OPA method modified by use of N,N-dimethyl-2-mercaptoethylammonium chloride as thiol component

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Modifizierte OPA-Methode mit N,N-Dimethyl-2-mercaptoethylammoniumchlorid als Thiolkomponente

Summary. In the analysis of biological matrices, the application of o-phthaldialdehyde in the presence of a thiol component (OPA-method) for the quantitative determination of free α- and ε-amino groups in amino acids, peptides and proteins as well as their hydrolytic or proteolytic products, respectively, is well known. The most frequently used thiol compound is mercaptoethanol. The use of this substance has the disadvantage that the initially generated isoindole often undergoes intramolecular rearrangement which often leads to a decrease in extinction at the monitoring wavelength of 340 nm. The present paper describes a modified method in which mercaptoethanol was replaced by N,N-dimethyl-2-mercaptoethylammonium chloride. The use of the new thiol component in the OPA reaction results in long-term stability of the extinction values („plateauing" > 1 h). The modified method is further characterized by high accuracy and precision.

Introduction

In the last 10–15 years, o-phthaldialdehyde (OPA) has been widely used [1–4] for quantitative determination of free α- and ε-amino groups in amino acids, peptides and proteins as well as their hydrolytic and proteolytic products in biological materials. The topics involved range from biochemistry and food analysis to automated pre- and post-column derivatization in high performance liquid chromatography of amino acids [1, 5].

Materials and Methods

Extinction measurements. Kontron Instruments Uvikon 860 and Shimadzu spectrophotometer UV-120-01.

Chemicals, amino acids, peptides and proteins were products of Aldrich-Chemie (Steinheim), Sigma-Chemie (Munich), Serva (Heidelberg), Merck (Darmstadt).

Standard amino acid solutions. The concentrations of the aqueous standard amino acid solutions (19 amino acids) were accurately adjusted to around 10.0 nmol × μl^{-1}.

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Determination of the extinction versus time diagram. 10 µl of a standard amino acid mixture (consisting of 19 amino acids) was mixed with 1 ml of freshly prepared OPA solution (40 mg o-phthaldialdehyde, 50 ml 0.1 mol × l^{-1} disodium tetraborate buffer, pH 9.3, 1 ml methanol, 100 mg N,N-dimethyl-2-mercaptoethylammonium chloride). Taking the point of mixing as $t = 0$, the variation with time of the extinction at 340 nm was follwed using a Kontron double beam spectrophotometer with OPA reagent solution as reference.

Determination of the extinction coefficients of the individual standard amino acid solutions. 1 ml of OPA solution was added to each of six 10 µl aliquots of the appropriate amino- and standard solution. The mixtures were allowed to stand for 2 min before determining the extinction at 340 nm using the Shimadzu double-beam spectrophotometer with OPA reagent solution as reference. The extinction coefficients were calculated using the arithmetic mean of the six extinction values and taking into account the initial concentration of the standard amino acid solution.

Concentrations and amounts of aqueous peptide and protein solutions used. Glu-Lys: 88 nmol × 5 μl^{-1}; Thr-Lys-Tyr: 62 nmol × 10 μl^{-1}; Ser-Ser-Ser: 95 nmol × 10 μl^{-1}; Thr-Lys-Pro-Arg (Tuftsin): 84 nmol × 10 μl^{-1}; β-lactoglobulin (bovine, type A): 4.7 nmol × 5 μl^{-1} β-lactoglobulin (bovine, type B): 5.3 nmol × 5 μl^{-1}; α-lactalbumin (bovine): 5.8 nmol × 5 μl^{-1}; α-lactalbumin (human): 5.7 nmol × 10 μl^{-1}.

Determination of extinction of the peptide and protein solutions before hydrolysis. 1 ml of OPA solution was added to each of six 5 µl or 10 µl aliquots of the substrate solution and the extinction at 340 nm after $t = 2$ min was determined using the Shimadzu photometer with OPA reagent solution as reference. For extinction measurement of the milk proteins 1.25 ml of an aqueous 20% (g/g) SDS solution was added to the initial OPA solution.

Hydrolysis of the peptides and proteins. Six samples of the original or diluted substrate solutions, each sample of maximum volume 10 µl, were mixed with 200 µl 6 mol × l^{-1} HCl solution in 5 ml glass vials which were then sealed under vacuum and kept for 24 h at 110° C in a laboratory sandbath. After cooling the hydrolyzed samples were neutralized with 200 µl 6 mol × l^{-1} NaOH solution.

Determination of extinction of the peptide and protein solutions after hydrolysis. 1 ml of OPA solution was pipetted

Fresenius Z Anal Chem (1988) 330:631–633

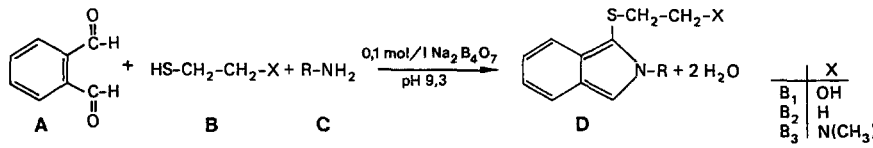

Fig. 1
Formation of 1-alkylthio-2-alkylisoindoles

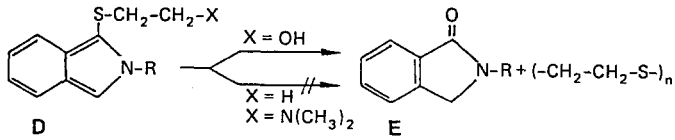

Fig. 2. Formation of isoindolones

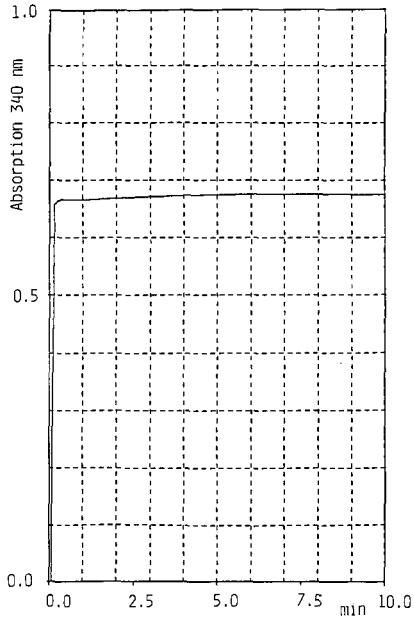

Fig. 3. Extinction versus time diagram of the OPA reaction of a mixture of 19 different OPA-sensitive amino acids

into each of the neutral hydrolyzed preparations and the extinction at 340 nm of 1 ml of the reaction solution was measured against the pure OPA solution after $t = 5$ min Parallel to this a blank value (after hydrolysis) was determined for calculating yield.

Results and discussion

The measuring principle is based on the formation of 1-alkylthio-2-alkylisoindoles (D; Fig. 1) generated by the reaction of a primary amino group with o-phthaldialdehyde (A) in the presence of a thiol component (B). The thiol component normally used is mercaptoethanol (B1).

However, the range of application of mercaptoethanol is limited because of the frequently occurring, rather rapid, matrix-dependent decrease in extinction. This decrease results from the conversion of the primarily formed bis-alkylated isoindole to 2,3-dehydro-1H-isoindole-1-one (E; Fig. 2). The formation of isoindolones is induced by an intramolecular nucleophilic attack on the C1-atom of the pyrrole system by the hydroxyl group of mercaptoethanol. This leads to false extinction values because isoindolones do not possess an absorption maximum at 340 nm [6, 7].

Table 1. Extinction coefficients of amino acids by use of N,N-dimethyl-2-mercaptoethylammonium chloride as thiol component

	ε_{340}	s	CV (%)
	($mmol^{-1} \times l \times cm^{-1}$)		
Glycine	7.09	0.08	1.13
Alanine	6.53	0.08	1.23
Valine	6.41	0.06	0.94
Leucine	6.29	0.06	0.95
Isoleucine	6.43	0.06	0.93
Serine	6.46	0.06	0.93
Threonine	6.40	0.08	1.25
Aspartic acid	6.45	0.02	0.31
Asparagine	6.48	0.02	0.31
Glutamic acid	6.54	0.05	0.76
Glutamine	6.39	0.05	0.78
Lysine	12.96	0.09	0.69
Histidine	6.24	0.07	1.12
Arginine	6.44	0.08	1.24
Tyrosine	6.31	0.08	1.27
Tryptophan	5.98	0.03	0.50
Phenylalanine	6.20	0.05	0.81
Methionine	6.42	0.08	1.25
O-Phosphoserine	6.57	0.07	1.07

$\varnothing\ \varepsilon_{340}$ for an OPA-sensitive amino group:
$6.42 \pm 0.20\ mmol^{-1} \times l \times cm^{-1}$

Substitution of mercaptoethanol by ethanethiol (B2; Fig. 1 [8, 9]) prevents the rearrangement of alkylthioalkylindole mentioned above and gives stable extinction values over a longer period of time [4]. A disadvantage remains a strong, repulsive odour formation and a plateau in the extinction versus time curve which is not always pronounced and constant.

We have modified the OPA method by using N,N-dimethyl-2-mercaptoethylammonium chloride (powder; does not give off a repulsive odour) which has not been applied so far for the OPA reaction (B3; Fig. 1). The new thiol component (B3) used for the determination of OPA-sensitive amino groups gives stable extinction values over a long measuring period (> 1 h). This stable extinction behaviour is demonstrated in Fig. 3 for the reaction of a mixture of 19 amino acids with OPA.

Spectroscopic measurements of the individual amino acid-OPA reaction products gave the extinction coefficients shown in Table 1.

Each of the 19 amino acids contain 1 α-NH_2-group which is OPA-sensitive. Lysine contains 2 OPA-sensitive NH_2-groups (α- and ε-terminal NH_2-group) and is, therefore, bifunctional. Proline (does not react with OPA) and cysteine (competitive reaction because of SH residue) have not been measured. For quantitation of amino acid mixtures and proteolytic products the mean extinction coefficient of an OPA-sensitive amino group was found to be $\varepsilon = 6.42 \pm 0.20\ mmol^{-1} \times l \times cm^{-1}$.

Table 2. OPA reaction of several substrates before and after hydrolysis with HCl

	OPA-sensitive NH_2-groups before hydrolysis[a]	Recovery ± s (% of the theor. calculated value)	OPA-sensitive NH_2-groups after hydrolysis[b]	Recovery ± s (% of the theor. calculated value)
Glu-Lys	2	91.2 ± 1.3	2.85	93.1 ± 0.6
Thr-Lys-Tyr	2	87.1 ± 0.8	3.80	87.9 ± 0.8
Ser-Ser-Ser	1	94.6 ± 2.1	2.85	95.2 ± 0.9
Thr-Lys-Pro-Arg	2	98.9 ± 1.3	3.80	107.8 ± 1.1
β-Lactoglobulin A (bovine)	16	97.7 ± 1.2	158.65	93.7 ± 1.3
β-Lactoglobulin B (bovine)	16	101.8 ± 0.9	158.65	100.0 ± 2.0
α-Lactalbumin (bovine)	13	95.5 ± 1.2	122.55	95.6 ± 0.7
α-Lactalbumin (human)	13	96.0 ± 0.6	123.50	93.3 ± 0.9

[a] Calculation: N-terminal α-NH_2-group + ε-terminal NH_2-groups of Lys

[b] Calculation: α-NH_2-groups of amino acids + ε-terminal NH_2-groups of Lys−Trp−Pro − 5% destruction of amino acids by HCl hydrolysis

Repeatability was determined for each of the 19 amino acids (6-fold analysis in series). The variation coefficient of the mean values ranges from 0.31 to 1.27% (Table 1).

Recovery of OPA-sensitive amino groups was determined for several peptides and proteins before and after HCl hydrolysis on the basis of the mean extinction coefficient calculated for an OPA-sensitive amino group (Tables 1, 2).

The number of OPA-sensitive amino groups before hydrolysis results from the lysine side chains (ε-terminal NH_2) as well as the α-terminal NH_2-group at the N-terminus.

The number of OPA-sensitive amino groups after hydrolysis results from:

a) the total number of amino acids,
b) the number of lysine (bifunctional) residues,
c) the number of proline (no OPA reaction) residues,
d) the number of tryptophan (complete destruction during hydrolysis) residues,
e) a general destruction rate of approxymate 5%.

In conclusion, the results obtained indicate that this modified OPA method is characterized by good repeatability and recovery in the analysis of free amino groups in biogenic amines, amino acids, peptides and proteins as well as their hydrolytic and proteolytic products. Furthermore, the stability of the extinction values, make the reaction particularly suitable for pre- and post-column derivatization in HPLC.

Acknowledgements. We wish to thank Mrs. Enken Jacobsen for technical assistance. Mrs. Karin Meyer-Bergatt and Dr. David Tait are acknowledged for their help with the English version of the manuscript. This work was financially supported by the European Commission (EWG VO No. 1150/86-14.5).

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Received October 14, 1987