Journal of Immunological Methods 387 (2013) 317–321

Contents lists available at SciVerse ScienceDirect

Journal of Immunological Methods

journal homepage: www.elsevier.com/locate/jim

Technical note

Determination of protein concentration for protein–protein conjugates using ultraviolet absorption

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ARTICLE INFO

Article history:
Received 19 July 2012
Received in revised form 12 September 2012
Accepted 16 October 2012
Available online 23 October 2012

Keywords:
Concentration of protein–protein conjugate
Molar ratio
Microsoft Excel solver
Amino acid analysis
Least squares analysis

ABSTRACT

The present study reports a method to determine the total protein concentration or concentration of a protein of interest in a protein–protein conjugate using ultraviolet absorption, after determining the molar ratio of proteins in the conjugates, from which an extinction coefficient can be calculated. A Microsoft Excel solver-based template using amino acid analysis data was developed for determining the molar ratio. The percent mass of each protein in the conjugate is calculated from the amino acid composition data using the least squares method in the Microsoft Excel solver function, and the percent mass is converted to molar portion of each protein using corresponding molecular weight. A molar ratio is obtained by dividing the molar portion of protein 1 by the molar portion of protein 2. A weighted extinction coefficient is calculated using the molar ratio, and the total protein concentration is determined using ultraviolet absorption at 280 nm. The accuracy of the method was verified using mixtures of known proteins. The present study provides a rapid, simple and accurate method for determining protein concentration in protein–protein conjugates.

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1. Introduction

Conjugating a poorly immunogenic compound to a highly immunogenic carrier protein to increase immunogenicity is a common practice in biological science field and has broad applications. Our previous studies demonstrated that significantly higher antibody titers could be achieved when Pfs25 or Pfs28, leading malaria transmission-blocking vaccine candidate antigens, was conjugated to the outer-membrane protein complex (OMPC) of *Neisseria meningitidis* serogroup B (Wu et al., 2006) or a recombinant nontoxic *Pseudomonas aeruginosa* ExoProtein A (rEPA) (Qian et al., 2007, 2009). An accurate assessment of the concentration of a conjugated protein is essential to downstream investigations. The most critical step in the determination is to assess the molar ratio of protein–protein conjugates, which can be used to calculate the extinction coefficient of the conjugate. A number of methods have been developed to estimate the molar ratio of protein–protein conjugates, including radioactively labeled protein (Green et al., 1982), sodium dodecyl sulfate (SDS) electrophoresis (Jones et al., 1989), spectrophotometric method (Jones et al., 1989; Sashidhar et al., 1994), matrix-assisted laser desorption/ionization time of light (MALDI-TOF) mass spectrometry (Pakarinen et al., 2002), and capillary electrophoresis (Safi et al., 2007). However, the ratios determined by these methods were rough estimates and may not be suitable for the accurate measurement of the protein concentration of a conjugate.

It appears that amino acid analysis is the most accurate method for determining the molar ratio of protein in protein–protein conjugates. Antoni and Presentini (1989) reported DOS- and least-squares-based methods for the determination of molar

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0022-1759/$ – see front matter. Published by Elsevier B.V.
http://dx.doi.org/10.1016/j.jim.2012.10.011

ratios of two different proteins in conjugates using the results of amino acid analysis. Shuler and co-workers presented a comprehensive Microsoft Excel- and least-squares-based methods to determine the ratios of small peptides to keyhole limpet hemocyanin (KLH) using amino acid analysis (Shuler et al., 1992). As technology rapidly evolves, the program written in basic language for the VAX 750 computer described in Antoni and Presentini's paper is no longer suitable for today's applications; and the method developed by Shuler depends on the method used to calculate protein composition from amino acid analysis data, which requires extensive verification.

In this communication, we present a simple and accurate method by which the molar ratio of protein–protein conjugates can be determined by a Microsoft Excel solver-based template using amino acid analysis data. The total protein concentration in the conjugate and the concentration of the individual protein components can be accessed using calculated extinction coefficients (Pace et al., 1995). The accuracy of this method was verified by calculating the molar ratios in known mixtures of proteins. This method should have general applications where the protein concentration of protein–protein conjugates must be estimated.

2. Material and method

2.1. Antigen and carrier proteins

The recombinant *Pichia pastoris* expressed Pvs25 (MacDonald and Narum, unpublished), Pfs28 (MacDonald and Narum, unpublished), and AMA1–FVO (Kennedy et al., 2002) proteins, as well as the *Escherichia coli* expressed ExoProtein A (rEPA) (Qian et al., 2007) protein were manufactured with methods developed at the Laboratory of Malaria Immunology and Vaccinology (LMIV), National Institute of Allergy and Infectious Diseases, National Institutes of Health, and the protein concentrations were determined by ultraviolet absorption at 280 nm. BSA was purchased from Thermo Fisher Scientific.

2.2. Protein mixture preparation

The known molar ratio of protein mixture was prepared according to Table 1. The % mass of protein 1 and protein 2, and the experimentally prepared molar ratio of protein 1/protein 2 were also summarized in Table 1.

2.3. Amino acid analysis

The amino acid composition was determined by the W.M. Keck Foundation Biotechnology Resource Laboratory at Yale University (New Haven, CT). The samples were hydrolyzed in vacuo for 16 h at 115 °C in 100 μL of 6 N HCl/0.2% phenol (with 1 nmol norvaline/100 μL as an internal standard) to digest the protein into free amino acids. After hydrolysis, the HCl was dried off in a vacuum-centrifuge and the resulting amino acids were dissolved in 100 μL of 0.02 N HCl (with 2 nmol taurine/100 μL as a second internal standard). Amino acid analysis was carried out on a Hitachi L-8900 PH Amino Acid Analyzer which used an ion-exchange column with pH and temperature gradients to separate the amino acids and post-column derivatization with ninhydrin for detection at 570 nm and 440 nm. EZChrom Elite (for Hitachi) software was used to operate the analyzer and collect and analyze the data.

2.4. Selection of amino acids used in the calculation

Fourteen amino acids were used in our calculation. During HCl hydrolysis, asparagine is converted to aspartic acid and glutamine is converted to glutamic acid; therefore, these amino acids were reported as aggregate Asx and Glx values. Cysteine, tryptophan, threonine and serine recoveries are typically low, methionine may be partially oxidized, and proline quantitation is often inaccurate due to interference from cysteine, so those amino acids were excluded from the calculation.

2.5. Calculation of the molar ratio of protein mixture

Table 2 is a Microsoft Excel template for molar ratio calculation. The percent composition of each amino acid in each protein (or conjugate) was obtained by dividing the experimental nmol of the amino acid of protein 1 (X1), protein 2 (X2), or conjugate (Y) with total nmol ($\sum$) of X1, X2, or Y to produce normalized *X*1 (protein 1), *X*2 (protein 2) and *Y* (conjugate), respectively. *Y* represents the experimentally determined percent composition of an amino acid in a conjugate. The theoretical percent composition (Υi) of an amino acid was calculated by formula: $\Upsilon i = (X1_i \times Z1) + (X2_i \times (1 - Z1))$, where i represents an amino acid and Z1 represents the % mass of protein 1 which is produced when $\sum (Y - \Upsilon)^2$ is the smallest as determined by the Microsoft solver (see Appendix A). Subse-

Table 1
Preparation of the known molar ratio of protein mixture.

Protein 1	Protein 2	Conc. of protein 1 (mg/mL)	Conc. of protein 2 (mg/mL)	Volume of protein 1 in mixture (μL)	Volume of protein 2 in mixture (μL)	% mass of protein 1[a]	% mass of protein 2[b]	Molar ratio of protein 1/protein 2[c]
Pfs25	BSA	0.770	0.752	37.5	12.5	75%	25%	1.111
				25	25	51%	49%	3.332
				12.5	37.5	25%	75%	9.995
AMA1–FVO	EPA	0.437	0.841	5	15	15%	85%	0.187
				10	10	34%	66%	0.562
				15	5	61%	39%	1.685
Pvs25	EPA	0.690	1.265	15	10	45%	55%	2.650
Pvs28	EPA	1.308	1.225	10	10	52%	48%	3.396

[a] % mass of protein 1 = [(volume of protein 1 in the mixture × concentration of protein 1) ÷ (volume of protein 1 in the mixture × concentration of protein 1 + volume of protein 2 in the mixture × concentration of protein 2)] × 100.
[b] % mass of protein 2 = [(volume of protein 2 in the mixture × concentration of protein 2) ÷ (volume of protein 1 in the mixture × concentration of protein 1 + volume of protein 2 in the mixture × concentration of protein 2)] × 100.
[c] Molar ratio of protein 1/protein 2 = (% mass of protein 1 ÷ molecular weight of protein 1) ÷ (% mass of protein 2 ÷ molecular weight of protein 2).