Protein determination
Automated assay
Marine chemistry

Détermination de protéines
Essai automatisé
Chimie marine

Automated assay for the quantification of dissolved proteins in natural seawater samples

Klaus NAGEL, Ferdinand LIEMANN
Institut für Biochemie und Lebensmittelchemie, Abteilung Biochemie der Universität Hamburg, Martin-Luther-King-Platz 6, D-2000 Hamburg, FRG.

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ABSTRACT

An assay is described that permits the simultaneous determination of concentrations of total dissolved proteins and total "dissolved free amino acids" in natural seawater samples. It can be performed automatically even during field experiments using an AutoAnalyzer system or a modified amino acid analyzer. Except filtration, no further pretreatment of the samples is necessary. The determination is based on the reaction of o-phthaldialdehyde (OPA) with proteins or amino acids, respectively, after separating both components by a column chromatography step. For routine analysis of protein concentrations in natural seawater samples, the lower detection limit is at least 0.5 ng/sample. As the sample volume can be varied between 25 µl and 2000 µl, concentrations below 0.5 µg/l can be analyzed. The determination of protein concentration in a single seawater sample takes about 30 minutes.

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RÉSUMÉ

Détermination quantitative automatisée des protéines dans des échantillons d'eau de mer naturelle

Une méthode de détermination simultanée des concentrations en protéines totales dissoutes et en acides aminés libres dissous dans des échantillons d'eau de mer est décrite. Les analyses automatiques peuvent être effectuées également à bord, à l'aide d'un système AutoAnalyzer ou d'un analyseur d'acides aminés modifié. A part la filtration, aucun traitement préliminaire des échantillons n'est nécessaire. La détermination est fondée sur la réaction de la o-phthaldialdéhyde (OPA) avec les protéines ou les acides aminés, respectivement, après séparation de ces deux composés par chromatographie sur colonne. Pour les analyses de routine des protéines, la limite inférieure de détection est de 0,5 ng/échantillon. Des concentrations inférieures à 0,5 ng/l peuvent être mesurées, car le volume des échantillons peut varier entre 25 et 2000 µl. La mesure de la concentration en protéines d'un échantillon d'eau de mer nécessite environ 30 minutes.

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INTRODUCTION

In many ecological studies, efforts have been made to analyze and quantify dissolved materials occurring in the aquatic environment. Because of its important role, the nitrogen cycle in aquatic ecosystems is one of the main topics of investigation. For this purpose, several methods are available to determine the concentrations of total nitrogen and of other nitrogen-containing substances – except dissolved proteins – by automated procedures, even during field experiments.

The quantification of dissolved proteins in natural seawater is sometimes difficult, mainly because of the rather low concentrations and because of problems arising from interfering materials usually present in these samples. Despite these difficulties, some experi-

ments show that concentrations of dissolved proteins in seawater lie in the range of 0-200 µg protein/l (Baier *et al.*, 1974; Dawson, Liebezeit, 1981; Dawson, Pritchard, 1978; Maita, Yamada, 1978; Siegel, Degens, 1966). However, methods used in these works are hardly suitable for analyzing a large number of samples aboard ship during field experiments.

In recent decades, various methods have been published for estimating concentrations of dissolved proteins in aqueous solution, mainly differing in sensitivity and specificity (Lowry *et al.*, 1951; Warburg, Christian, 1942; Robinson, Hogden, 1940; Schaffner, Weissmann, 1973; Bradford, 1976). Because they lack sensitivity and/or specificity, the quantification of proteins in natural seawater by these methods requires some kind of sample pretreatment. Apart from the question of the time necessary for this purpose, errors may be caused as a result of adsorption effects, precipitation, aggregation or contamination. Because of such problems and the fact that these procedures can not be performed automatically, the above-mentioned methods are hardly suitable for estimating protein concentrations in seawater samples during field experiments.

Some years ago, fluorescent reagents such as fluorescamine or o-phthaldialdehyde (OPA) were introduced into analytical biochemistry, predominantly for the detection of low concentrations of amino acids and other primary amines (Udenfriend *et al.*, 1972; Roth, 1971). As these dyes react in highly specific fashion with compounds containing primary NH_2-groups (Weigele *et al.*, 1972; Svedas *et al.*, 1980) they had already been used to establish a very sensitive and specific procedure for the quantification of proteins in dilute solutions (Böhlen *et al.*, 1973; 1974; Benson, Hare, 1975; Torres *et al.*, 1976; Creaser, Hughes, 1977).

The automated assay described in this paper has been developed for the determination of concentrations of total dissolved proteins and total "dissolved free amino acids" in natural seawater samples during field experiments immediately after sampling. The determination is based on the reaction of OPA with proteins or amino acids, respectively. The determination of "dissolved free amino acids" in natural seawater samples by an automated assay comparable with that described here has already been published by Josefsson *et al.* (1977). It should be pointed out, however, that the term "dissolved free amino acids" in Josefsson's work includes – besides free amino acids – all components reacting with OPA, *e.g.* proteins, ammonia, amino sugars, *etc.*, whereas in the assay presented here, the term "dissolved free amino acids" is restricted to substances with molecular weights of about 100-250 d, and which are thus mainly free amino acids, amino sugars and very small peptides, and excludes proteins and ammonia.

Advantages of this assay are its high sensitivity and high specificity for proteins after removal of amino acids and other low molecular weight components. Furthermore, the concentration of "dissolved free amino acids" can be estimated simultaneously without additional experimental expenditure. With slight modifications, nearly all of the commonly used AutoAnalyzer or amino acid analyzer systems can be employed for this procedure.

MATERIALS AND METHODS

All proteins used to establish this assay were obtained from Boehringer (Mannheim), E. Merck (Darmstadt), Serva (Heidelberg), or Sigma (München). Before use all proteins were desalted and checked for purity by SDS-acrylamide electrophoresis according to Laemmli (1970). Amino acid standard H (Hamilton, Reno, USA) was used to calibrate the quantification of free amino acids. O-phthaldialdehyde was obtained in a concentrated solution as "Fluescin" from E. Merck. Sephadex was supplied by Pharmacia Fine Chemicals (Freiburg). All other chemicals (analytical grade) were obtained from E. Merck.

OPA-reagent buffer contains 600 mM boric acid, 60 mM 2-mercaptoethanol, 0.001% Brij 35, and 4 ml Fluescin/l buffer. With solid KOH the pH is adjusted to 10.6. Synthetic seawater consists of 480 mM NaCl, 32 mM $MgSO_4$, 30 mM boric acid, and 0.005% $HgCl_2$. Buffer A contains 240 mM NaCl, 25 mM $MgSO_4$, 30 mM boric acid, and 0.005% $HgCl_2$. The pH of synthetic seawater and of buffer A is adjusted to 8.0 with 5 N KOH. To prevent adsorption of ammonia from air, all buffers are purged with N_2.

All measurements were carried out at least as triplicates. For establishing the calibration curves, at least two separately prepared standards were used. Estimation of protein concentration in seawater samples was throughout calibrated using bovine serum albumin (BSA) as standard protein.

The principal scheme for the simultaneous estimation of concentrations of dissolved proteins and "dissolved free amino acids" is given in Figure 1. Buffer A is continuously pumped by a peristaltic pump (*e.g.* Gilson Minipuls) via a sample injector and a Sephadex G-10 column (250 × 10 mm) into a mixing device. Here the

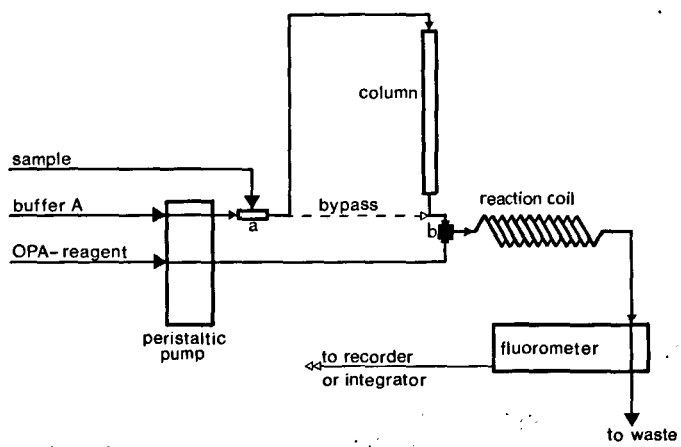

Figure 1

Flow scheme for the procedure of simultaneous determination of concentrations of dissolved proteins and "dissolved free amino acids" in natural seawater samples. For further details see Materials and Methods. a: sample injector; b: mixing coil.

effluent of the column is mixed with an equal volume of OPA-reagent buffer. The mixture is subsequently pumped via a reaction coil directly into the fluorometer. To yield a fluorescence independent of minor variations in temperature and instrumental equipment, delay time in the coil should be 6 min. (± 2 min.). The peak areas registrated by the fluorometer (*e.g.* Gilson GLO Filter Fluorometer, excitation 350 nm, emission 455 nm) are directly proportional to the concentrations of proteins and amino acids, respectively.

RESULTS

By the assay procedure described in this paper, the simultaneous estimation of total concentrations of dissolved proteins and "dissolved free amino acids" in natural seawater samples can be performed automatically, even during field experiments. Proteins, "free amino acids", and other low molecular weight compounds were separated by gel-filtration prior to measurement (Fig. 2). If concentrations of pure proteins in the absence of interfering materials are to be determined, the gel-filtration step may be omitted (shown as "bypass" in Fig. 1). Using the bypass, the assay can be run at a rate of 40 samples per hour.

By this method, proteins can be accurately estimated within a concentration range between 0.5 and 400 ng/l using different electronical range settings of the fluorometer. Standard deviations for this assay are below 5% for protein concentration greater than 25 ng/l and below 10% in the concentration range between 1 ng/l and 25 ng/l.

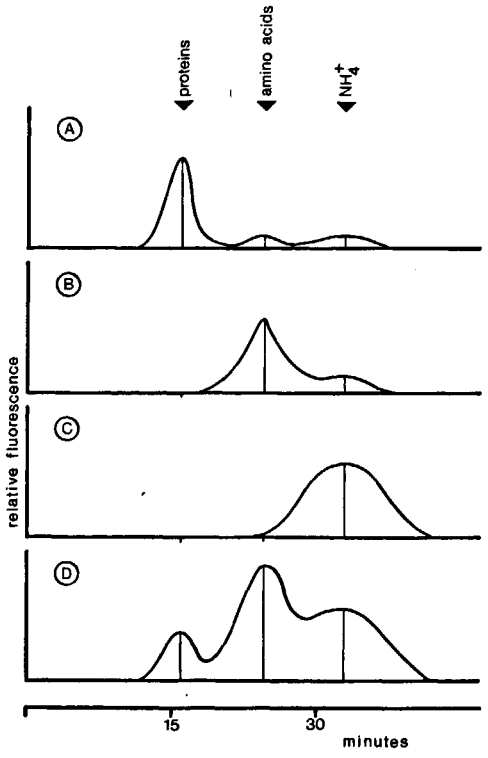

Figure 2

Separations of proteins, free amino acids, and ammonia. A: 50 ng BSA without "desalting" prior to measurement; B: 250 pmoles of amino acid standard mixture; C: Buffer containing ammonia (concentration not calibrated); D:. Sample of natural seawater collected in the German Bight.

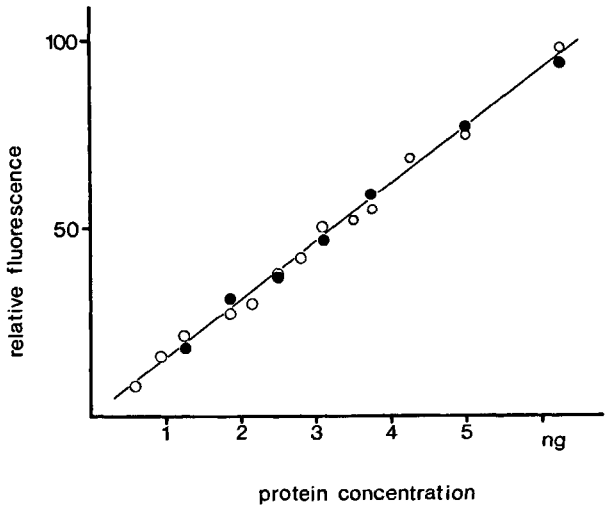

Figure 3

Influence of natural seawater upon the calibration curve of BSA: ● - ●: calibration curve of BSA in the presence of 30 mM borate buffer; ○ - ○: calibration curve of BSA dissolved in a natural seawater sample collected in the German Bight. The fluorescence of the seawater without added BSA was subtracted from each measured value.

To demonstrate that natural seawater has no effect on this protein assay, two calibration curves were measured. For the first, the BSA standard was dissolved and diluted with 30 mM boric acid (pH 8.0); in the second case the protein was dissolved and diluted with a natural seawater sample collected in the German Bight. For better comparison, the fluorescence of the seawater alone without added proteins was subtracted from each value of the second curve. As shown in Figure 3, the components of natural seawater do not influence the estimation of protein concentration at all. As already reported by Josefsson *et al.* (1977), the quantification of "dissolved free amino acids" is not affected either by the substances normally occurring in natural seawater.

In further control experiments it has been proved that the concentrations determined for dissolved proteins are not affected by variable amounts of free amino acids or other low molecular weight interfering materials in the sample. Concentrations of "dissolved free amino acids" are also independent of those of the other components separated by the gel-filtration step.

No significant differences between the integration values of the recorded fluorescence peaks are found if equal amounts of pure proteins are measured after the column chromatography step or via the bypass. Using the bypass, peaks are very high and narrow, whereas peaks after the column chromatography step become much lower and broader. The advantage of correlating protein concentration to peak areas rather than to peak heights is that the measured protein concentration is almost independent of the sample volume injected (greatest volume tested was 2.0 ml). However, as an increase of sample volume results in a broadening of separated peaks during the column chromatography step, for routine analysis of natural seawater samples using a column with the dimensions given above, sample sizes of 0.5 or 1.0 ml were found to be optimal in most cases.

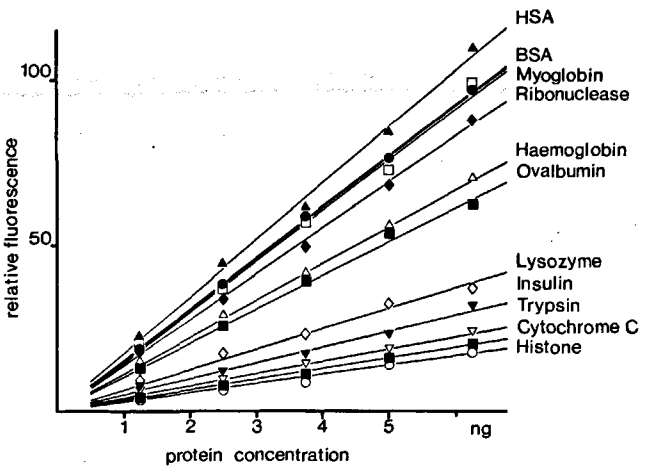

Figure 4
Comparison of the relative fluorescence responses of several pure proteins.

Flow rate is not critical for the reaction of proteins or amino acids with OPA-reagent, if a delay time of about 6 min is maintained. However, by varying the flow rate, the separation of proteins, amino acids and other low molecular weight materials during the column chromatography step may be affected, as optimal separation depends on flow rate, temperature and column dimensions. Using a column with the dimensions given above, best results have been obtained with flow rates of 1 ml/min. The influence of the temperature was found to be negligible in the range between 15 and 25°C.

To reduce background fluorescence and prevent precipitation of seawater compounds during mixing of reagent and sample in the mixing device, OPA-reagent is buffered at pH 10.6. The high salt concentration of buffer A was chosen, because some precipitation was occasionally observed at lower salt concentrations.

As known from other procedures for the estimation of protein concentration, individual proteins yield different responses in the assay reaction. In Figure 4, fluorescence responses of several pure proteins in the OPA-reaction are shown. Although there are considerable differences in the relative fluorescence of equal amounts of the individual proteins, linear calibration curves are obtained for all proteins examined in a range from 1 to 400 ng.

DISCUSSION

Apart from the fact that it can be performed automatically, the described method for the simultaneous determination of total concentrations of dissolved proteins and "dissolved free amino acids" has the advantage of being highly specific and very sensitive. Apart from the injection of the filtered seawater sample, no further experimental expenditure is required. As protein concentration as low as 5 µg/l can be determined accurately in routine analysis during field experiments, the method is sensitive enough to quantify proteins even in those samples that contain less proteins than usually found in seawater.

Differences in the relative fluorescence of equal amounts of individual pure proteins certainly constitute a severe disadvantage of this procedure. However, similar differences have been reported for other protein assays (Weidekamm *et al.*, 1973; Pierce, Suelter, 1977; Van Kley, Hale, 1977; Lewis, 1979). In contrast to most other methods, linear calibration curves were found for all proteins tested. Since differences in the fluorescence of individual proteins are mainly based on the amino acid composition and on protein structure, they can hardly be circumvented. For a more precise estimation of protein concentration in natural seawater samples, it would be necessary to use those proteins for calibration, that occur in seawater. However, to follow the isolation process of such proteins, one must have at least an approximate value for protein concentration at each isolation step.

Another advantage of the procedure described here is, that it will permit – besides quantification – the classification of several classes of proteins by only slight modifications. For example, the concentration of "glycoproteins" in natural seawater samples may be easily analyzed by replacing the Sephadex G-10 column by an appropriate ConA-affinity column. Although some modifications in the buffer system will be necessary, this determination can also be performed according to the same principal scheme shown in Figure 1.

By the procedure described here, protein concentrations in several series of natural seawater samples collected in different field experiments have been analyzed. Using BSA for calibration, protein concentrations in these samples varied between less than 0.5 and 15 µg/l. In samples collected in the German Bight, dissolved proteins were found to be one of the major components of nitrogen – containing substances (Fig. 5). It was estimated that proteins contribute to the amount of total dissolved nitrogen by about 25% [for further details see Kattner *et al.* (1985)]. Even if one must admit some errors arising from the fact that BSA may not be the best choice for calibrating the assay, these relative values may contribute to a better understanding of nitrogen cycle in marine ecosystems.

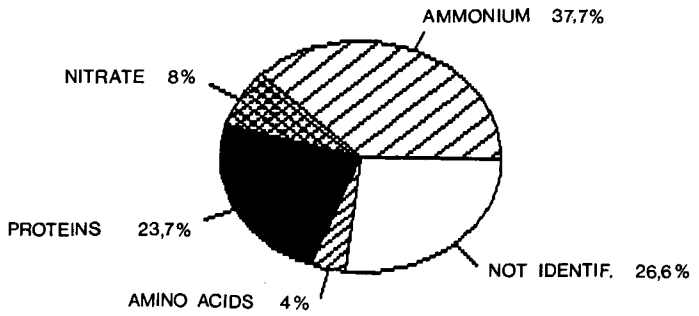

Figure 5
Mean of the relative proportions of dissolved nitrogen containing substances collected during the NOFF 77 experiment in the German Bight [for details see Kattner et al. (1985)].

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