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PII: S0308-8146(97)00164-7

Food Chemistry, Vol. 62, No. 3, pp. 363–367, 1998

Printed in Great Britain
0308-8146/98 $19.00+0.00
Analytical, Nutritional and Clinical Methods Section

Determination of α-amino nitrogen in pea protein hydrolysates: a comparison of three analytical methods

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(Received 3 April 1997; revised version received and accepted 13 August 1997)

Three spectrophotometric methods, using 2,4,6-trinitrobenzenesulphonic acid (TNBS), *o*-phthaldialdehyde (OPA) or ninhydrin, for the determination of α-amino nitrogen in pea protein isolates and hydrolysates were compared. The determined amounts of α-amino nitrogen differed greatly, depending on the method used. The TNBS and OPA methods produced comparable results, whereas the data obtained with the ninhydrin method were only half of the TNBS or OPA values. Colour stability, recovery of the standard from the protein matrix and reproducibility of the results were determined. The methods showed good accuracy (SE 1–3%) with recovery values of the standard (L-leucine) from the protein matrix of 91, 111 and 75% for the OPA, ninhydrin and TNBS method, respectively. © 1998 Elsevier Science Ltd. All rights reserved.

INTRODUCTION

Peas are grown all over the world but pea protein is only to a limited extent used in food applications. The quality of proteins, and, thereby their utilisation can be improved by enzymatic hydrolysis. One of the prerequisites for obtaining a standard product by enzymatic hydrolysis of proteins is adequate control of the degree of substrate hydrolysis (DH). The DH determination should be based on a selective and appropriately sensitive and rapid analytical method. A widely used method is the method reported by Adler-Nissen (1986), relying on the reaction of α-amino groups with 2,4,6-trinitrobenzenesulphonic acid (TNBS). However, this method is not completely selective for primary amino groups because of colour development due to hydroxy ions present in the medium (Adler-Nissen, 1979). Although this light-catalysed reaction is slow, it causes instability of aqueous TNBS solutions and high blank values. Furthermore, TNBS cannot be applied if mercaptoethanol has been used to disrupt disulphide bonds in the protein molecule, because the reaction products also absorb at the measured wavelength of 340 nm (Kotaki *et al.*, 1964).

Alternatively, ninhydrin (Wiewiórowski *et al.*, 1958) and *o*-phthaldialdehyde (OPA) (Church *et al.*, 1983), which are more selective for α-amino groups than TNBS, may be used as these reagents only react with amino groups or its derivatives. The nitrogen atom is the essential factor for colour development in these colour reactions. A further advantage of the ninhydrin method is its specific reaction with α-amino groups (analytical wavelength, $\lambda_{max} = 570$ nm). Its reaction with ε-amino groups and ammonium ions gives products that absorb at another wavelength, $\lambda_{max} = 440$ nm (Yemm and Cocking, 1955; Schilling *et al.*, 1963; Samejima *et al.*, 1971).

According to reports by Benson and Hare (1975) and Goyal *et al.* (1988), OPA also forms a fluorescent adduct with ammonium ions. Although the authors did not indicate that the complex formed absorbs at analytical wavelength, it is likely. Furthermore, β-mercaptoethanol, widely used as a thiol compound in the OPA assay, initiates a secondary reaction leading to decomposition of the coloured amino-OPA complex (Frister *et al.*, 1986). They reported that as early as 2 min from the start of the reaction the absorbance markedly decreases, causing serious errors in the readings. This reaction, however, may be eliminated and thereby the measuring stability improved, by using

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ethanethiol (Frister *et al.*, 1986) or *N*-acetyl-L-cysteine (Alvarez-Coque *et al.*, 1989).

Although the methods for α-amino nitrogen determination as described above have been used individually frequently, only few comparative studies have been reported (Bertrand-Harb *et al.*, 1993). In this study the TNBS, OPA and ninhydrin methods have been compared to establish accurate and reliable DH measurements for pea protein hydrolysis. Colour stability, reproducibility and recovery of a standard from the hydrolysed protein matrix have been determined and the correlation between the data obtained by the three methods is presented.

MATERIALS AND METHODS

Scope of experiments

The absorbance, at given wavelength, obtained after reactions of L-leucine or amino groups of protein hydrolysate with TNBS, OPA or ninhydrin was recorded for 30 min at 30-s intervals. The amounts of standard or hydrolysate were selected in such a way that the absorbance value was between 0.5 and 0.8. For the OPA assay the absorbance was measured directly after the reagents were mixed and for the TNBS and ninhydrin assays after the period required for colour developement, as described in the assay description. Recovery of L-leucine from the hydrolysed protein matrix was determined by standard addition of L-leucine to the protein hydrolysate with a DH of 4%. Two levels of standard addition were used, resulting in an increase in the absorbance values of 20 and 50% as compared to the values found for unfortified samples. The number of samples in each series was 10. The accuracy of particular analytical methods, expressed as the relative standard error ($n = 15$), was determined with the protein hydrolysate with a DH of 4%. The correlation between the results of the three methods was calculated and presented as linear regression equations. Also, the results were compared using Student's *t*-test for the correlated samples (Bożyk and Rudzki, 1977). The presence of systematic constant and variable errors between the methods was tested using Student's *t*-test (eqns 1–4) for the hypothesis verification that a and b in regression equations do not differ from $A = 1$ and $B = 0$ (Bożyk and Rudzki, 1977).

$$t_{acalc} = \frac{a - A}{s_a} \tag{1}$$

$$t_{bcalc} = \frac{b - A}{s_b} \tag{2}$$

$$s_a = \sqrt{\frac{\sum_{i=1}^{n}(y_i - y_i')^2}{(n-2)\left[\sum_{i=1}^{n} x_i^2 - \frac{1}{n}\left(\sum_{i=1}^{n} x_i\right)^2\right]}} \tag{3}$$

$$s_b = \sqrt{\frac{\sum_{i=1}^{n}(y_i - y_i')^2 \cdot \sum_{i=1}^{n} x_i^2}{(n-2)\left[n.\sum_{i=1}^{n} x_i^2 - \frac{1}{n}\left(\sum_{i=1}^{n} x_i\right)^2\right]}} \tag{4}$$

where x_i is the result obtained using method X; y_i is the result obtained using method Y; and y_i' is the result calculated from the regression equation.

Enzymatic hydrolysis

For enzymatic hydrolysis the pea protein isolate, *Propulse* (Cosucra, Momalle, Belgium) was used. A suspension of protein isolate (25 g/200 ml water, pH 8) was prepared and heated to 50°C. Then 50 ml of trypsin solution (proteolytic activity of 0.375 Anson's Unit/50 ml) was added and the solution mixed. Enzymatic hydrolysis was performed at 50°C and kept at pH 8 by constant addition of a 1 M NaOH solution. Samples were taken after 0, 10, 30, 50, 70 and 90 min of incubation time. To the aliquot of the hydrolysate (2.5 ml), 5 ml of a 1% sodium dodecyl sulphate (SDS) solution was added and the mixture heated at 90°C for 15 min to inactivate the enzyme. The solution was then carefully transferred to a volumetric flask and adjusted to a final volume of 25 ml. This solution will be referred to as 'the examined sample'. The degree of hydrolysis (DH) of the substrate was determined by the TNBS method.

TNBS assay

The assay was performed according to the method reported by Adler-Nissen (1979) with the reagent volume being reduced to 50% of the original volume. In short, to a 0.125-ml aliquot of the examined sample, diluted 2.5-fold, 1 ml of a phosphate buffer (pH 8.2, 0.212 M) was added along with 1 ml of a freshly prepared aqueous solution of TNBS (0.1%). Tightly secured tubes were shaken in the dark at 50°C for 1 h. The reaction was stopped by the addition of 2 ml of a 100 mM HCl solution. After 20 min, 4 ml of water was added and after another 10 min the absorbance was read at 340 nm. The blank was prepared identically and L-leucine was used as the standard ($0–5 \times 10^{-7}$ mol/0.125 ml of sample).

OPA assay

The assay was carried out according to the method reported by Frister *et al.* (1986). In contrast to the

original method, reactions were carried out in tubes instead in quartz cuvetes. To the aliquot, 0.25 ml of examined sample, 10 ml of the solution freshly prepared, of the following composition: 160 mg OPA, 4 ml MeOH, 0.4 ml ethanethiol, and 200 ml 0.1 mol litre^{-1} $Na_2B_4O_7$, was added. After the tubes were tightly secured and then mixed, they were left at room temperature for 20 min. The absorbance was measured at 340 nm. A blank was prepared identically. The standard curve with L-leucine was prepared for concentrations from 0 to 2×10^{-6} mol/0.25 ml of sample.

Ninhydrin assay

The analysis was performed according to the procedure published by Gronowska and Prończuk (1981). To the tubes, containing 2 ml of the examined sample and 2 ml of a phosphate buffer (pH 7.0, 0.080 M), a 1 ml freshly prepared aqueous solution of ninhydrin (1%) was added. The tubes were loosely secured with a stopper and heated in a boiling water bath for 30 min. After cooling, the samples were carefully transferred to volumetric flasks and adjusted to a final volume of 100 ml. The absorbance was read at 570 nm. Blanks were prepared identically. The standard curve was prepared with L-leucine ($0–1\times10^{-5}$ mol/2 ml of sample).

Apparatus and reagents

Absorbance measurements were made in a Beckman DU-7500 spectrophotometer with a diode array detector. The pH control was performed with a Radiometer PHM95 pH-meter. The reagents were: trypsin, L-leucine, ninhydrin (Sigma), sodium dodecyl sulphate, 2,4,6-trinitrobenzenesulphonic acid (Serva), ethanethiol (Merck), *o*-phthaldialdehyde, methanol, buffer components (Polskie Odczynniki Chemiczne, Gliwice, Poland), water 18.2 MΩ cm^{-1} (Millipore Q5 plus).

Table 1. Recovery (%) of standard from the protein matrix using the TNBS, OPA, and ninhydrin assays ($n = 10$)

Standard addition[a]	TNBS	OPA	Ninhydrin
1/2	97 ± 15	100 ± 8	95 ± 2
1/5	74 ± 55	111 ± 11	91 ± 11

[a]See Materials and methods.

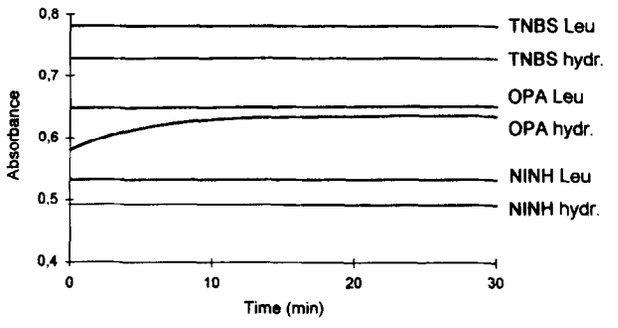

Fig. 1. Colour stability of the reaction mixtures in time for the TNBS, OPA and ninhydrin assays.

RESULTS AND DISCUSSION

In kinetic studies, the stability of coloured products formed by reaction of L-leucine or α-amino groups from the hydrolysate with TNBS, OPA or ninhydrin was

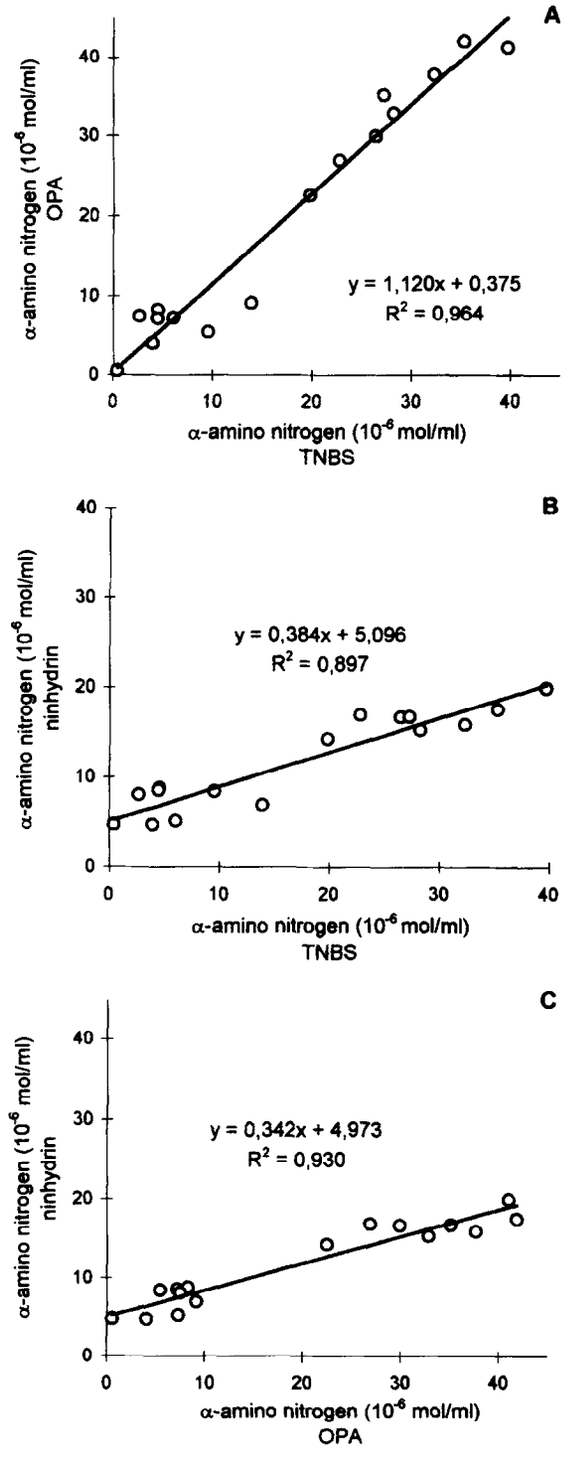

Fig. 2. Correlation of the results of α-amino group determinations in the hydrolysed material obtained with the OPA, ninhydrin and TNBS assays.

determined. The absorbance values obtained by reaction of the samples with ninhydrin or TNBS were stable during 30 min after the end of the reaction (Fig. 1). For the OPA assay, the absorbance of the hydrolysate was stabilised after 20 min, indicating the importance of inclusion of this time period in the analytical procedure. By measuring the amount of amino groups with TNBS, a very high blank value (0.8) was obtained. Therefore, this assay was modified in such a way that the reaction mixture was two-fold diluted after colour development, by addition of 4 ml water (see Materials and methods).

For the ninhydrin and OPA methods the simple measurement errors were similarly low, 0.83 and 0.87%, respectively, whereas for the TNBS method the error was found to be higher (2.49%). Also, good precision of the ninhydrin and OPA methods was found, and good standard recovery values from the protein matrix were measured (Table 1). The relatively high errors found for the recovery of L-leucine from the protein matrix by using the TNBS assay probably result from the heterogeneity of the protein material present in the samples taken for analysis, particularly when small volumes (0.125 ml) are used.

Table 2. Results of the statistical evaluation of the three methods

(A) Significance of the differences between the obtained results using Student's *t*-test

Methods	t_{calc}	t_0
TNBS vs OPA	2.821	2.110
TNBS vs ninhydrin	2.212	2.110
OPA vs ninhydrin	2.763	2.110

(B) Student's *t*-test for the *a* and *b* coefficients in regression equations

Methods	t_{acalc}	t_{bcalc}	t_0
TNBS vs OPA	2.207	0.339	2.120
TNBS vs ninhydrin	18.98	7.736	2.120
OPA vs ninhydrin	27.94	9.103	2.120

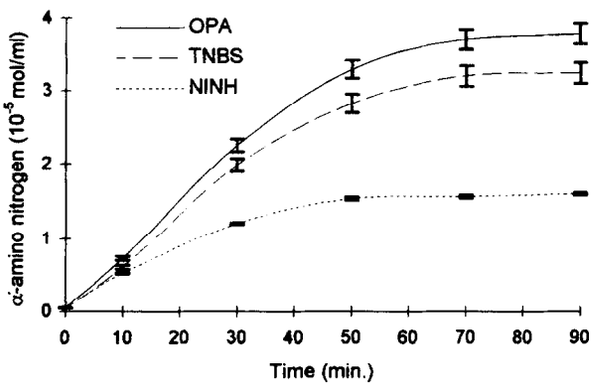

Fig. 3. Changes in the content of α-amino nitrogen during hydrolysis of pea protein as monitored with the OPA, TNBS and ninhydrin methods.

The correlation between the results of the three methods of α-amino nitrogen determination in pea protein hydrolysate is shown in Fig. 2. The results obtained by the OPA assay were highly correlated with those obtained by the TNBS assay (correlation coefficient of 0.964). A weaker correlation of 0.897 was found between the results of TNBS and the ninhydrin assay. The slope index in the regression equation for the OPA and TNBS results was equal to 1.12, close to the value of 1, whereas comparison of the ninhydrin and the TNBS method, remarkably, yielded a slope index of only 0.38. The higher values obtained with OPA compared to the TNBS assay (Fig. 2) are in good agreement with the results of milk protein studies reported by Bertrand-Harb *et al.* (1993). However, the much lower values obtained by the ninhydrin assay are remarkble and could be caused by steric hindrance in the step of oxidative deamination of the N-ends of the large polypeptide molecules (Lamothe and McCormick, 1973).

Statistical evaluation of the methods demonstrated that the results obtained by each method significantly differ from each other (Table 2A). The results of Student's *t*-test (Table 2B) indicate that the differences between the TNBS and ninhydrin methods, and the ninhydrin and OPA methods are due to both systematic variable ($t_{acalc} > t_0$) and stable ($t_{bcalc} > t_0$) errors. The difference between the TNBS and the OPA methods depends only on systematic variable errors ($t_{acalc} > t_0$, $t_{bcalc} < t_0$).

Application of the three analytical methods for the evaluation of pea protein hydrolysis gives very different results for DH (Fig. 3). The curves following the points determined with TNBS and OPA are rather close, whereas the values obtained by the ninhydrin assay are remarkably low. Comparison of the presented results with those obtained with the pH-stat method and consideration of electrophoretic patterns of hydrolysis (Kramać *et al.*, 1996), suggest that the most correct results are obtained with the TNBS and OPA methods. The ninhydrin method gives values that are too low compared to the actual content of α-amino groups liberated during hydrolysis of pea proteins.

ACKNOWLEDGEMENTS

This work was supported by the European Commission within the framework of the Cooperation in Science and Technology with Central and Eastern European Countries, COPERNICUS (CIPA CT 94-0177).

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