Isolation of Low Molecular Weight Digestion Products of the Human Platelet Thromboxane A_2 Receptor by Tricine Continuous Elution Preparative Gel Electrophoresis

Joseph W. Turek and Guy C. Le Breton, Department of Pharmacology, University of Illinois at Chicago, College of Medicine, Chicago, IL 60612. Address correspondence to: Joseph W. Turek, 835 South Wolcott, Department of Pharmacology M/C 868, University of Illinois at Chicago, Chicago, IL 60612. E-mail: jturek1@icarus.uic.edu

Introduction

The human platelet thromboxane A_2 (TXA_2) receptor, in addition to its normal role in hemostasis, has been implicated in the pathogenesis of multiple cardiovascular diseases.[1] Studies suggests that increased platelet production of the natural ligand, TXA_2, may lead to enhanced platelet aggregatory activity, predisposing an individual to spontaneous platelet aggregation, followed by thrombus formation. Attempts to develop specific antagonists to block the TXA_2 receptor have been hampered by a lack of information concerning the receptor ligand-binding domain.

Labeling of receptors, followed by sequential receptor digestion, accumulation, and sequence identification of labeled fragments, has become a commonly used approach to identify receptor ligand-binding domains.[2–6] However, the initial digestion product contains a wide mixture of proteins, from smaller peptides which cannot be separated by traditional glycine gel electrophoresis, to larger proteins not effectively purified using reversed-phase or size exclusion HPLC. In addition, the limited availability of certain receptor proteins (including the TXA_2 receptor) has hindered the isolation of sufficient amounts of labeled fragments. To circumvent these problems, we have coupled continuous elution preparative gel electrophoresis with the technique of tricine gel electrophoresis.

In this regard, the recent development of continuous elution preparative gel electrophoresis has provided significant benefits to the protein biochemist. The Mini Prep Cell enables fractionation and isolation of a mixture of proteins with minimal sample loss. In addition, complete isolation can be achieved within a shorter time frame, since commonly used electro-elution techniques for the recovery of proteins from gel slabs are not needed. The development of tricine gel electrophoresis has also provided significant advantages in analytical protein chemistry, since it allows effective resolution of low molecular weight proteins at relatively moderate acrylamide concentrations. Thus, substitution of tricine for glycine as the trailing ion,[7] results in low molecular weight resolution which cannot be achieved using the conventional glycine gel electrophoresis procedure of Laemmli.[8] An added advantage of this approach is that the use of tricine versus glycine prevents signal disruption during subsequent amino acid sequencing. In light of these considerations, we have developed a procedure for casting tricine tube gels in the continuous elution preparative gel electrophoresis Mini Prep Cell. This procedure was then used to successfully isolate and accumulate low molecular weight digestion products of the human platelet TXA_2 receptor.

Methods

SAMPLE PREPARATION

Purified TXA_2 receptor protein was chemically digested with cyanogen bromide according to the method described by Gross.[9] In short, receptor protein was suspended in 70% formic acid, resulting in a final protein concentration of 1 mg/ml. Cyanogen bromide (CNBr) crystals were then added in 100 times molar excess over methionine residues in the protein, with digestion carried out at room temperature, while tilting the solution for 24–36 hours in the dark. Digestion was terminated by diluting the sample solution to 7% formic acid with deionized water, immediately followed by lyophilization. Digested protein was then resuspended in 600 µl deionized water and added to 150 µl of 5x sample buffer (125 mM Tris-base, 30% glycerol, 10% SDS, 5% β-mercaptoethanol, and 0.25% Coomassie® blue G-250, pH to 6.8 with HCl). Samples were denatured at 50 °C for 45 minutes prior to loading onto the preparative electrophoresis gel.

Table 1. Gel Composition for Tricine Continuous Elution Electrophoresis

	Stacking Gel 4% T, 3% C	Spacer Gel 10% T, 3% C	Separating Gel 16.5% T, 6% C
49.5% T, 3% C solution*	250 µl	1.5 ml	—
49.5% T, 6% C solution*	—	—	2.5 ml
Gel Buffer**	775 µl	2.5 ml	2.5 ml
Glycerol	—	—	1.0 ml
Water	2.1 ml	3.5 ml	750 µl
10% Ammonium Persulfate	13 µl	25 µl	25 µl
TEMED	1.3 µl	2.5 µl	2.5 µl

* *Where T=acrylamide+bisacrylamide (w/v)* $C = \frac{bisacrylamide}{(acrylamide+bisacrylamide)}$ *(w/v)*

** *3 M Tris-base, 0.3% SDS, pH 8.45 adjusted with HCl*

BIO-RAD

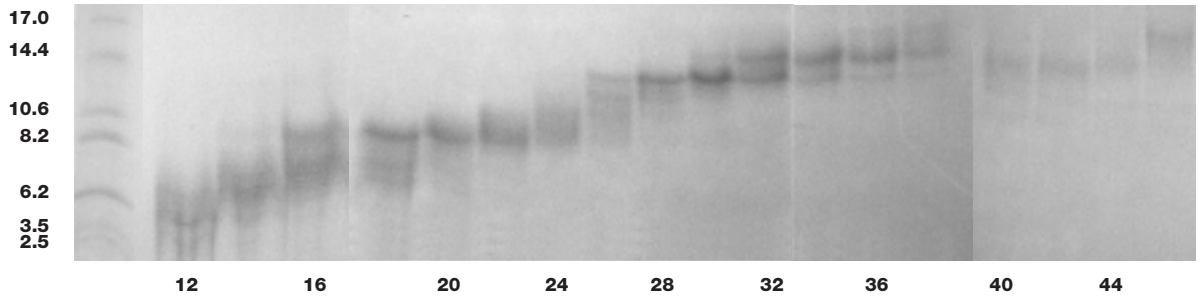

Fig. 1. Coomassie-stained tricine SDS-PAGE slab gel (16.5%) of elution fractions from tricine continuous elution preparative gel electrophoresis of CNBr digested TXA_2 receptor.

TRICINE CONTINUOUS ELUTION ELECTROPHORESIS

The Mini Prep Cell was assembled with a single modification to the manufacturer's instructions. A dialysis membrane of 100 Dalton molecular weight exclusion limit was used to replace the 3.5 or 6 kD membrane supplied by the manufacturer. This modification prevents desirable low molecular weight proteins from passing through the dialysis membrane. Table 1 shows the gel composition for the 16.5% tricine gels used in these experiments. The separating gel was poured to a height of 6.67 cm and immediately followed by a 1.33 cm spacer gel. After these layers had polymerized sufficiently, a 2 cm stacking gel was cast over the spacer gel. Following complete polymerization, the gels were stored at 4 °C overnight to allow equilibration. The next day, electrophoresis was carried out in the coldroom (4 °C) using tricine cathode buffer (100 mM Tris-base, 100 mM tricine, 0.1% SDS, pH 8.25) in the upper chamber, and anode buffer (200 mM Tris-base, pH to 8.9 with HCl) in the lower and elution chambers. The apparatus was operated at 4 watts constant power until the dye front migrated to the bottom of the tube gel. At this time, the tube gel was connected to a peristaltic pump (flowing at a rate of 0.1 ml/min), and 1 ml fractions were isolated for the next 16 hours. Fractions were concentrated by lyophilization and subsequently resuspended in a small volume of buffer (250 µl).

Results

Tricine SDS-PAGE slab gels (16.5%) were stained by Coomassie brilliant blue and used to identify fractions containing CNBr digested TXA_2 receptor protein in the eluate from the Mini Prep Cell. These gels have been described by Schagger and von Jagow to optimally separate low molecular weight proteins.[7] As shown in Figure 1, this procedure resulted in the separation of low molecular weight CNBr digested TXA_2 receptor fragments (3.5–17 kD) into discrete bands. Under these conditions, proteins with a molecular weight difference as low as 2–3 kD could be effectively resolved. The bands corresponding to labeled fragments can then be sequenced to reveal their amino acid composition.

Conclusions

The procedure outlined in this bulletin offers a highly effective method for separating low molecular weight proteins using the Mini Prep Cell. In this system, the substitution of tricine for glycine in the running buffer and the use of a low pH gel buffer prevents the low molecular proteins from spreading as they unstack within the gel, resulting in sharper bands as low as 3.5 kD. Additionally, since the proteins are obtained in solution, traditional electro-elution techniques for recovering the proteins from gels are not needed. Finally, microsequencing can be performed directly from these solutions since tricine, unlike glycine, does not interfere with Edman degradation.

Acknowledgements

This work was supported by National Institutes of Health Grant HL-24530, NATO Grant CRG-940595, The Blowitz Foundation, and the American Heart Association Student Scholar in Cardiovascular Disease and Stroke Award. This work was conducted under the auspices of the Association for United States-French Biomedical Cooperation.

References

1 *Moncada, S. and Vane, J. R.,* Pharmacol. Rev., ***30**, 293–331 (1978).*

2 *Kurtenbach, E., Curtis, C. A. M., Pedder, E. K., Aitken, A., Harris, A. C. M. and Hulme, E. C.,* J. Biol. Chem., ***265**, 13702–13708 (1990).*

3 *Dohlman, H. G., Thorner, J., Caron, M. G. and Lefkowitz, R. J., Annu. Rev. Biochem., **60**, 653–688 (1991).*

4 *Cohen, J. B., Sharp, S. D. and Liu, W. S., J. Biol. Chem., **266**, 23354–23364 (1991).*

5 *Boyd, N. D., Kage, R., Dumas, J. J., Krause, J. E. and Leeman, S. E.,* Proc. Natl. Acad. Sci., ***93**, 433–437 (1996).*

6 *Servant, G., Laporte, S. A., Leduc, R., Escher, E. and Guillemette, G.,* J. Biol. Chem., ***272**, 8653–8659 (1997).*

7 *Schagger, H. and von Jagow, G.,* Anal. Biochem., ***166**, 368–379 (1987).*

8 *Laemmli, U. K.,* Nature *(London), **227**, 680-685 (1970).*

9 *Gross, E.,* Methods in Enzymology, ***11**, 238-253 (1967).*

Bio-Rad Laboratories

Life Science Group

***Website** www.bio-rad.com **U.S.** (800) 4BIORAD **Australia** 02 9914 2800 **Austria** (01)-877 89 01 **Belgium** 09-385 55 11 **Canada** (905) 712-2771 **China** 86-10-62051850 **Denmark** 45 39 17 99 47 **Finland** 358 (0)9 804 2200 **France** (01) 43 90 46 90 **Germany** 089 318 84-0 **Hong Kong** 852-2789-3300 **India** (91-11) 461-0103 **Israel** 03 951 4127 **Italy** 02-21609.1 **Japan** 03-5811-6270 **Korea** 82-2-3473-4460 **The Netherlands** 31 318-540666 **New Zealand** 64-9-4152280 **Singapore** 65-2729877 **Spain** (91) 661 70 85 **Sweden** 46 (0)8 627 50 00 **Switzerland** 01-809 55 55 **United Kingdom** 0800-181134*