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Application News

Protein sequencer

Analysis of the N-terminal Amino Acid Sequence of IgG Antibodies Using a Protein Sequencer (PPSQ™) Isocratic System

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User Benefits

- Edman degradation allows for the reliable determination of amino acid sequences.
- With optimized analytical conditions and simple, user-friendly software, the system is easy to operate.
- The use of an isocratic system helps reduce operating costs.

■ Introduction

In recent years, biopharmaceuticals, including antibody drugs, have become important treatment options across a wide range of areas, such as cancer, autoimmune diseases, and rare diseases, and are further increasing their presence in the pharmaceutical market. While these biopharmaceuticals are highly effective, their molecular structures are complex, and even slight differences in manufacturing processes or culture conditions can affect their quality characteristics.

For this reason, rigorous quality assessment through structural and characterization analyses is essential in the development and manufacturing of biopharmaceuticals. In particular, N-terminal amino acid sequence analysis, which verifies the primary structure of proteins, is used as a key analytical method for confirming the identity of the target protein and ensuring quality. Protein sequencers that utilize the Edman degradation method are employed for N-terminal amino acid sequence analysis, enabling the reliable determination of the N-terminal sequences of proteins and peptides.

Here, we present an example of N-terminal amino acid sequence analysis of humanized IgG monoclonal antibodies derived from Chinese hamster ovary cells, using the PPSQ-51A/53A isocratic protein sequencer equipped with an SPD-40 detector.

■ Edman Degradation

Fig. 1 illustrates the principle of a protein sequencer. The protein sequencer is a device that cleaves amino acids from the N-terminus of a protein one by one by Edman degradation and analyzes the phenylisothiocyanate derivatives (PTH-amino acids) by HPLC. By repeating this process and arranging the obtained PTH-amino acids in order, the amino acid sequence from the N-terminus of a protein or peptide can be determined easily and reliably. It can also determine the amino acid sequences of proteins not registered in databases. It is useful not only for basic research but also in various applications, such as the analysis of proteins and peptides in antibody drugs and food products.

■ Protein Sequencer PPSQ-51A/53A

The PPSQ-51A/53A protein sequencer used in this paper is an isocratic system equipped with an SPD-40 detector (Fig. 2). Table 1 shows the analytical conditions for PTH-amino acids obtained by Edman degradation, and Fig. 3 shows the chromatogram of a PTH-amino acid standard mixture. It is possible to easily separate and identify the amino acids that make up proteins and peptides, with the exception of cysteine.

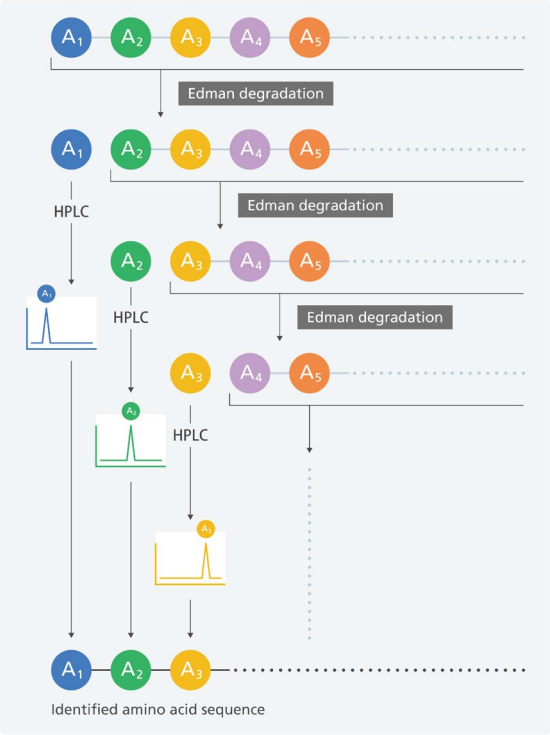

Fig. 1 Principle of Edman degradation

Fig. 2 Protein Sequencer PPSQ™-51A/53A

Table 1 Analytical Conditions for PTH Amino Acids

Column	:	Wakopak Wakosil PTH-Ⅱ (250 mm L, 4.6 mm I.D.)
Mobile phase	:	PTH-amino Acids Mobile Phase
Flow rate of mobile phase	:	1.0 mL/min
Column temp.	:	40˚C
Detection	:	SPD-40(269 nm)with STD cell

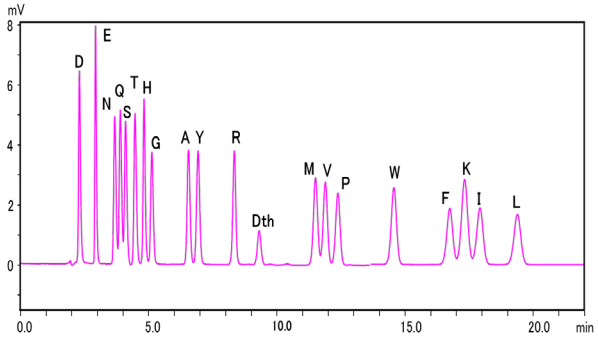

Fig. 3 Analysis of a PTH-amino Acids Mixture Standard(25 pmol)

■ The Structure of Immunoglobulins

Immunoglobulins are composed of two types of polypeptide chains: heavy chains (H-chains), which have a high molecular weight, and light chains (L-chains), which have a low molecular weight. They are classified into five classes based on the type of heavy chain. Fig. 4 shows the basic structure of immunoglobulin G (IgG), which is central to antibody therapeutics. IgG has a complex tertiary structure, and the heavy and light chains must be separated as a pretreatment step for analyzing the N-terminal amino acid sequence using a protein sequencer.

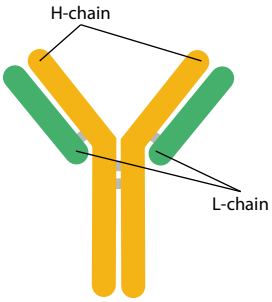

Fig. 4 Structure of IgG

■ IgG Analysis

The pretreatment protocol is shown in Table 2 and Fig. 5. The sample used was humanized IgG1κ derived from Chinese hamster ovary cells (National Institute of Advanced Industrial Science and Technology). The sample was diluted with Sample Buffer supplemented with BPB to a final concentration of approximately 3 pmol/μL.

Table 2 Details of the Sample Preparation Protocol

SDS-PAGE			
Sample Buffer	Dissolve the following in ultrapure water to a total volume of 100 mL		
	Tris((Hydroxymethyl) aminomethane)	0.76 g	Bio-Rad
	SDS(Sodium Dodecyl Sulfate)	2.3 g	Bio-Rad
	Glycerol	10 mL	FUJIFILM Wako
	2-Mercaptoethanol	5 mL	Nacalai Tesque
Staining	Bromophenol Blue(BPB)		FUJIFILM Wako
Gel	Novex TBE Gels 4–20 %		Thermo Fisher Scientific
Running Buffer	NuPAGE MOPS SDS Running Buffer		Thermo Fisher Scientific
Molecular Weight Marker	LMW Marker Kit		Cytiva
Electrophoresis Conditions	100 V, 1 hr		
Electroblotting			
Transfer Buffer	NuPAGE Transfer Buffer (20X)		Thermo Fisher Scientific
PVDF membrane	FluoroTrans PVDF membrane		PALL
Staining	CBB-R250 (Coomassie Brilliant Blue-R250)		Nacalai Tesque
Destaining	50 % methanol(v/v)		
Electrophoresis Conditions	25 V, 2 hrs		

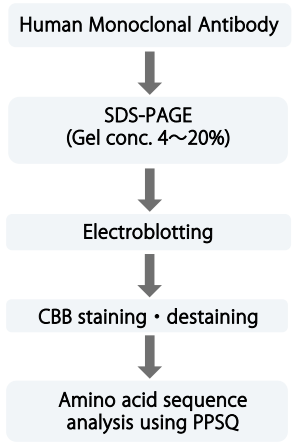

Fig. 5 Sample Preparation Protocol

Immediately prior to SDS-PAGE, the samples were heat-treated at 95 ˚C for 25 minutes. A 10 μL sample solution was loaded onto the gel, and electrophoresis was performed. After electrophoresis, electroblotting was performed onto a PVDF membrane. The transferred PVDF membrane was immersed in CBB staining solution and shaken for 1 minute to stain it, and then destained in the same manner with 50% methanol for 1 minute. Fig. 6 shows the PVDF membrane after electroblotting. The bands corresponding to the L-chain and H-chain were excised and analyzed using PPSQ-51A/53A.

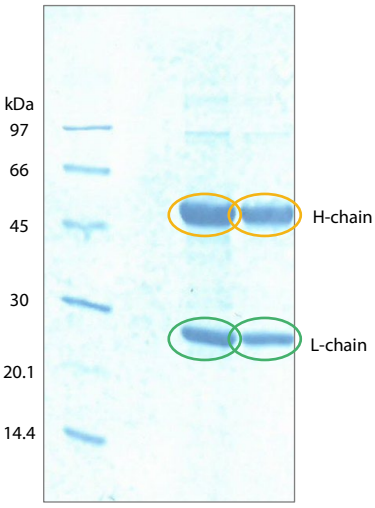

Fig. 6 Results of Electroblotting

Figs. 7 and 8 show the chromatograms of the amino acid sequence results. Fig. 7 presents the results of the N-terminal amino acid sequence analysis of the IgG light chain. The first cycle is shown as a raw chromatogram, while the second cycle and subsequent cycles are shown as subtracted chromatograms. In each cycle, it can be observed that the PTH-amino acids obtained from Edman degradation increase specifically. For example, the N-terminal amino acid residue was identified as aspartic acid (Asp), and in the fourth cycle, it was identified as methionine (Met). The amino acid sequence from the N-terminus up to the 21st residue was confirmed to be Asp-Ile-Gln-Met-Thr-Gln-Ser-Pro-Ser-Ser-Leu-Ser-Ala-X-X-Gly-Asp-Arg-X-Thr. For cycles 14, 15, and 19, no PTH amino acids showing a specific increase were detected, or multiple PTH amino acids were increased in those cycles; therefore, it was not possible to estimate the amino acid sequence. Based on the results of a database search, this sequence was identified as originating from the variable region of the Human immunoglobulin κ light chain.

Similarly, Fig. 8 shows the results of the amino acid sequence analysis for the H-chain from the first to the fifth cycle. This sequence was confirmed to be Glu-Val-Gln-Leu-Val-Glu-Ser-Gly-Gly-Gly-Leu-Val-Gln-Pro-Gly-Gly-Ser-Leu-Arg-Leu from the N-terminus, and a database search identified it as a sequence derived from the Human immunoglobulin heavy chain variable region gene IGHV3-72.

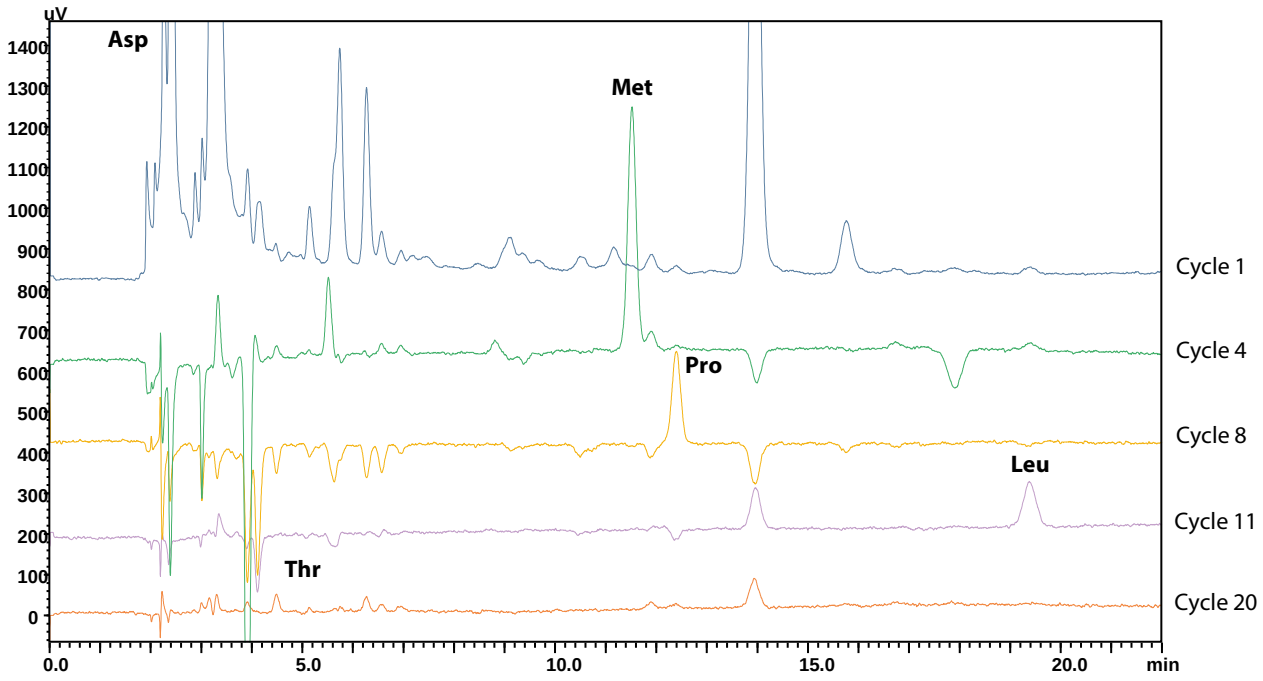

Fig. 7 Chromatograms of L-chain

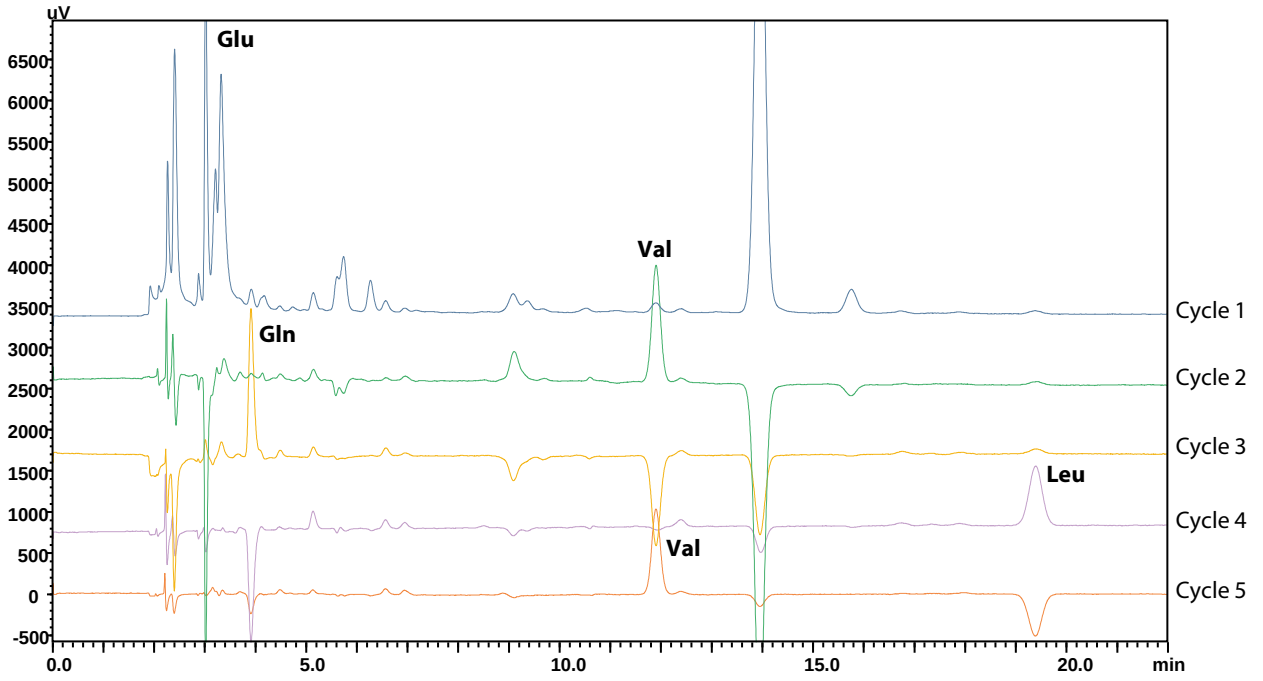

Fig. 8 Chromatograms of H-chain

■ Conclusion

As demonstrated in this application, the use of a protein sequencer makes it possible to easily and accurately determine the N-terminal amino acid sequence of IgG. The software used for analysis is simple and user-friendly, and the isocratic system helps reduce running costs.

Based on the above features, the protein sequencer can be considered a useful analytical system for the identity verification and N-terminal structure evaluation of biopharmaceuticals.

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