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Edman Protocols for High Yield Covalent and Adsorptive Sequence Analysis on the ProSequencer™

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INTRODUCTION

For the last few years, our research has focused on improving the state-of-the-art in covalent ("solid-phase"*) sequence analysis. This work has resulted in the introduction of the 6625 Pro-Sequencer (1), Sequelon-AA and -DITC covalent attachment membranes (2) and the SequeNet covalent entrapment process (3). Using the covalent attachment strategy it is now possible to analyze picomole quantities of peptides and proteins and more easily study important polypeptide modifications (e.g., phosphorylation and glycosylation sites (4,5)).

One drawback to the covalent strategy is the fact that occasionally a sample does not contain a residue (e.g., lysine, aspartic, glutamic acid or a carboxyl terminus) necessary for covalent attachment. In addition, the identification of attachment residues can be problematic since a majority of the residue may be covalently bound to the solid support. This is particularly important when small quantities of material are analyzed (< 20 pmol). To circumvent these problems, we undertook the development of cycle protocols for the 6625 ProSequencer that would allow for analysis of adsorptively bound samples. The goals of the project were:

1. Develop a set of adsorptive cycles that would allow the instrument operator to switch between covalent and adsorptive analysis without the need to change sequencer chemicals or make hardware modifications.

2. Improve the existing covalent cycles.

3. Improve the HPLC system used to analyze the PTH derivatives to provide more reliable sequencing at high sensitivity.

The result would be a versatile sequencer that could be used for analysis of virtually any type of sample.

*Note about the terminology used in this poster

All modern sequencers use some type of sample support or "solid-phase" (e.g. PVDF membrane or glass fiber disk). To more accurately describe the mechanism by which the sample is immobilized on the support, we have chosen to employ the terms covalent and adsorptive to reflect the presence or absence of a covalent bond between the polypeptide and the sample matrix. Use of the terms "solid-phase" and "gas-phase" is sometimes confusing since the former indicates a covalent bond to the support while the latter actually describes the method by which reagents are delivered to the sample support regardless of how the sample is immobilized.

SYSTEM DESCRIPTION

The 6625 Prosequencer consists of three components; a microfluidics unit, an HPLC system and a computer.

The microfluidics unit performs the Edman chemistry according to cycle protocols downloaded from the computer. The fluidics unit is comprised of a reaction chamber and an autoconverter vial connected to a series of precision syringe pumps and pneumatically actuated low dead volume valves. The entire system volume is less than 500 ul such that excess PITC and its reaction by-products can be removed from the system with minimal amounts of wash solvents.

PTH amino acid derivatives are transferred in over 95% yield from the microfluidics unit to a Waters 625 HPLC system. The derivatives are separated using gradient elution on a SequeTag PTH analysis column (2.0 x 30 cm or 3.9 x 30 cm). Following separation, the residues are detected at 269 nm with a Waters 486 detector. The dehydro (delta) products of PTH-Ser and PTH-Thr can also be observed by programming the detector to briefly switch to 313 nm just before these breakdown products elute (see Figure 4 and the discussion section).

In addition to providing control of the microfluidics unit, a 386 based computer serves to collect and process chromatographic data. The computer display can be used overlay chromatograms for visual sequence calling. Alternatively, successive chromatograms can be mathematically aligned and subtracted to provide easily interpreted difference traces. The raw or processed data can be printed or plotted in a variety of formats dependent on the needs of the operator.

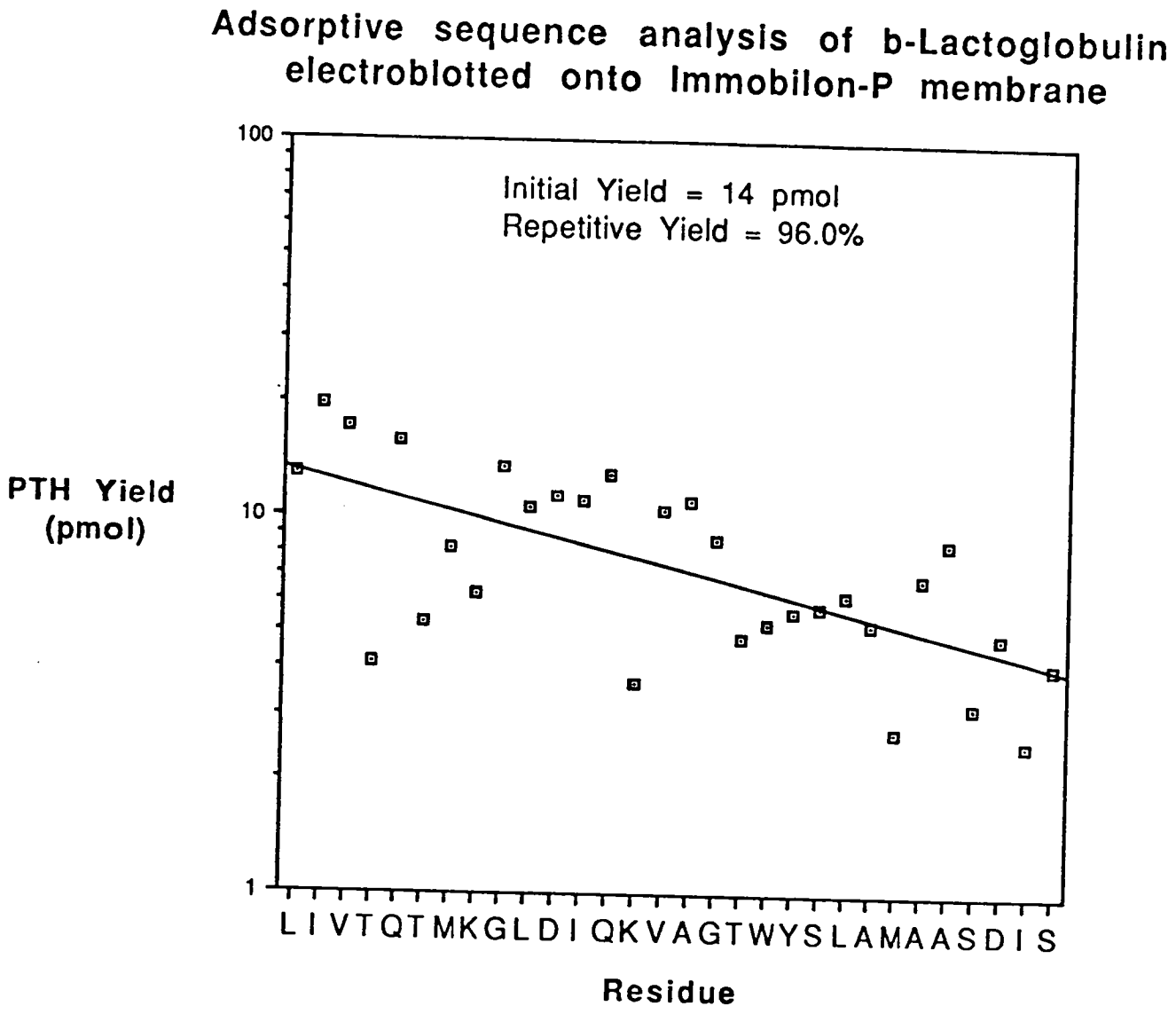


Figure 1. Yield plot from the adsorptive sequence analysis of b-lactoglobulin. Approximately 50 pmol of b-lactoglobulin was purified by gel electrophoresis and electrotransferred to Immobilon-P as described by Matsudaira (6).

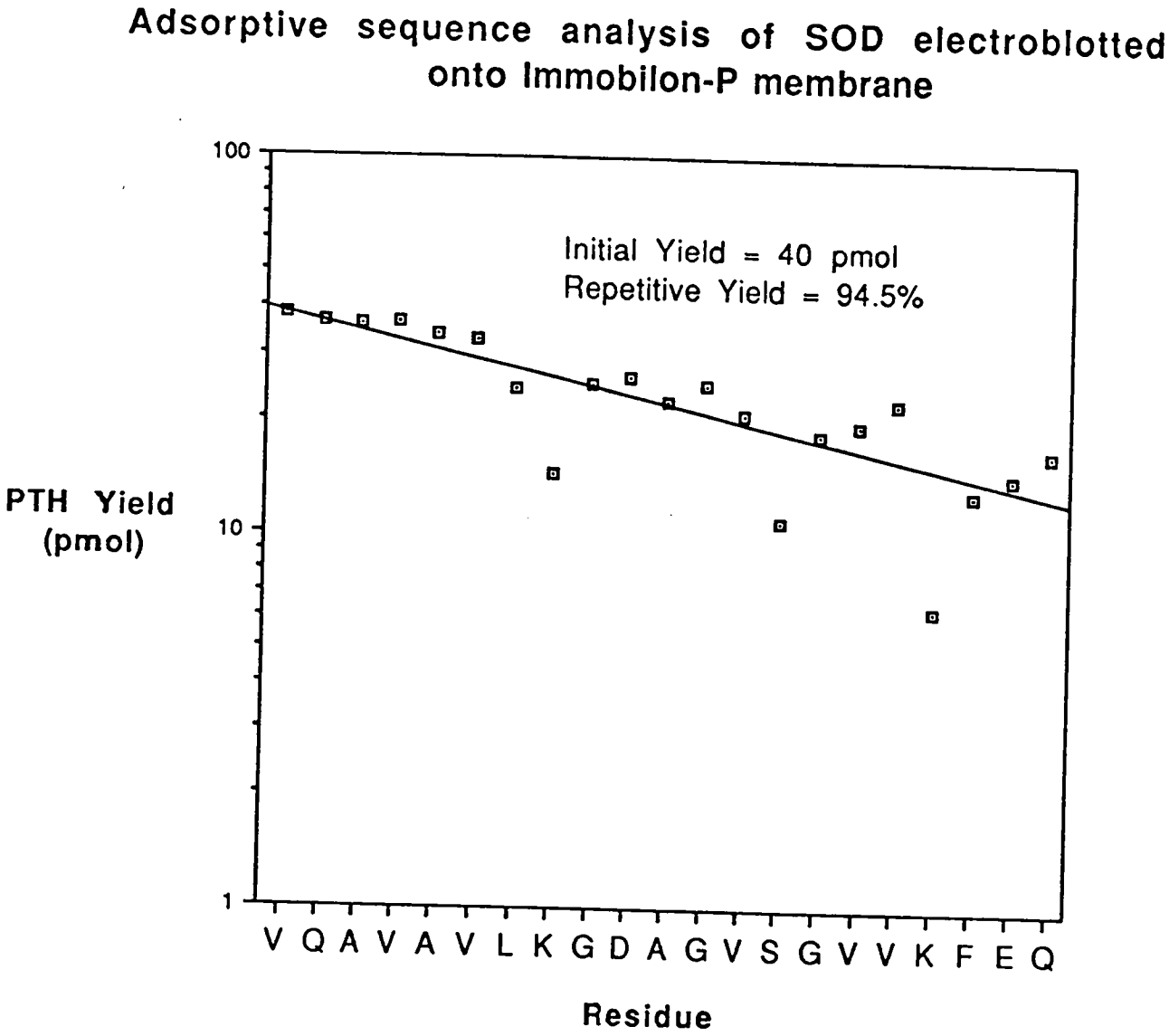
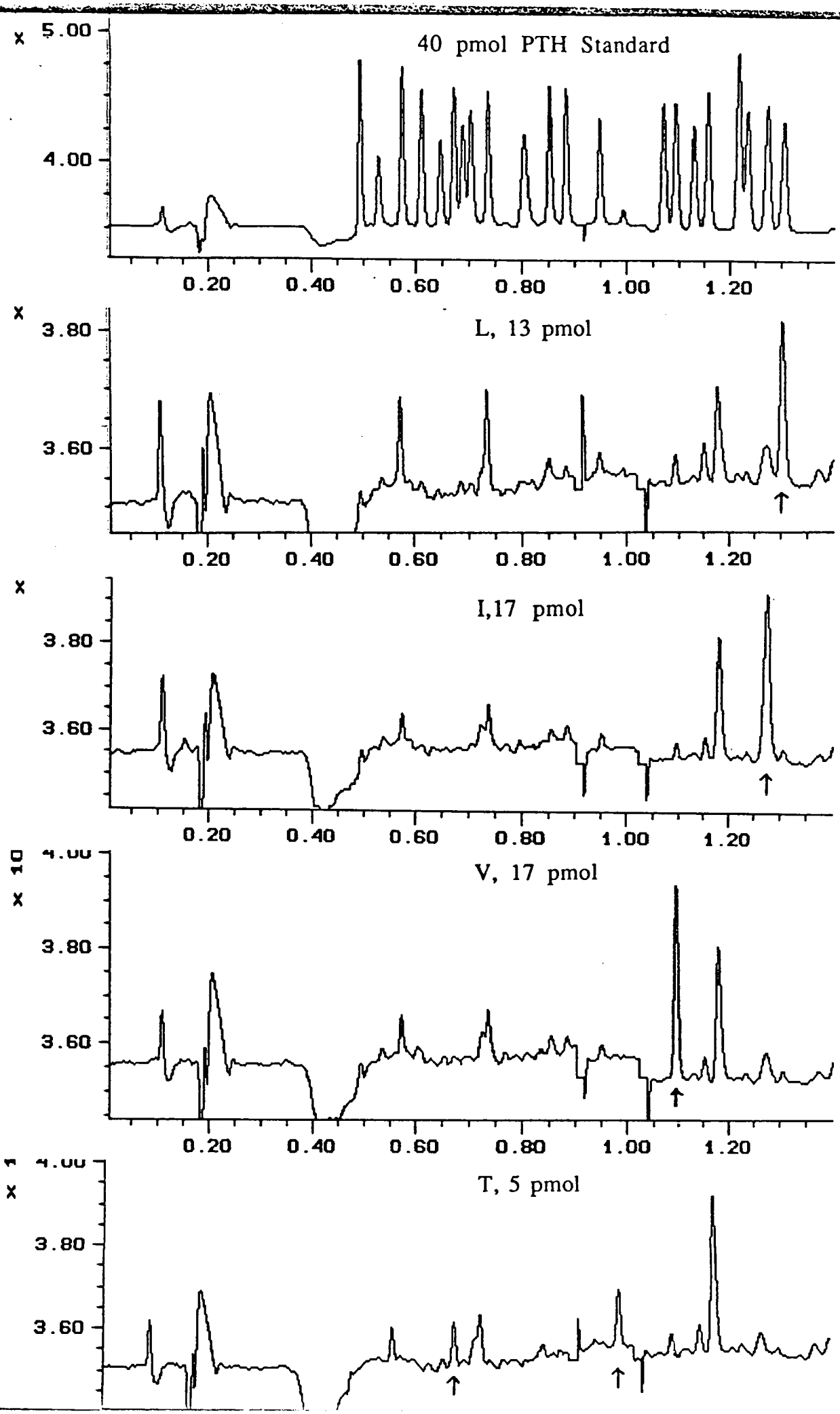
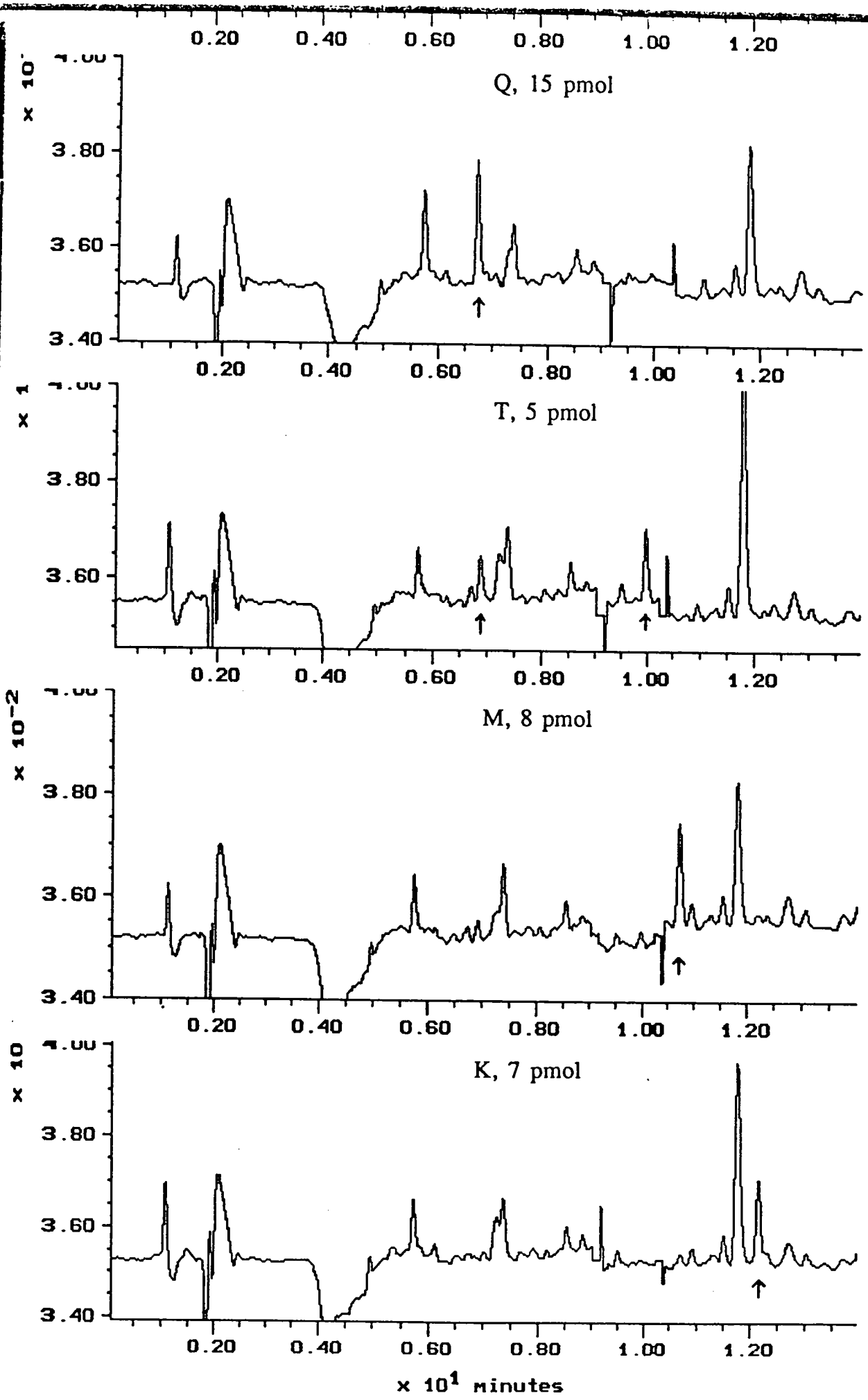
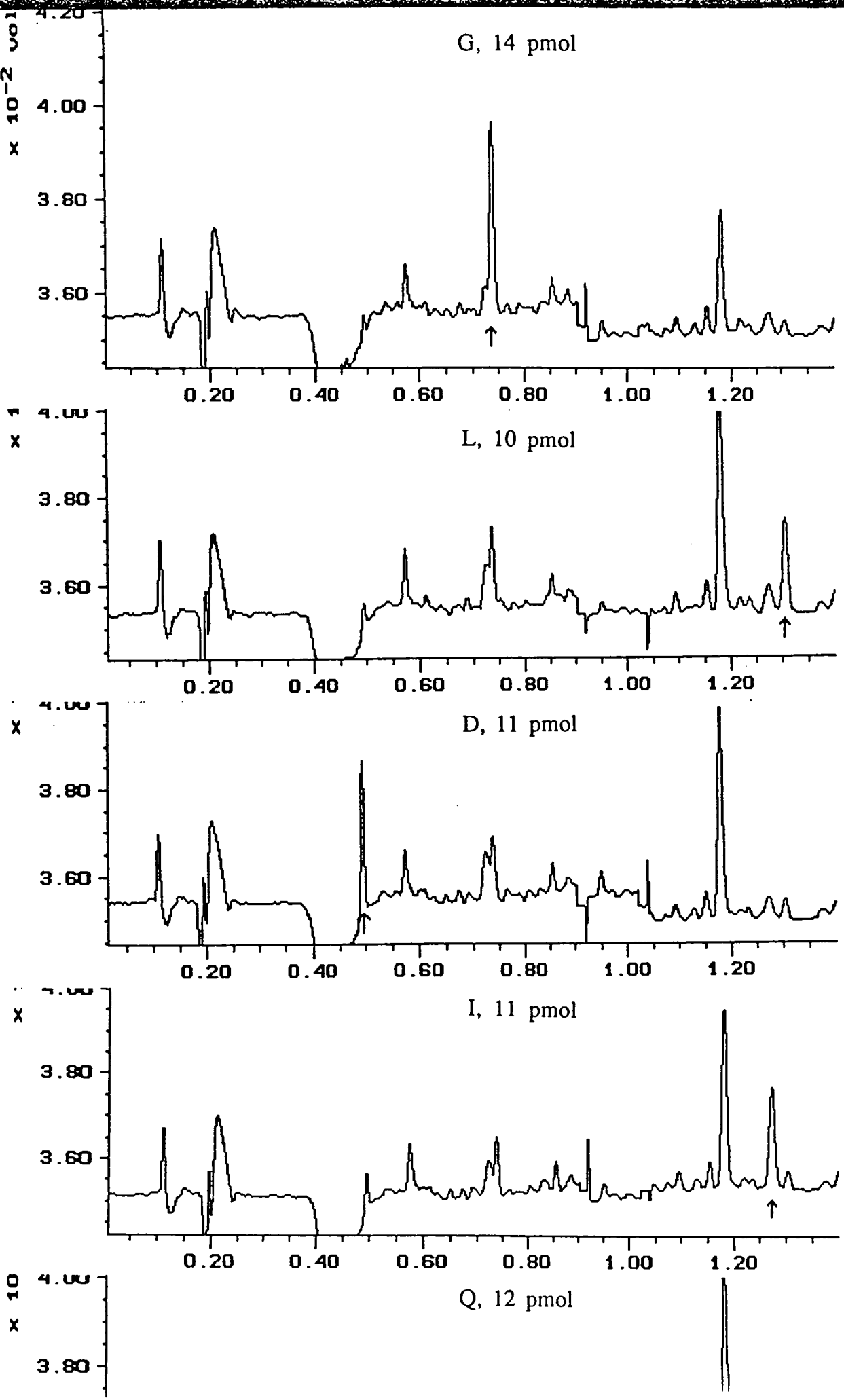


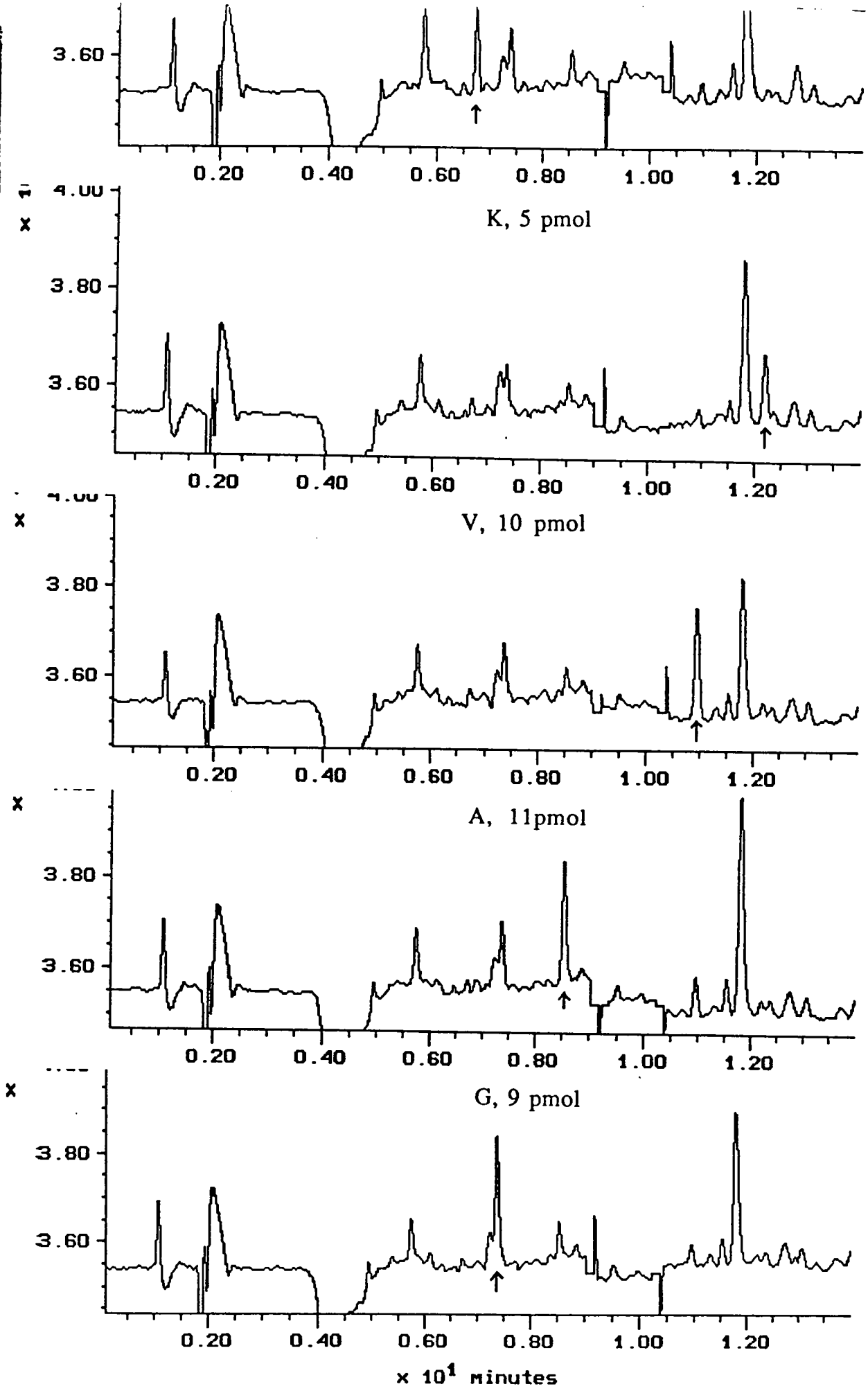
Figure 2. Yield plot obtained from the adsorptive sequence analysis of super oxide dismutase (SOD). Approximately 100 pmol of b-lactoglobulin was purified by gel electrophoresis and electro-transferred to Immobilon-P as described by Matsudaira (6).

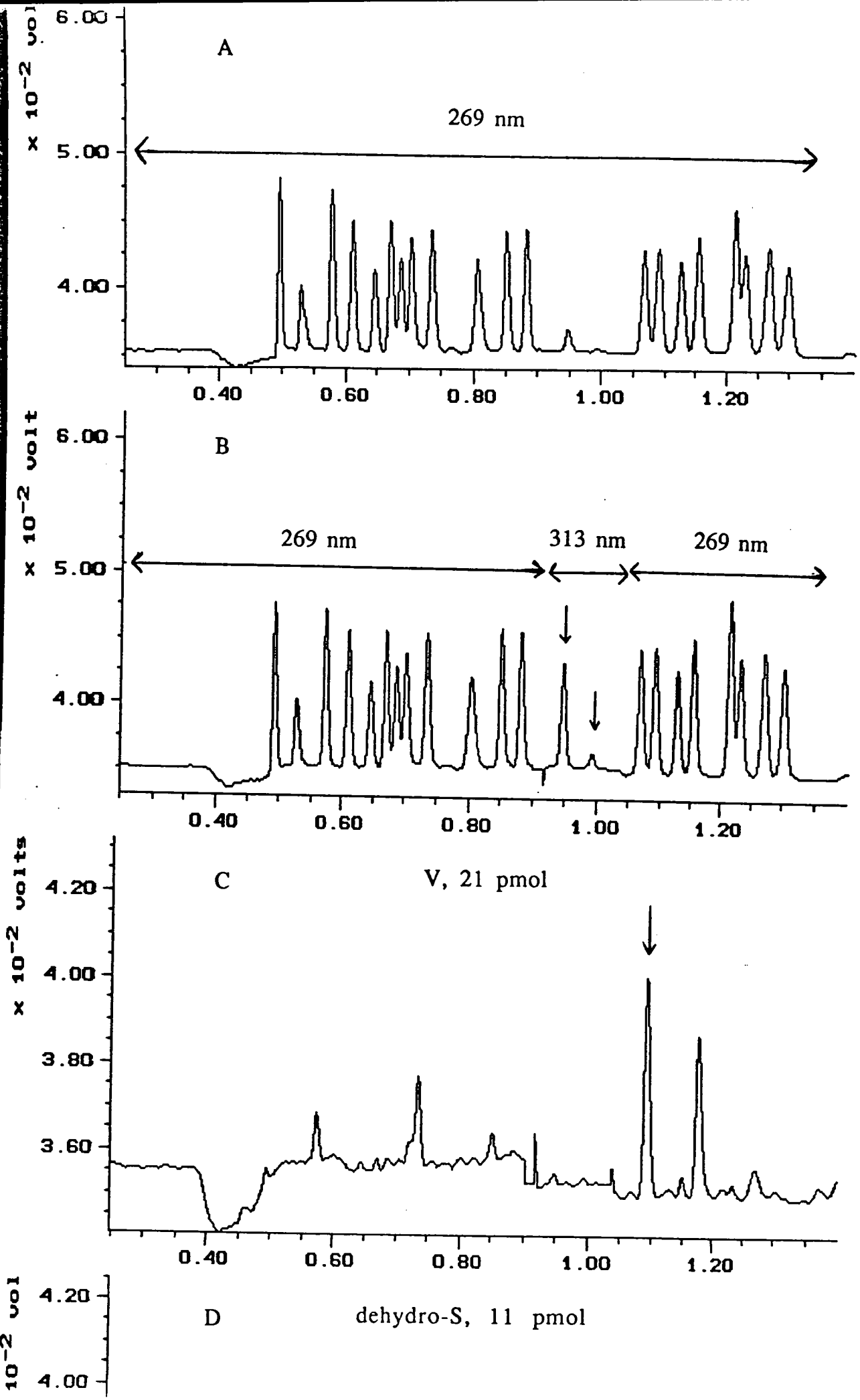
Figure 3. Raw data traces from the adsorptive analysis of b-lactoglobulin electroblotted onto Immobilon-P. The yield plot for this sequence run is presented in Figure 1. Wavelength switching was used to identify serine and threonine residues as shown in Figure 4.


40 pmol PTH Standard
L, 13 pmol
I,17 pmol
V, 17 pmol
T, 5 pmol
x
x 10
5.00
4.00
3.80
3.60
0.20
0.40
0.60
0.80
1.00
1.20


Q, 15 pmol
T, 5 pmol
M, 8 pmol
K, 7 pmol
x 10
x 1
x 10⁻²
3.80
3.60
3.40
0.20
0.40
0.60
0.80
1.00
1.20
x 10¹ minutes


G, 14 pmol
L, 10 pmol
D, 11 pmol
I, 11 pmol
Q, 12 pmol
4.20
4.00
3.80
3.60
0.20
0.40
0.60
0.80
1.00
1.20


3.60
0.20
0.40
0.60
0.80
1.00
1.20
K, 5 pmol
3.80
V, 10 pmol
A, 11pmol
G, 9 pmol
x 10¹ minutes


A
269 nm
x 10^-2 vol
6.00
5.00
4.00
0.40
0.60
0.80
1.00
1.20
B
269 nm
313 nm
269 nm
x 10^-2 volt
6.00
5.00
4.00
0.40
0.60
0.80
1.00
1.20
C
V, 21 pmol
x 10^-2 volts
4.20
4.00
3.80
3.60
0.40
0.60
0.80
1.00
1.20
D
dehydro-S, 11 pmol
10^-2 vol
4.20
4.00

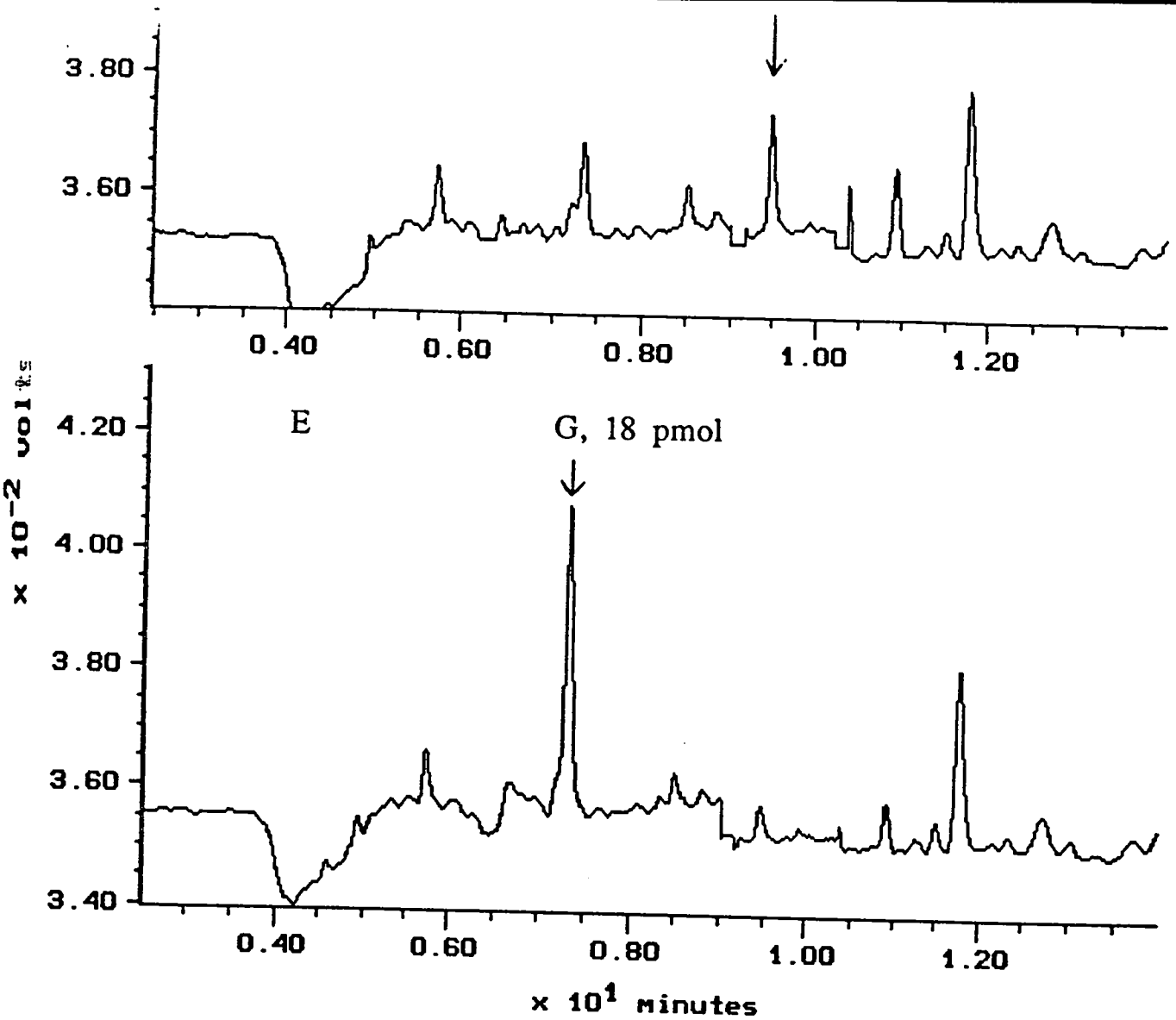


Figure 4. Wavelength switching was used to observe the dehydro elimination products of PTH-Ser and PTH-Thr at 313 nm. At the top (A) is shown a 40 pmol PTH standard acquired at 269 nm. Below (B) is the same standard but the detector wavelength was switched to 313 nm between 9.0 and 10.5 minutes. The following chromatograms (C-E) show the data from cycles 13-15 of the analysis of SOD electroblotted onto Immobilon-P. Dehydro PTH-Ser was easily visible in 313 nm window of cycle 13 (D).

TABLE 1

COMPARISON OF CYCLE PROTOCOLS

Subcycle	"Old" Covalent	New Covalent	New Adsorptive	Typical "Gas-Phase"
Coupling	PITC/Liquid base	PITC/Liquid base	PITC/Liquid base	PITC/Base vapor
Wash	EtAc/Methanol	EtAc/Methanol	EtAc	Heptane and/or EtAc
Cleavage	Liquid TFA	Liquid TFA	Liquid TFA	TFA vapor
Extraction	Liquid TFA	EtAc	EtAc	BuCl or EtAc
Conversion	Aqu. TFA	Aqu. TFA	Aqu. TFA	Aqu. TFA
HPLC Transfer	Aqu. ACN	Aqu. ACN	Aqu. ACN	Aqu. ACN
Total Cycle Time:	36 min	31.5 min	44 min	45 min

TABLE 2

REAGENTS AND BOTTLE POSITIONS FOR COVALENT/ADSORPTIVE SEQUENCE ANALYSIS ON THE 6625 PROSEQUENCER

Bottle Position	"Old" Covalent Mode	Covalent/Adsorptive Dual Mode
1	20% Aqu. acetonitrile	10% Aqu. acetonitrile
2	Trifluoroacetic acid	Trifluoroacetic acid
3	20% Aqu. TFA	20% Aqu. TFA
4	Auxiliary	Auxiliary
5	10% PITC in acetonitrile	2% PITC in heptane
6	5% N-methyl morphiline in aqu. methanol	5% N-methyl morpholine in aqu. methanol
7	Ethyl acetate/heptane	Ethyl acetate
8	Methanol	Methanol

TABLE 3

REAGENT USEAGE FOR THE NEW COVLAENT/ADSORPTIVE CYCLES

	"OLD" COVALENT	NEW COVALENT	NEW ADSORPTIVE
PITC Solution	85 uL	50 uL	50 uL
NMM Buffer	135 uL	16 uL	16 uL
Ethyl Acetate Wash	5 mL	5 mL	3.5 mL
Methanol Wash	6.5 mL	3 mL	n/a
TFA	160 uL	16 uL	65 uL
Conversion Acid	100 uL	100 uL	100 uL
Transfer Solution	600 uL	400 uL	400 uL

TABLE 4

INITIAL AND REPETITIVE YIELDS FOR PROTEIN A AND INSULIN A CHAIN APPLIED TO IMMOBILON AND SEQUELON MEMBRANES

SAMPLE	MODE	SUPPORT	AMOUNT* (pmol)	INIT. YIELD (%)	REPET. YIELD (%)
Protein A	Adsorptive	Immobilon-P	20	85	95.2
	Covalent	Sequelon-DITC	20	85	95.8
Insulin A (ox.)	Adsorptive	Immobilon-N	20	55	92.9
	Covalent	Sequelon-AA	20	65	93.6

* The amount of applied sample was determined by ninhydrin or Pico-Tag based amino acid analysis. Samples were spotted directly onto Immobilon membrane disks for adsorptive analysis. For covalent analysis, samples were spotted onto Sequelon membranes and attached according to ref. (2).

TABLE 5

RECOMMENDED SEQUENCING MODES AND MEMBRANES SUPPORT FOR VARIOUS SAMPLE TYPES

MODE	SAMPLE TYPE	SOURCE	SUPPORT
Covalent	Protein	Chromatography	Sequelon-DITC, -AA
		Eluted from gel	Sequelon-DITC, -AA
		Capillary electrophoresis	Sequelon-DITC, -AA
		Electroblot	Immobilon-P/SequeNet
	Peptide*	Chromatography	Sequelon-AA
Adsorptive	Protein	Chromatography	Immobilon-P, -N
		Eluted from gel	Immobilon-P, -N
		Capillary Electrophoresis	Immobilon-P, -N
		Electroblot	Immobilon-P
	Peptide	Chromatography	Immobilon-N

* The use of Sequelon-AA is strongly recommended for analysis of hydrophobic peptides and peptides containing modified amino acid residues (e.g. phosphopeptides)

DISCUSSION

Early experiments during the development of asdsorptive protocols for the 6625 Prosequencer indicated it might be possible to utilize a common set of reagents for both high yield covalent and adsorptive sequence analysis. A study was undertaken to understand the factors responsible for wash out of protein samples bound to Immobilon-P membrane (data not shown). We were then able to minimize wash out and optimize the coupling and cleavage reaction yields of the Edman chemistry for samples that were adsorptively bound to the membrane surface.

Table 1 shows a comparison of the new covalent/adsorptive protocols with our earlier covalent cycle as well as a typical gas-phase adsorptive protocol. The new covalent/adsorptive protocols are similar to our original covalent cycle yet differ considerably from a typical gas-phase protocol since they utilize liquid reagents to effect the coupling and cleavage reactions. Unlike our previous covalent cycle, however, we now use ethyl acetate to extract the ATZ-amino acid from the solid support. This has led to an effective increase in initial sequence yields due to the fact that our previous use of TFA for extraction led to considerable destruction of amino acid ATZs. Cycle times for the new covalent and adsorptive protocols are 31.5 and 44 minutes, respectively.

Tables 2 and 3 list reagent bottle positions, compositions and usage for the adsorptive/covalent protocols as compared to our previous covalent cycle. The solvent used for PITC delivery has been changed from acetonitrile to heptane and the concentration has been lowered from 10% to 2% (v/v). As a result chemical background in the HPLC traces has been minimized in both the covalent and adsorptive sequencing modes and wash out of adsorptively bound samples has been reduced. The

new cycles utilize 2-10 fold smaller quantities of TFA, coupling buffer and PITC. In addition to lowering reagent consumption and cycle cost, reduction in the use of these reagents appears to give an increase in repetitive yields over those previously observed (5), presumably due to reduced exposure of the sample to trace impurities in the PITC and/or TFA (data not shown).

The new adsorptive protocols provide excellent results for electroblotted samples as evidenced by the yield plots obtained from analysis of b-lactoglobulin and super oxide dismutase (SOD) blotted onto Immobilon-P (Figures 1 and 2). Repetitive yields for b-lactoglobulin and SOD were 94.5% and 96.0%, respectively, from initial yields of 14 and 40 pmol. Figure 3 shows the raw data traces for the b-lactoglobulin sequence run. Levels of contaminants (e.g. DPTU, and aniline) were less than 10 pmol allowing for unambiguous sequence assignment.

Sequencing results from the covalent and adsorptive analysis of protein A and porcine insulin A chain are presented in Table 4. Initial yields of 85% were obtained for 20 pmol of protein A applied to Immobilon-P (adsorptive) or Sequelon-DITC (covalent). Repetitive yields were greater than 95% for both modes of analysis. Adsorptive analysis of 20 pmol of (oxidized) insulin A chain applied to Immobilon-N, a cationic PVDF membrane, gave initial and repetitive yields of 55% and 92.9%. Immobilon-N is superior to Immobilon-P for preventing wash out of peptides during adsorptive sequence analysis (see Poster T38 for a detailed study on the use of this membrane). Yields for this peptide were higher in the covalent mode following attachment to Sequelon-AA. In the covalent mode it was possible to assign 20 of 21 amino acids, the exception being the glutamic acid residue at position 17. The adsorptive run allowed identification of all residues except for the C-terminal glutamine and the highly polar cysteic acid residues which could not be extracted from the membrane surface.

We have also investigated the use of HPLC detector wavelength switching to increase the reliability of assignment of serine and threonine residues during sequence runs. Serine and threonine are sometimes difficult to assign during

high sensitivity analysis (< 20 pmol) since the PTH derivatives of these amino acids undergo undergo elimination during the conversion reaction. The dehydro products absorb much less strongly at 269 nm than the parent PTH compounds. However, the dehydro products absorb strongly at 313 nm and can be easily detected at this wavelength. Figure 4 shows the use of wavelength switching during data collection for detection of the dehydro products of the hydroxy amino acids.

SUMMARY

We have developed a set of reagents and chemical protocols that provide for high yield covalent and adsorptive sequence analysis on the 6625 ProSequencer. As illustrated in Table 5, this system allows the protein chemist to conveniently choose the analysis mode and sequencing support most suited to the characteristics and method of isolation of the polypeptide sample.

ACKNOWLEDGMENTS

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