was applied to the analysis of amino acids in a koji liquid, which was used as a flavor aid in sauce products. Analysis of amino acids in the koji liquid can help fine tuning of the fermentation process and the final product flavor. The koji liquid was a complex mixture containing not only pro-

Fig. 5. SRM electropherograms for 20 dabsyl-AA in a koji liquid. Peak intensities are expressed relative to the highest one, which is that of dabsyl-Pro.

teinaceous materials (amino acids, peptides and proteins) but also other soy and wheat constituents. Analysis of dabsyl-AA in the koji liquid by traditional HPLC-UV method may pose a problem in resolution and identification, because a preliminary experiment performed on a mixture of equal concentrations of each of the 20 common amino acids plus a dipeptide (Gly)₂ and a pentapeptide (Gly)₅ resulted in a surged content of an amino acid (data not shown). On the other hand, only the dabsyl-AA with a few additional peaks were observed in the SRM electropherograms (Fig. 5) by the established method and the identification of the dabsyl-AA was believed to be more reliable. Controlling the production processes of the koji liquid for sauce application will definitely benefit from the established method and will be reported in another paper.

Conclusion

This article has depicted a method to analyze dabsyl-AA by HPLCMS/MS with high selectivity. For most of the dabsyl-AA, except dabsyl-Cys and dabsyl-Lys, good linear correlation of concentration and response peak area with $R^2>0.99$ was achieved between 10 and 250 μM . Signals of dabsyl-Cys and dabsyl-Lys showed up at higher concentrations. Their regression curves lost a bit of linearity but still had at least 95%

confidence. Nevertheless, it is well understood that the MS/MS analysis offers higher selectivity than UV or fluorescent detectors. This advantage of MS/MS renders it an excellent tool for the analysis of amino acids in a protein hydrolysate, where peptides and proteins may interfere if only an UV or a fluorescent detector coupling to a HPLC is used. The established method was proved useful in the analysis of amino acids in a complex medium like koji liquid, and it is expected that even wider application can be achieved.

Acknowledgment Financial support for this study from the Ministry of Economic Affairs, Taiwan, the Republic of China, is highly appreciated (88-EC-2-A-17-237).

References

- Chang, C.-S. and Liu, C.-S. (1988). A picomole-level amino acid analysis by means of gasphase hydrolysis and DABS-Cl/HPLC method. *J. Chin. Biochem. Soc.*, **7**, 12–19.
- Chen, C.-C., Chien, F.-N. and Chen, C.-C. (1993). Free amino acid analysis by dabsylchloride derivatization and reversed-phase high-performance liquid chromatography. (1) Completeness of dabsyl-Cl derivatization and recovery. Res. Report Food Ind. Res. Develop. Inst., Hsinchu, Taiwan, No. 799.
- Chien, F.-N., Chen, C.-C., Su, N.-C. and Chen, C.-C. (1993). Free amino acid analysis by dabsyl-chloride derivatization and reversed-phase high-performance liquid chromatography. (2) Effect of pre-treatments on free amino acid content. Res. Report Food Ind. Res. Develop. Inst., Hsinchu, Taiwan, No. 801.
- Cohen, S.A. and Strydom, D.J. (1988). Amino acid analysis utilizing phenylisothiocyanate derivatives. *Anal. Biochem.*, **174**, 1–16.
- Cooper, D. and Morris, M. (2000). LC-MS-MS analysis of amino acids using AccQ-Tag derivatisation. Application Brief AB25. Micromass UK Ltd., Manchester, UK.
- Duncan, M.W. and Poljak, A. (1998). Amino acid analysis of peptides and proteins on the femtomole scale by gas chromatography/mass spectrometry. *Anal. Chem.*, **70**, 890–896.
- Einarsson, S., Josefsson, B. and Lagerkvist, S. (1983). Determination of amino acids with 9-fluorenylmethyl chloroformate and reversed phase high performance liquid chromatography. *J. Chromatogr.*, **282**, 609–618.
- Fujii, K., Shimoya, T., Ikai, Y., Oka, H. and Harada, K. (1998). Further application of advanced Marfey's method for determination of absolute configuration of primary amino compound. *Tetrahedron Lett.*, **39**, 2579–2582.
- Gartenmann, K. and Kochhar, S. (1999). Short-chain peptide analysis by high-performance liquid chromatography coupled to electrospray ionization mass spectrometer after derivatization with 9-fluorenylmethyl chloroformate. *J. Agric. Food Chem.*, **47**, 5068–5071.
- Heinrikson, R.L. and Meredith, S.C. (1984). Amino acid analysis by reverse-phase high-performance liquid chromatography: precolumn derivatization with phenylisothiocyanate. *Anal. Biochem.*, **136**, 65–74.
- Herbert, P., Barros, P., Ratola, N. and Alves, A. (2000). HPLC determination of amino acids in musts and port wine using OPA/FMOC derivatives. *J. Food Sci.*, **65**, 1130–1133.
- Johnson, S.K., Houk, L.L., Johnson, D.C. and Houk, R.S. (1999). Determination of small carboxylic acids by capillary electrophoresis with electrospray-mass spectrometry. *Anal. Chim. Acta*, **389**, 1–8.
- Lafont, F., Aramendia, M.A., Garcia, I., Borau, V., Jimenez, C., Marinan, J.M. and Urbano, F.J. (1999). Analyses of phenolic compounds by capillary electrophoresis electrospray mass spectrometry. *Rapid Commun. Mass Spectrom.*, **13**, 562–567.
- Lin, J.K. and Wang, C.H. (1980). Determination of urinary amino acids by liquid chromatography with dabsyl chloride. *Clin. Chem.*, **26**, 579–583.
- Liu, H.J., Chang, B.Y., Yan, H.W., Yu, F.H. and Liu, X.X. (1995). Determination of amino acids in food and feed by derivatization with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate and reversed-phase liquid chromatographic separation. *J. AOAC Int.*, **78**, 736–744.
- Lu, W., Poon, G.K., Carmichael, P.L. and Cole, R.B. (1996). Analysis of tamoxifen and its metabolites by on-line capillary electrophoresis-electrospray ionization mass spectrometry employing nonaqueous media containing surfactants. *Anal. Chem.*, **68**, 668–674.
- Moore, S. and Stein, W.H. (1951). Chromatography of amino acids on sulfonated polystyrene resins. *J. Biol. Chem.*, **192**, 663–681.
- Pawlowska, M., Chen, S. and Armstrong, D. (1993). Enantiomeric separation of fluorescent, 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate, tagged amino acids. *J. Chromatogr.*, **641**, 257–265.
- Schmidt, G.J., Olson, D.C. and Slavin, W.J. (1979). Amino acid profiling of protein hydrolysates using liquid chromatography and fluorescence detection. *J. Liq. Chromatogr.*, **2**, 1031–1045.
- Soga, T. and Heiger, D.N. (2000). Amino acid analysis by capillary electrophoresis electrospray ionization mass spectrometry. *Anal. Chem.*, **72**, 1236–1241.
- Song, X. and Budde, W.L. (1996). Capillary electrophoresis-electrospray mass spectra of the herbicides paraquat and diquat. *J. Am. Soc. Mass Spectrom.*, **7**, 981–986.
- Spackman, D.H., Stein, W.H. and Moore, S. (1958). Automatic recording apparatus for use in the chromatography of amino acid. *Anal. Chem.*, **30**, 1190–1206.
- Terabe, S., Ishihama, Y., Nishi, H., Fukuyama, T. and Otsuka, K. (1991). Effect of urea addition in micellar electrokinetic chromatography. *J. Chromatogr.*, **545**, 359–368.
- van Leuken, R.G.J., Duchateau, A.L.L. and Kwakkenbos, G.T.C. (1995). Thermospray liquid chromatography/mass spectrometry study of diastereomeric isoindole derivatives of amino acids and amino acid amides. *J. Pharma. Biomed. Anal.*, **13**, 1459–1464.