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A TECHNIQUE FOR THE REMOVAL OF PYROGLUTAMIC ACID FROM THE AMINO TERMINUS OF PROTEINS USING CALF LIVER PYROGLUTAMATE AMINO PEPTIDASE

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SUMMARY

An improved technique has been developed which can quickly and easily unblock proteins and peptides containing pyrrolidone carboxylic acid (PCA) or pyroglutamic acid in the amino terminal position utilizing commercially available pyroglutamate amino peptidase isolated from calf liver. The successful application of the technique to five immunoglobulin polypeptide chains including two heavy chains and three light chains is demonstrated. Description of the deblocking procedure under stabilizing enzyme conditions and biochemical properties of the enzyme are given.

INTRODUCTION

The primary structural analyses of many naturally occurring proteins and peptides including gastrin (1), fibrinopeptides (2,3,4), collagen (5), bradykinin-potentiating peptides (6), and immunoglobulins (7,8,9) have been hindered by the presence of pyrrolidone-carboxylic acid (PCA) or pyroglutamic acid as an amino-terminal residue. It is believed that pyroglutamic acid is formed after cyclization of terminal glutaminyl or glutamyl residues. In addition, Sanger and Thompson (10) found that pyroglutamyl containing peptides can spontaneously arise during the isolation and proteolysis of peptides containing glutamine in the amino-terminal position. Since the automated Edman primary amino acid sequence analysis depends on the availability of an alpha amino group (11), those proteins and peptides which begin with pyroglutamic acid pose problems in their direct primary structural analysis.

Takahashi and Cohen (12) have used diborane in tetrahydrofuran or tetramethylurea to reduce the pyrrolidone ring of NH_2-terminal pyroglutamyl

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residues to the pyrrolidine ring. Although this method provides an easy and rapid qualitative identification of such N-terminal residues, the authors point out that diborane reduction shows limited selectivity since carboxyl and peptide groups are partially reduced. Furthermore, there is only 35 to 46% conversion of pyroglutamic acid into proline with concurrent loss of peptide bonds by reduction. Thus, the procedure is of limited use when studying proteins available in limited quantity.

Doolittle and Armentrout (13), and Szewczuk and Mulczyk (14) were successful in isolating enzyme preparations from Bacillus subtilis and Pseudomonas fluorescens, respectively, that could liberate free pyroglutamic acid from N-terminal pryoglutamyl containing proteins and peptides. Although both enzyme preparations functioned successfully, their isolation and use pose serious problems. Doolittle and Armentrout demonstrated that, although the activity of their purified enzyme from Pseudomonas was 100 times greater than crude extract preparations, the enzyme activity is highly unstable and storage under ammonium sulfate or by freezing was unsatisfactory. Further, the isolation procedures for both bacterial enzymes involves considerable time and is not practical in most laboratories.

We have developed a technique which is able to unblock proteins with pyroglutamic acid in the amino-terminal position utilizing a commercially available pyroglutamate amino peptidase isolated from calf liver. This selective removal of pyroglutamyl residues from proteins is now able to be done easily and quickly under enzyme stabilizing conditions. Moreover, the active enzyme can be stored in a lyophilized state at -20°C for an indefinite time period.

MATERIALS AND METHODS

Isolation of cryoglobulins: Purified IgG cryoglobulins with heavy and/or light chains containing N-terminal pyroglutamic acid were isolated from patients' serum by centrifugation at 4°C and washed three times with ice-cold phosphate buffered saline, pH 7.4. The washed proteins were further purified by DEAE-cellulose chromatography at 37°C (0.015 M phosphate buffer pH 7.3). Studies establishing the restricted heterogeneity and immuno-chemical properties of these antibodies have been presented elsewhere (9,15,16).

Preparation of heavy and light chains for deblocking and primary structural studies: The purified cryoglobulins were reduced and alkylated as described by Fleishmann et al (17) in 0.2 M Tris 4 M in urea pH 8.6, and heavy and light chains were prepared in gel filtration on sephadex G-100 in 1 M acetic acid. The heavy and light chains were left in solution until the deblocking procedure was performed since lyophilization caused severe solubility problems of the proteins in deblocking buffer (DBB).

Deblocking with calf liver pyroglutamyl amino peptidase: Pyroglutamate amino peptidase from calf liver (L-pyroglutamyl peptide hydrolase, EC 3.4.11.8) was purchased from Boehringer Mannheim Biochemicals, Indianapolis, Indiana, and was stored as a lyophilisate at -20°C. 10 ml of totally reduced and alkylated protein preparation (1 mg/ml) were first dialyzed at 4°C vs deblocking buffer (DBB). DBB was prepared by adjusting a solution of 0.1 M disodium phosphate to pH 8.0 with 0.1 M monosodium phosphate. This buffer was made 5 mM in dithiothreitol (DTT), 10 mM in disodium EDTA, 5% in glycerin (v/v) and adjusted to pH 8.0 with 1 N NaOH. The protein preparation was transferred to a screw-top vial and 0.5 mg of a lyophilized crude protein extract (containing approximately 0.025 mg of active enzyme) was added, the tube flushed with nitrogen, capped and mixed. Protein and enzyme were allowed to incubate at 4°C for 9 hours with occasional mixing. After 9 hours the mixture was brought to room temperature, 0.5 mg of lyophilized crude enzyme was added a second time, the vial flushed with N_2 again, and mixed. This was incubated at room temperature for 14 hours with occasional mixing. The treated protein was then dialyzed vs 0.05 M acetic acid, lyophilized, and utilized for automated amino acid sequence analysis.

N-terminal analysis of the intact cryoimmunoglobulins: Dansylation of the cryoglobulins was carried out according to the procedure of Gross and Labouesse (18). N-terminal dansyl amino acid derivatives were chromatographed on 5 x 5cm Cheng Chin polyamide thin layer plates in two dimensions using the following solvents (19,20): I. 1.5% formic acid; II. Benzene:acetic acid (9:1); and III. ethyl-acetate:methanol:acetic acid (20:1:1).

Protein sequencing techniques: Five mg of purified heavy or light chain, representing 100 or 200 nM of protein respectively, were subjected to automated amino acid sequencing on an updated Beckman model 890 B protein-peptide sequencer with nitrogen flush and dry systems. A modified 0.5 M quadrol single cleavage program provided sequential additions of phenylisothiocyanate and quadrol buffer (Pierce Chemical Co.), washing with benzene and acidified ethylacetate, cleavage with heptafluorobutyric acid and extraction of the thiazolinone derivatives with butyl chloride, 1 mM ethanethiol. The cleaved amino acid derivatives were converted to the stable phenylthiohydantoins by addition of 1 N HCl 1 mM in ethanethiol to the dried residue and incubation at 80°C for 10 minutes under nitrogen. The PTH-amino acid derivatives were extracted in acidified ethylacetate, nitrogen dried and identified.

Identification of PTH amino acids: Phenylthiohydantoin derivatives were identified by 1) gas liquid chromatography using a Packard Model 419 gas chromatograph with dual columns containing 10% SP-400 bonded to Chromosorb W-HP (Supelco), 2) high pressure liquid chromatography using an Altex model 310 single pump system with methanol and water solvent systems as described elsewhere (21), and 3) by thin layer chromatography on 5 x 5 cm polyamide thin layer plates utilizing solvent systems in two dimensions (22,23).

RESULTS

In order to investigate the nature of the amino termini of the cryoglobulin polypeptide chains, N-terminal analysis was performed by the

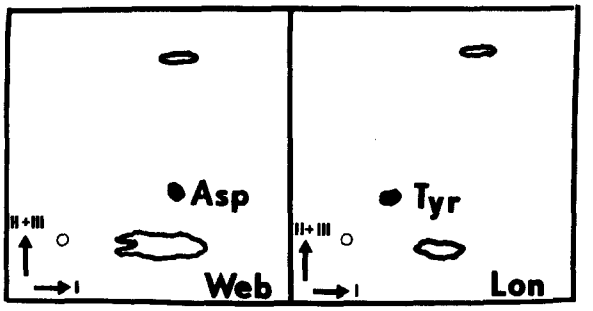

FIGURE 1: Representative drawings of two-dimensional chromatograms of the amino terminal dansyl derivatives of intact IgGs WEB and LON. Solvents II and III are run in the same direction. The lower and upper unlabeled non-specific spots represent dansyl-OH and dansyl-NH_2 respectively.

microscale dansylation reaction. Figure 1 shows representative drawings of typical two-dimensional chromatograms of the amino terminal dansyl derivatives of two intact IgG cryoproteins, WEB and LON. A single amino acid derivative was observed for each intact protein. These were identified as aspartic acid for protein WEB and tyrosine for protein LON, and represented the light chain amino termini of these cryoglobulins whose sequences have been presented elsewhere (9).

Automated amino acid sequence analysis was then performed on the WEB and LON isolated heavy chains for six sequenator cycles. No PTH-amino acids were identified by gas, high pressure liquid and thin layer chromatography. These findings assured us of the presence of blocked amino termini on these heavy chains.

Each heavy chain preparation was then deblocked by the removal of pyroglutamic acid as described in Methods, and subjected again to automated amino acid analysis for three cycles. Figure 2 shows representative identifications by high pressure liquid and thin layer chromatography of selected PTH-amino acid residues from heavy chain of protein LON. Pyroglutamate amino peptidase used in DBB cleaves the N-terminal PCA and allows automated amino acid sequence analysis to begin with the second residue. The percent

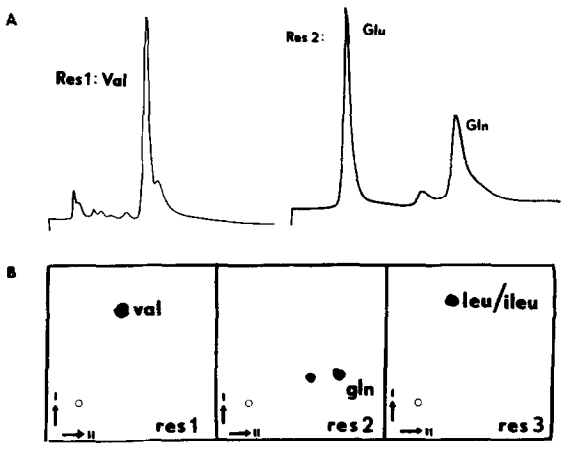

FIGURE 2: Identification of PTH-amino acid derivatives, A. High performance liquid chromatograms of deblocked heavy chain from IgG LON. Residue 1 was eluted with a 40:60 H_2O:MeOH solvent system. Residue 2 was eluted with a 50:30 H_2O:MeOH solvent system. The presence of PTH-glutamic acid is due to partial deamidination of PTH-glutamine during acid cleavage. B. Thin layer chromatograms of protein LON deblocked heavy chain. Solvent I contained toluene:pentane:acetic acid (60:30:16, v/v) and 25 mg of butyl PBD. Solvent II contained 25% (v/v) acetic acid.

yield for the first PTH-amino acid residues obtained varied from 83 to 85% and is similar to the percent yield of unblocked proteins (24). This indicated that deblocking was nearly 100% complete.

Table I lists the N-terminal amino acid sequences for heavy chains WEB and LON and additional proteins containing N-terminal pyroglutamic acid thus far sequenced in our laboratory. In addition, the technique exactly as described above has been successfully applied to deblock the amino terminus of immunoglobulin variants in another laboratory which has allowed their automated amino terminal sequence, thus confirming the utility of the method.*

It had been known previously that pyroglutamyl amino peptidases isolated from bacteria (13,14) possessed certain biochemical properties. Specifically, the activity of the enzyme was extremely sensitive to temperature, oxidation, length of incubation with substrate, bivalent metal ions and

*Personal communication: Dr. E.C. Franklin and F.C. Prelli, New York University Medical Center.

TABLE I

N-TERMINAL AMINO ACID SEQUENCE OF PCA BLOCKED IMMUNOGLOBULINS

PROTEIN	POLYPEPTIDE CHAIN	SEQUENCE
		1 2 3
WEB	GAMMA (heavy)	(PCA)-VAL-GLN-LEU 82.7 81.9*
LON	GAMMA (heavy)	(PCA)-VAL-GLN-LEU 83.5 74.0*
BAK	LAMBDA (light)	(PCA)-SER-VAL-LEU
PIE	LAMBDA (light)	(PCA)-VAL-LEU
BUL	LAMBDA (light)	(PCA)-LEU-THR-ILE

* Numbers indicate per cent yield for residues of proteins WEB and LON

alkylating agents. Therefore, in order to determine the optimal conditions for deblocking polypeptide chains of considerable lengths (M.W. 25,000-50,000), it was necessary to investigate the influence of these parameters on the activity of the calf liver pyroglutamyl amino peptidases to deblock our proteins. Table II illustrates the effects of various incubation conditions on the ability of the enzyme to deblock a polypeptide gamma chain termed WEB. Other preliminary studies indicated that the enzyme is similar to bacterial pyroglutamyl amino peptidase previously isolated (13,14) since it requires an SH-compound for activation.† Thus, when the enzyme and proteins are incubated with the SH-group blocking compound Iodoacetamide, complete enzyme inactivation was observed. While 2-mercaptoethanol is somewhat effective, we routinely employ dithiothreitol in the deblocking buffer due to its greater stability and resistance to oxidation. The presence of bivalent metal ions such as Hg^{++} led to complete enzyme inactivation and necessitated the addition of a chelating compound such as disodium EDTA. Oxidation upon exposure to room air led to a 59%

†Personal communication: Boehringer Mannheim Biochemicals, Indianapolis, Indiana.

TABLE II

EFFECTS OF INCUBATION CONDITIONS ON ENZYME ACTIVITY

CONDITIONS	PERCENT YIELD - RESIDUE 1*
Normal Procedure: 9 hr incubation 4°C, 14 hr incubation R.T. } under N_2	82.7
9 hr incubation 4°C, 4.5 hr incubation R.T. } under N_2	33.5
9 hr incubation 4°C under N_2	0
14 hr incubation R.T. under N_2	39.0
9 hr incubation 4°C, 14 hr incubation R.T. } under N_2 in presence of 50 mM Iodoacetamide	0
9 hr incubation 4°C, 14 hr incubation R.T. under room air	48.7
9 hr incubation 4°C, 14 hr incubation R.T. } under N_2 in the presence of 2 mM $HgCl_2$	0

*Prototype blocked polypeptide heavy chain WEB was utilized in these experiments.

reduction in enzyme activity. Since we noted that the enzyme was extremely unstable above room temperature and found that incubation at 37°C did not allow deblocking of the test protein (see discussion below), the influence of temperature on enzyme activity was also determined. Table II demonstrates that deblocking of the WEB heavy chain was most complete when a double incubation procedure at 4°C and at room temperature was utilized. Elimination of either the room temperature or the 4°C incubation led to complete or partial inactivation of the enzyme respectively. Therefore, an initial incubation at 4°C followed by a second incubation at room temperature is suggested in order to insure maximum enzyme stabilizing conditions.

DISCUSSION

The strategy for deblocking with pyroglutamate amino peptidase was

heavily influenced by obstacles previously realized by others (13,14,25). In the present method, the temperature of the reaction, concentrations of protein and the protein:enzyme ratio, and conditions which effect the solubility of the protein prior to enzyme treatment are of importance.

The first and probably the most crucial factor is the instability of the enzyme preparations. Doolittle and Armentrout (13) noted increased instability of their enzyme from Pseudomonas during purification procedures. Thus, they utilized a crude sephadex G-200 extract of the enzyme at room temperature for 12 hours, to deblock PCA containing peptides in order to retain enzyme activity. Szewczuk and Mulczyk (14) found that although pyrrolidonyl peptidase isolated from Bacillus subtilis showed no change in activity with preincubation at 45°C for 30 minutes, at 55° and 65°C the enzyme was rapidly inactivated. Storage of the enzyme at -10°C for 2 months decreased the activity by about 50%. Their procedure for removing PCA from human seromucoid involved a 16 hour incubation at 37°C with a protein:enzyme ratio of 3000:1. Furthermore, it was also necessary to precipitate the protein with hot ethanol and isolate the purified unblocked protein by high voltage electrophoresis.

Our first attempts at using the pyroglutamate peptidase isolated from calf liver with blocked human gamma chains at 37°C for 16 hours (protein: crude enzyme ratio, 3000:1) were unsuccessful. At this time and prior to its use the enzyme was reconstituted with DBB and stored under nitrogen at 4°C. Since it was possible that the enzyme could be inactivated at 37°C, we decided to lower the initial incubation temperature to 4°C and incubate for 9 hours. The reaction vial was then allowed to equilibrate to ambient temperature. Furthermore, the enzyme was found to be more stable if stored lyophilized at -20°C rather than reconstituted with DBB. We also decreased the protein:crude enzyme extract ratio to 100:1 and then to 10:1 without any difficulty. After incubation the protein and enzyme mixture is dialyzed directly into 0.05 M acetic acid, lyophilized and subjected to automated

primary amino acid sequence determination without any problems of contaminating sequences.

In addition, we found the calf liver pyroglutamyl amino peptidase to be extremely sensitive to the SH-group blocking compound Iodoacetamide and to the presence of bivalent metal ions such as Hg^{++}. Air oxidation caused severe reduction in enzyme activity and we therefore advise the incubation to proceed under N_2.

We also found it helpful to dialyze the heavy chains isolated from sephadex G-100 column chromatography directly into DBB without lyophilization. Lyophilized heavy chains are relatively insoluble in DBB and are in an apparently unsatisfactory conformation for complete cleavage of amino-terminal pyroglutamic acid by the enzyme. Further, aggregation of the proteins utilized was reduced and their solubility improved if their concentration in DBB was kept at approximately 1 mg/ml. We thus recommend incubation of proteins with the enzyme before they have been subjected to severely denaturing conditions, and their treatment with enzyme at low protein concentrations.

Applications of this improved method of deblocking, which has thus far allowed initial amino-terminal sequence analysis on five proteins in our laboratory should facilitate analogous studies of other proteins and peptides which have been hindered by blocked amino termini.

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