Available online at www.sciencedirect.com
SCIENCE DIRECT®
Analytical Biochemistry 342 (2005) 120–125
ANALYTICAL BIOCHEMISTRY
www.elsevier.com/locate/yabio

The removal of pyroglutamic acid from monoclonal antibodies without denaturation of the protein chains

William E. Werner *, Sylvia Wu, Michael Mulkerrin

Abgenix Corporation, Fremont, CA 94555, USA

Received 24 February 2005
Available online 27 April 2005

Abstract

Typically, the removal of pyroglutamate from the protein chains of immunoglobulins with the enzyme pyroglutamate aminopeptidase requires the use of chaotropic and reducing agents, quite often with limited success. This article describes a series of optimization experiments using elevated temperatures and detergents to denature and stabilize the heavy chains of immunoglobulins such that the pyroglutamate at the amino terminal was accessible to enzymatic removal using the thermostable protease isolated from *Pyrococcus furiosus*. The detergent polysorbate 20 (Tween 20) was used successfully to facilitate the removal of pyroglutamate residues. A one-step digestion was developed using elevated temperatures and polysorbate 20, rather than chaotropic and reducing agents, with sample cleanup and preparation for Edman sequencing performed using a commercial cartridge containing the PVDF membrane. All of the immunoglobulins digested with this method yielded heavy chain sequence, but the extent of deblocking was immunglobulin dependent (typically > 50%).

Keywords: Protein sequencing; Pyroglutamate removal; Blocked N-terminal sequencing; Antibody sequencing; Antibody analysis

When the amino terminal residue of a protein is glutamine, it often spontaneously undergoes a cyclization reaction and forms pyroglutamic acid. This reaction effectively blocks the amino terminus to the chemical determination of the amino acid sequence by Edman degradation. Immunoglobulins frequently contain a blocked amino terminus on either the heavy chain or the light chain, or sometimes on both, due to this reaction. As a class of molecules, immunoglobulins are being developed for numerous indications that require extensive confirmation of the identity and sequence of the specific protein. The pyroglutamic acid typically found on the amino terminus of the light or heavy chain complicates this characterization because Edman degradation is one of the "workhorse" methods used to confirm the amino acid sequences of proteins. Thus, the development of robust methods for the removal of pyroglutamic acid is desirable for the subsequent characterization of therapeutic antibodies.

The enzymatic removal of pyroglutamate is an activity widely observed in nature. It appears to be part of the cellular machinery for the degradation and recycling of proteins. This enzyme, pyroglutamate aminopeptidase, has been purified and commercialized from several sources, and methodologies for its use have been developed [1–4]. In general, this enzyme has not proved to be as robust in vitro as have enzymes such as trypsin. In particular, the removal of pyroglutamate from relatively large proteins has been problematic and often not repeatable from laboratory to laboratory. Typically, digestions occur over long periods of time (24–48 h) and may employ enzyme-to-substrate ratios greater than 1. One factor that could influence the removal of pyroglutamate from large proteins is the accessibility of the amino terminus in the native structure. Several of the methodologies cited above call for the

* Corresponding author. Fax: +1 415 920 4461.
E-mail address: bill.werner@abgenix.com (W.E. Werner).

0003-2697/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.ab.2005.04.012

denaturation of the blocked proteins with chaotropes, reduction of disulfide bonds, and alkylation of cysteine residues prior to digestion with pyroglutamate aminopeptidase. Takara Biochemicals (Shiga, Japan) sells a thermostable enzyme isolated from *Pyrococcus furiosus* [5,6] that in theory should allow for the exposure of the amino terminal pyroglutamate residue by employing an elevated digestion temperature. This potential had not yet been realized for monoclonal antibodies [4].

Even methodologies employing protein denaturation to expose the amino terminal pyroglutamate residues have not proved to be universal in their application. One potential reason for this inconsistency might be the fact that the solubility of denatured and alkylated proteins could be significantly less than that of the native protein. If it were possible to prevent the aggregation and precipitation of denatured proteins and keep them in solution, the consistent removal of pyroglutamate residues might be improved. This article describes a series of experiments that employed detergents to improve the removal of pyroglutamate from monoclonal antibodies without the use of chaotropic agents. The use of detergents and elevated temperatures was employed to denature immunoglobulins and take advantage of the thermostable nature of the *Pyrococcus* enzyme.

Materials and methods

Unless otherwise noted, the chemicals used in these experiments were obtained from either Sigma (St. Louis, MO, USA) or Fisher (Santa Clara, CA, USA) without any noticeable differences between the sources. Thermostable pyroglutamate aminopeptidase (10 mU) and digestion buffer were purchased from Takara Biochemicals. The enzyme was initially resuspended in 100 μl of 1× digestion buffer and was then aliquoted into 10-μl aliquots (1.0 mU) and frozen at −80 °C until used.

Edman sequencing was performed on an Applied Biosystems 490 series Procise sequencer (Foster City, CA, USA) employing the manufacturer's reagents. The standard pulse–liquid cycles were employed. Samples were desalted and immobilized onto the PVDF membrane using the ProSorb sample preparation cartridge (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's recommendations with the exception that biorene was not always employed with whole antibody samples [7]. Typically, 2 μg of antibody was digested and immobilized for sequence analysis, but up to 10 μg was used in initial studies.

Monoclonal antibodies

Fully human antibodies were generated against target antigens employing proprietary XenoMouse technology. The cell hosts for these antibodies were either murine hybridoma or Chinese hamster ovary (CHO)[1] cells. The antibodies used for these method development studies were designated as Mab numbers. These antibodies and their properties are listed in Table 1. Initial sequence analysis revealed that all of these antibodies possessed blocked (>95%) amino termini on the heavy chains and free amino termini on the light chains.

Table 1
XenoMouse antibodies employed

Antibody name	Type	Cellular background
Mab1	IgG2	Hybridoma
Mab2	IgG2	Hybridoma
Mab3	IgG2	CHO
Mab4	IgG2	CHO
Mab5	IgG1	CHO
Mab6	IgG1	CHO
Mab7	IgG1	Hybridoma
Mab8	IgG4	Hybridoma
Mab9	IgG4	Hybridoma
Mab10	IgG4	Hybridoma

Note. CHO, Chinese hamster ovary.

General assay conditions

The range of assay volumes employed in this study was between 50 and 150 μl (50–70 μl eventually became the standard reaction volume). Detergents to be tested were made up in deionized water and mixed with the appropriate amount of the manufacturer's 5× digestion buffer. Approximately 2 μg of antibody was added to the buffer along with the pyroglutamate aminopeptidase. A 500-μl snap-cap tube was used to incubate the reaction, and the temperature was controlled with a standard heating block. At the end of the reaction, 10 μl of methanol (optional) was added to the reaction and the entire mixture was placed into a ProSorb cartridge in which the PVDF membrane had been previously wetted with methanol and 100 μl of 0.1% trifluoroacetic acid (TFA) had been placed in the reservoir. After the solution had passed through the membrane, an additional 100 μl of 0.1% TFA was passed through the membrane to wash the sample. The PVDF membrane was allowed to dry and then was placed directly onto the cartridge seal inside the reaction cartridge of the Edman sequencer. Pyroglutamate removal was estimated as a percentage by comparing the amount of light chain sequence observed with the amount of heavy chain sequence observed.

Results

Initial experiments attempting to digest Mab1 at elevated temperatures in the absence of detergent failed to

[1] *Abbreviations used:* CHO, Chinese hamster ovary; TFA, trifluoroacetic acid.