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Matrix-assisted laser desorption ionization mass spectrometry of membrane proteins: Demonstration of a simple method to determine subunit molecular weights of hydrophobic subunits

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Abstract

Matrix-assisted laser desorption ionization (MALDI) mass spectrometry has been used to obtain accurate molecular weight information for each subunit of several hydrophobic integral membrane proteins: cytochrome bo_3 (4 subunits) and cytochrome *bd* (2 subunits) from *E. coli*, and the bc_1 complex (3 subunits) and the cytochrome *c* oxidase (3 subunits) from *Rhodobacter sphaeroides*. The results demonstrate that the MALDI method is a convenient, quick, sensitive and reliable means for obtaining the molecular masses of the subunits of purified multisubunit membrane proteins. © 1997 Elsevier Science B.V.

Keywords: MALDI; Hydrophobic; Protein; Molecular mass

1. Introduction

The introduction of the technique of matrix-assisted laser desorption ionization (MALDI) mass spectrometry, has led to the accurate measurements of the molecular masses of a variety of biological macromolecules, including proteins [1,2]. The technique, which is capable of ionizing intact proteins and providing molecular masses with a high degree of accuracy, has been particularly successful with water-soluble proteins and polypeptides with molecular weights up to 300 KDa [3,4]. In this work, the use of MALDI is demonstrated for determining the molecular masses of very hydrophobic integral membrane proteins. Membrane-bound hydrophobic proteins, such as respiratory complexes, receptors, channels and transporters, present well known problems in sample-handling due to their propensity to aggregate, to bind detergent and due to incomplete unfolding, even in the presence of sodium dodecylsulfate (SDS) [5]. Often, the molecular weight estimates by SDS–PAGE of very hydrophobic proteins are artifactually low due to abnormal protein–detergent interactions in relation to water-soluble polypeptide standards. However, the application of mass spectrometry methods to determine molecular weights of hydrophobic proteins has been very limited [6–8].

Matrix-assisted laser desorption ionization (MALDI) mass spectrometry is applied in the current work to determine the molecular weights of each subunit of four different purified multisubunit inte-

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gral membrane protein complexes. The results demonstrate that MALDI is a convenient and reliable means to determine the molecular weights of membrane proteins.

2. Materials and methods

2.1. Protein purification

Purification of the cytochrome *bd* oxidase was performed as described previously [9], but with minor modifications which are described by Kaysser et al. [10]. Purification of cytochrome bo_3 was performed as described previously [11], whereas the His-tagged version of this enzyme was purified on a Qiagen Ni^{2+}–nitrilotriacetic acid (NTA) agarose column by low pressure nickel affinity chromatography [12]. Purification of the His-tagged bc_1 complex from *Rhodobacter sphaeroides* (Salcedo-Hernandez et al., manuscript in preparation) and of the His-tagged aa_3-type cytochrome *c* oxidase from *R. sphaeroides* [13] were also performed using the Qiagen Ni^{2+}–NTA resin. MALDI was performed with two different versions of the bc_1 complex, one in which the six-histidine tag was introduced at the C-terminus of the cytochrome *b* subunit and another where the tag was at the end of the cytochrome c_1 subunit of the complex.

2.2. Mass spectrometry

Molecular mass determination was done using a laser desorption VG TofSpec 'time-of-flight' spectrometer. The purified protein solutions were as follows. Cytochrome *bd*: 30 μM enzyme in 0.05% sarcosyl, 5 mM EDTA, 10 mM sodium phosphate buffer, pH 7.5; cytochrome bo_3: 30 μM enzyme in 0.025% sarcosyl, 1 mM EDTA, 50 mM potassium phosphate buffer, pH 8.3; cytochrome bc_1 complex: 30 μM enzyme in 100 mM histidine, 0.01% dodecyl maltoside, 50 mM MOPS, 30 mM sodium sulfate, 5 mM magnesium sulfate, and 20% w/v glycerol, pH 7.0; and cytochrome *c* oxidase: 50 μM enzyme in 0.05% lauryl maltoside, 100 mM potassium phosphate buffer, pH 8.0. For all of the MALDI samples, the matrix was prepared by dissolving 1.5 mg of HABA [2-(4-hydroxyphenylazo (benzoic acid)] (Aldrich), in 1 ml of solvent containing 66% acetonitrile, 33% water, 0.1% TFA (trifluoroacetic acid).

Two different methods were used to prepare the matrix-embedded proteins. For cytochrome *bd*, cytochrome bo_3, and cytochrome bc_1, 33 μl of each protein solution (30 μM) was precipitated by adding 200 μl of 10% trichloroacetic acid (TCA) solution, and the pellet was re-dissolved in 1 ml 99% formic acid, and then diluted to a final concentration of 45 pmol ml^{-1} in 99% formic acid. For the aa_3-type cytochrome *c* oxidase, the 50 μM solution of the protein was simply diluted 50:1 with water. Finally, 1 μl of each sample plus 1 μl of the matrix solution was placed on the target. All mass spectra were externally calibrated. It is noted that formylation of peptides in the presence of formic acid has been previously reported, but the use of trifluoroacetic acid in the matrix should eliminate this problem [14].

The calculated molecular weights of the subunits from translated nucleotide sequence were obtained using Version 8.0 of the Genetics Computer Group (GCG) Wisconsin Package, September 1994 (Madison, WI), using the PepSort.

The analysis of independent samples gave a standard deviation (2 to 4 samples) ranging from 0.1% to 0.5%. Independent protein preparations yielded the same results.

3. Results and discussion

3.1. Cytochrome bd

SDS–PAGE analysis of the purified enzyme indicates the presence of two subunits (Fig. 1). Subunit I and subunit II migrate with M_r values of ca. 52000 and 37000 Da, and the calculated molecular weights deduced from the amino acid sequences are 58180 and 42460 Da, respectively [15,16]. These two subunits are proposed to contain, 7 and 8 transmembrane helical spans, respectively [17,18]. Fig. 2 shows a MALDI mass spectrum of the purified cytochrome *bd*. Subunits I and II (singly protonated molecular ions) are easily identified in the spectrum as the sharp peaks at m/z 58187 and 42565, respectively. The doubly charged monomers of both subunits were also easily assigned to peaks at m/z 29107 and 21314, respectively. Subunit I differs from the calculated weight by only 7 Da, whereas subunit II differs from the calculated value by 105 Da (Table 1). It is noted that the N-termini of both subunits are blocked though

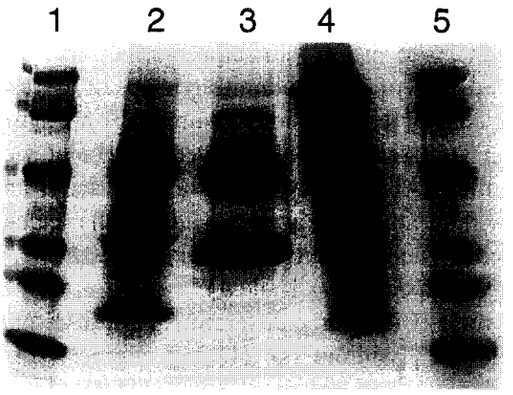

Fig. 1. SDS–PAGE analysis of purified proteins. Lanes 1 and 5 are low molecular weight standards with masses 100.8, 80, 50.9, 34, 27.3 and 16.9 kDa respectively. Lanes 2, 3 and 4 are purified protein samples of cytochrome bo_3, cytochrome *bd*, and cytochrome aa_3, respectively.

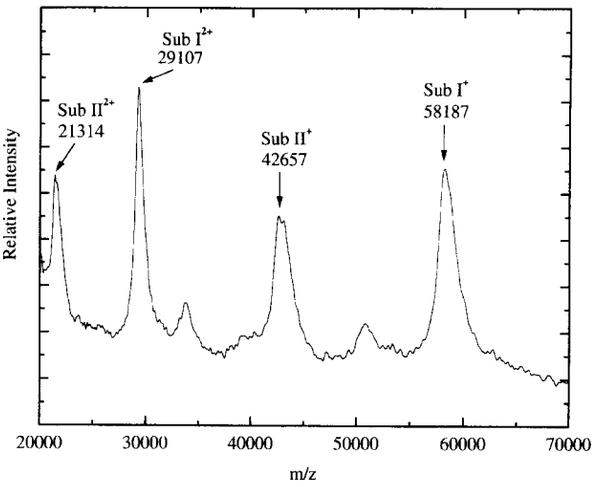

Fig. 2. MALDI mass spectrum of the purified cytochrome *bd* oxidase complex from *E. coli*. MALDI analysis was done using a laser desorption VG TofSpec 'time-of-flight' mass spectrometer.

the chemical nature of the block has not been defined. Two very minor peaks at m/z 50 831 and 33 706 are also shown in the mass spectrum. These may arise from contaminants or from degradation of subunits I and II, respectively.

3.2. Cytochrome bo_3

SDS–PAGE analysis of the purified enzyme indicates the presence of four subunits [11]. Subunit IV,

Table 1
Comparison of the molecular weights determined by MALDI with the calculated values

	Calculated (Da)	Observed (Da)	(observed − calculated) (Da)
Cytochrome *bd* oxidase			
Subunit I	58 180	58 186	6
Subunit II	42 460	42 564	104
Cytochrome bo_3 oxidase			
Subunit I	74 377	74 267	−110
Subunit II	34 914	32 985	−1929
Subunit II (His-tagged)	36 018	34 347	−1671
Subunit III	22 625	22 487	−138
Subunit IV	12 030	11 924	−106
Cytochrome bc_1			
Rieske	19 779	19 637	−142
Cytochrome c_1	28 488	28 779	291
Cytochrome c_1 (His-tagged)	29 797	30 105	308
Cytochrome *b*	49 918	48 666	−1252
Cytochrome *b* (His-tagged)	51 227	50 432	−795
Cytochrome *c* oxidase			
Subunit I (His-tagged)	63 986	63 760	−226
Subunit II	32 940	29 946	−2994
Subunit III	30 139	30 529	−390

which has a calculated molecular weight of 12030 Da [19], is usually very faint and difficult to detect by SDS–PAGE using Coomassie staining [11,20]. From the SDS–PAGE analysis, subunits I, II and III migrate to yield M_r values of 52000, 38000 and 23000 Da, respectively (Fig. 1), but the molecular weights calculated from the DNA sequences of these three subunits are subunit I, 74377; subunit II, 34914; and subunit III, 22625 [19]. The large discrepancy for subunit I, which is proposed to contain 15 transmembrane helical spans [21], is not uncommon for SDS–PAGE analysis of membrane proteins and may result from excessive SDS-binding and/or incomplete unfolding of the protein resulting in rapid electrophoretic mobility.

Mass spectrometry analysis was performed with cytochrome bo_3 purified using DEAE chromatography [11], and also with the His-tagged version of the oxidase, where six histidines and an arginine were introduced at the C-terminus end of subunit II and then purified by the Qiagen Ni^{2+}–NTA resin method [12]. Fig. 3 shows MALDI mass spectra of DEAE-purified cytochrome bo_3. In addition to the singly charged protonated molecular ions, the doubly charged ions of each subunit and a peak assigned to the quadruply charged ion of subunit II are observed in the two spectra. The observed molecular weights from the MALDI analysis for subunits I, III and IV are within a single amino acid of the calculated molecular weights, differing only by 109, 137, and 105 Da, respectively (Table 1).

Subunit II is clearly different. The DEAE-purified sample (i.e., without the His-tag engineered at the C-terminus) is expected to have a calculated molecular weight of 34914 Da, whereas the Ni^{2+}–NTA purified protein (i.e., with the His-tag) has a calculated molecular weight of 36018 Da, due to the addition of six histidine and an arginine (total molecular weight of about 1104 Da). For the DEAE-purified sample (Fig. 3a), the peaks corresponding to m/z 32986, 16506 and 8275 were assigned to the singly, doubly and quadruply charged ions of subunit II of cytochrome bo_3, though the assignment of the quadruply charged ion is dubious since the triply

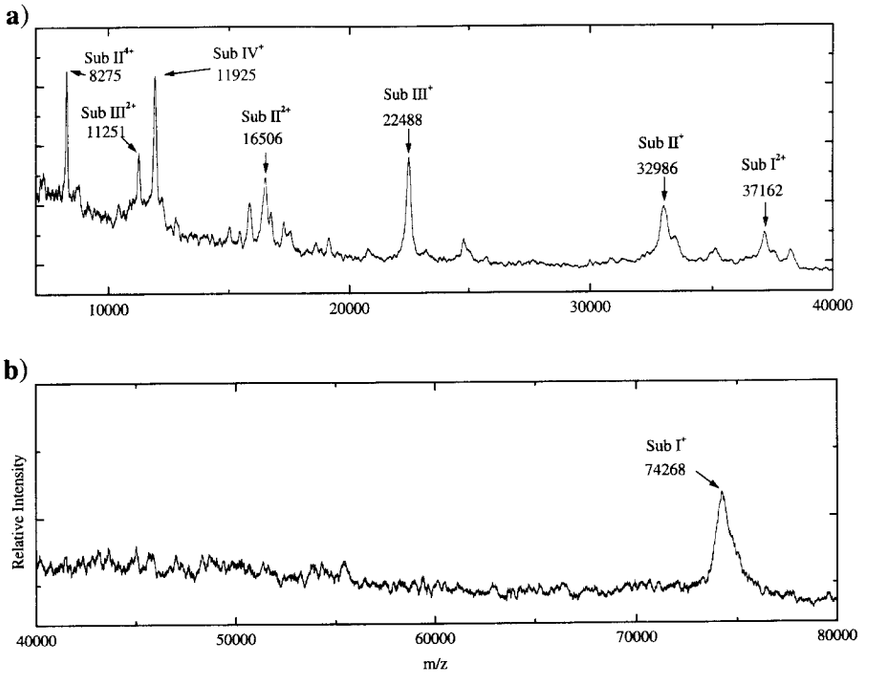

Fig. 3. MALDI mass spectrum of the DEAE purified cytochrome bo_3 oxidase complex from *E. coli*. (a) MALDI data for subunits with less than 40000 Da molecular weight. (b) MALDI data for subunits larger than 40000 Da molecular weight. MALDI analysis was performed using a laser desorption VG TofSpec 'time-of-flight' mass spectrometer.

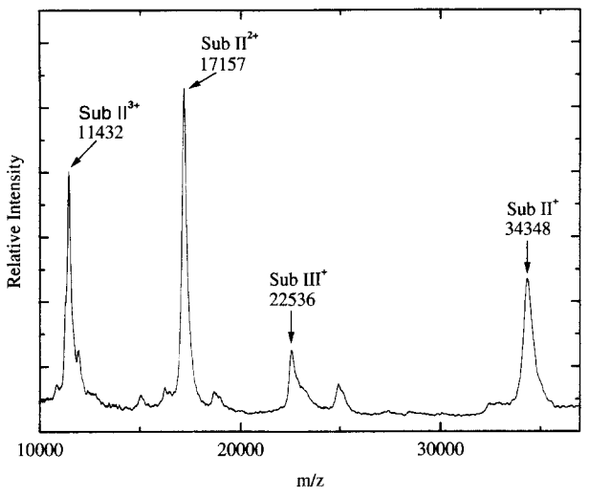

Fig. 4. MALDI mass spectrum of the Ni^{2+}–NTA resin-purified His-tagged cytochrome bo_3 oxidase complex from *E. coli*. After purification, the sample was denatured and then reloaded into the column in order to isolate subunit II of the oxidase. MALDI analysis was done using a laser desorption VG TofSpec 'time-of-flight' mass spectrometer.

charged species is not observed in the spectrum. The singly charged protonated molecular ion has a mass that is 1928 Da lower than the calculated value (Table 1). This discrepancy has been shown to be due to post-translational processing (Ma et al., manuscript in preparation) resulting in the loss of 24 amino acid residues and the addition of lipid at the N-terminus of subunit II.

The purified sample of cytochrome bo_3 with the His-tag at the C-terminus of subunit II was used to isolate subunit II. The sample was denatured using urea plus SDS and, after removal of SDS by dialysis, was re-loaded onto the Ni^{2+}–NTA column in the presence of urea in order to isolate subunit II for closer examination. The mass spectrum (Fig. 4) has three peaks that can be assigned to subunit II (m/z 34 348, 17 157 and 11 432), which correspond to the singly, doubly and triply charged protonated ions. The mass difference between the singly charged ion and the calculated molecular weight is about 1700 Da. This, again, is evidence of the same post-transla-

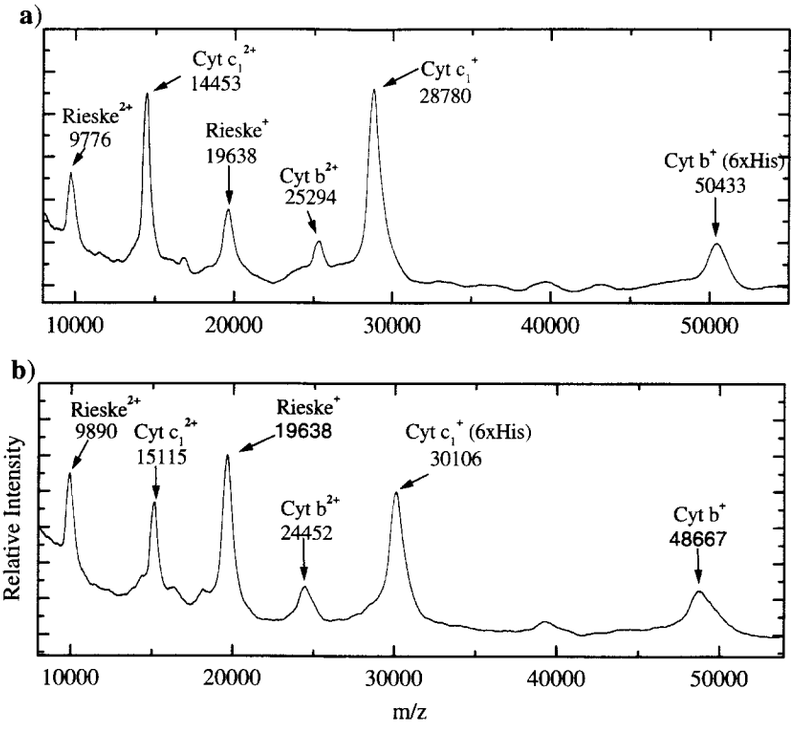

Fig. 5. MALDI mass spectrum of the Ni^{2+}–NTA resin purified bc1 complex from *R. sphaeroides*. (a) MALDI spectrum of the preparation with His-tagged cytochrome. (b) MALDI spectrum of the preparation with His-tagged cytochrome c_1. MALDI analysis was performed using a laser desorption VG TofSpec 'time-of-flight' mass spectrometer.

tional processing mentioned above. Another peak in the MALDI spectrum corresponding to m/z 22536 was assigned to contamination from singly charged subunit III. Besides the expected increase in the molecular weight of subunit II due to the C-terminal His-tag, the other subunits of this preparation of cytochrome bo_3 are identical in the MALDI analysis as with the enzyme lacking the His-tag.

3.3. The cytochrome bc_1 complex

SDS–PAGE analysis of the Ni^{2+}–NTA purified bc_1 complex from aerobically grown *R. sphaeroides* has three major bands (not shown) that migrate with M_r values of ca. 40000, 32000 and 25000 Da, identified as the cytochrome *b*, cytochrome c_1 and the Rieske subunits of the enzyme [22]. However, the calculated molecular weights (without the His-tags) of these subunits are 49918, 28488 and 19779 Da, respectively (Table 1). Subunit IV [22–25] is not present in these preparations of the enzyme (Salcedo-Hernandez et al., manuscript in preparation). MALDI mass spectrometry was performed with two different preparations, where six histidines were introduced at the C-terminus of the cytochrome *b* subunit (calculated molecular mass 51227 Da) or on the cytochrome c_1 subunit (calculated molecular mass 29797 Da). Fig. 5a and b show MALDI mass spectra of the preparations of the cytochrome bc_1 complex containing respectively, the His-tagged cytochrome *b* and His-tagged cytochrome c_1. In both of these spectra, the singly and doubly charged molecular ions of each of the three subunits are shown. The observed molecular weights from the MALDI analysis for the Rieske and cytochrome c_1 subunits are within one or two amino acids of the calculated molecular weights, differing by 141 and 300 Da, respectively (Table 1). The cytochrome *b* subunit in both preparations is observed to have significantly less than the expected mass, by 794 Da (His-tagged) and 1251 Da (non-

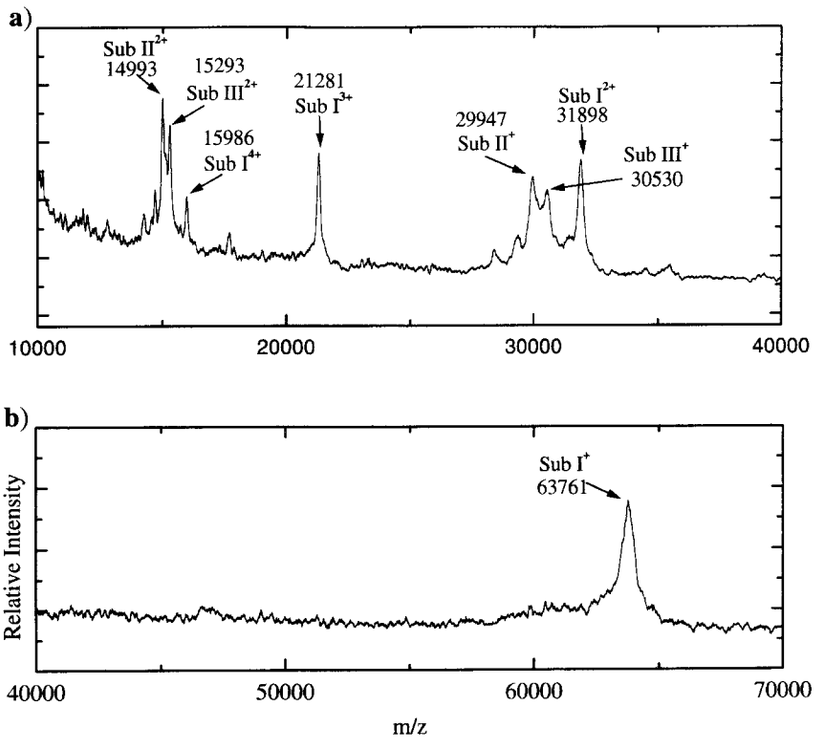

Fig. 6. MALDI mass spectrum of the Ni^{2+}–NTA resin purified His-tagged aa_3-type cytochrome *c* oxidase complex from *R. sphaeroides*. (a) MALDI spectrum of subunits with less than 40000 Da molecular weight. (b) MALDI spectrum of subunits larger than 40000 Da molecular weight. MALDI analysis was done using a laser desorption VG TofSpec 'time-of-flight' mass spectrometer.

His-tagged). Whether this is due to post-translational modification or proteolysis during purification remains to be seen.

3.4. The aa_3-type cytochrome c oxidase

SDS–PAGE of the Ni^{2+}–NTA purified aa_3-type oxidase shows four bands [13]. The band with the least migration appears to be an aggregate of the protein. The other three bands correspond to the three subunits of the oxidase. Subunits I, II and III migrate with M_r values of ca. 52 000, 37 000 and 21 000 Da (Fig. 1), even though the calculated molecular weights (Da) of the three subunits are: subunit I (with the addition of the His-tag), 63 986; subunit II, 32 940; and subunit III, 30 139.

Fig. 6 shows the mass spectrum of the aa_3-type cytochrome *c* oxidase. Subunit I (singly charged) is easily identified in the mass spectrum as the sharp peak at m/z 63 761 (Fig. 6b), which differs from the predicted mass by only 224 Da (Table 1). The doubly and triply charged species of subunit I are assigned to peaks at m/z 31 898 and 21 281, respectively. A small peak at m/z 15 986 may represent the quadruply charged subunit I species of the oxidase.

Singly charged subunit III can be reasonably assigned to the peak at m/z 30 530, and its doubly charged species to the peak at m/z 15 293, leaving one other major peak at m/z 29 947 as a possible subunit II species. This is less than the expected mass of subunit II by 2993 Da (Table 1), corresponding to the possible loss of 25 amino acid residues, perhaps a proteolytic artifact occurring during the purification. Evidence for proteolysis of subunit II has been noted previously [26] and this subunit often appears as a doublet by SDS–PAGE analysis [27].

4. Conclusion

The successful mass analysis of a series of hydrophobic proteins has been clearly achieved by matrix-assisted laser desorption ionization mass spectrometry. This method should be routinely applicable to other purified membrane proteins, avoiding the artifacts that are often observed with SDS–PAGE analysis of hydrophobic polypeptides. The technique is simple, rapid and reliable and appears to be accurate to within 100–300 ($< 0.5\%$ in most cases) Da over a wide range of subunit molecular weights.

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