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Simultaneous multiple analyte detection using fluorescent peptides and capillary isoelectric focusing

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Abstract

Analyte-specific detection based on the isoelectric point of the detection moiety is a new concept that is under investigation at Vysis. We have developed methods for the synthesis of fluorescent synthetic peptides that can be conjugated to bioanalytes such as nucleic acids and antibodies, processed in a hybridization or binding assay, and then chemically released prior to detection by capillary isoelectric focusing (cIEF)–laser-induced fluorescence (LIF) detection. A two-step cIEF method in coated capillaries using salt mobilization has been used that produces high peak efficiencies and good assay reproducibility. The concentration by focusing aspect of cIEF, which allows for the entire capillary to be filled with sample, enables detection limits in the p*M* as opposed to sub-n*M* level for conventional capillary electrophoresis (CE)–LIF. The simultaneous multiple detection of eleven different focusing entities has been achieved.

Keywords: Isoelectric focusing; Derivatization, electrophoresis; Peptides

1. Introduction

Because of the number and complexity of molecular events responsible for the progression of human disease, molecular diagnostics is moving towards the development of tests that are capable of detecting many different analytes at one time. Exciting possibilities exist for detection systems that allow for the simultaneous detection of many analytes when used in conjunction with miniaturized electrophoresis devices. We have begun work on a new type of diagnostic assay detection system that uses the concentrative and resolving powers of capillary isoelectric focusing (cIEF), and which has the potential to simultaneously detect many different analytes in a single electrophoretic run, with single-color fluorescence. The assay concept is summarized in Fig. 1.

Our first attempts at generating multiple detection moieties employed phycoerythrin molecules, which are complex, fluorescent, naturally occurring proteins. These molecules are attractive as isoelectric point (p*I*) detection entities because of their high, pH-independent, fluorescence quantum yields and amphoteric nature [1]. Although we were successful in developing chemistries that would modify the p*I* of these proteins, without markedly affecting overall fluorescence properties, attempts to fractionate the

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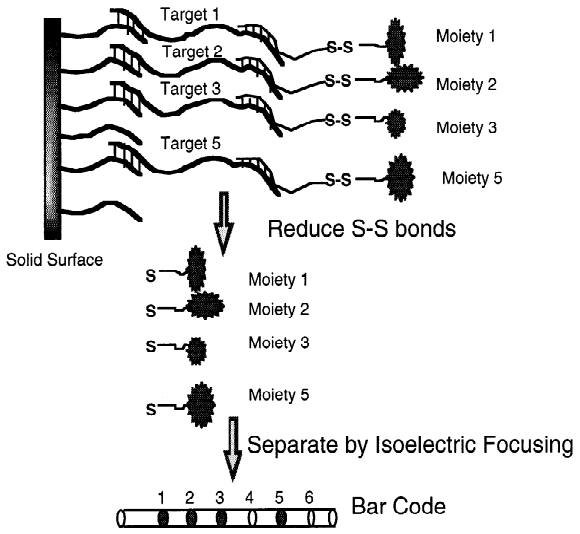

Fig. 1. Illustration of the concept of multiple analyte detection based on p*I* using a nucleic acid hybridization assay as an example. Upon separation by IEF, the identification of a peak at a specific p*I* value indicates the presence of an analyte in a specimen.

modified fluorescent proteins into usable detection moieties proved to be difficult and time-consuming. Fortunately, we found that small synthetic peptides, from three to eight residues long could be converted by fluorescent labeling into sharply focusing species. RhodamineGreen conjugated to even a single amino acid (lysine) formed a usable focusing peak. Peptides were prepared that contained a cysteine residue for reversible conjugation by disulfide bond formation and a lysine residue for dye attachment. By using simple fluorescently labeled peptides, we present an expedient route to many different detection moieties that is inexpensive and amenable to large-scale manufacture.

IEF has found limited application to small peptide analysis, probably because there are many different liquid chromatographic methods that can be applied to that type of separation problem. Mazzeo et al. [2] described the focusing of protein fragments, using UV detection and a one-step focusing method. More relevant to our work are publications by Shimura et al. [3,4] describing the slab-gel IEF and cIEF of tetramethylrhodamine-labeled peptides. Significant differences from our work are that labeling at the amino-terminus of non-cysteine-containing peptides was used and no attempt was made to use the peptides in a diagnostic assay.

2. Experimental

2.1. Reagents

RhodamineGreen carboxylic acid, succinimidyl ester (RG–NHS, cat. no. R-6107), RhodamineGreen X, succinimidyl ester (RGX–NHS, cat. no. R-6113), 6-carboxyfluorescein, succinimidyl ester (6CF–NHS, cat. no. C-6164) and tris(2-carboxyethyl)phosphine (TCEP, cat. no. T-2556) were obtained from Molecular Probes (Eugene, OR, USA). Dithiothreitol (DTT), lysine hydrochloride and cysteine hydrochloride were from Sigma (St. Louis, MO, USA). Cysteine- and lysine-containing peptides were custom synthesized by either Genemed Synthesis (South San Francisco, CA, USA) or Research Genetics (Huntsville, AL, USA). The vendor supplied mass spectra and/or liquid chromatographic data that characterized the structures. Amino-functionalized oligonucleotides were obtained from Midland Certified Reagent (Midland, TX, USA). Aminated genomic DNAs were prepared by bisulfite amination [5], from male or female gender-specific genomic DNAs or from mixed-gender human placental DNA obtained from Sigma. Ultralink immobilized N,N′-bis(3-aminopropyl)amine (DADPA) amino beads (cat. no. 53147) and SPDP–NHS (cat. no. 21657) were obtained from Pierce (Rockford, IL, USA). For derivatization, a 2.0-ml aliquot of the commercial DADPA amino bead suspension was repeatedly washed in water, then in borate buffer, followed by centrifugation and removal of supernatant. The beads were then suspended in sodium borate solution (9.0 ml) and reacted with 3-(2-S-pyridyldithio)propionate succinimidyl ester (SPDP–NHS) (50 mg, 0.16 mmol) pre-dissolved in dimethyl sulfoxide (DMSO; 3.0 ml). After rotary mixing at room temperature overnight, the beads were isolated by repeated rinsing in borate buffer, water and ethanol and, finally, were stored as a suspension in 5.0 ml of ethanol at −20°C. Attachment of SPDP was confirmed by the release of 2-thiopyridine upon treatment of an aliquot of beads with DTT (19 μmol of 2-thiopyridine/ml bead suspension).

RG–NHS is obtained as a mixture of isomers from the vendor. These isomers can be readily separated by reversed-phase liquid chromatography (RPLC) using the same methods as used for peptide analysis [6] (0.1% trifluoroacetic acid in water, eluting with a gradient of 0.1% trifluoroacetic acid in acetonitrile; Supelcosil SPLC-18-DB column, cat. no. 5-8358). The early and late-eluting isomers, termed RG1–NHS and RG2–NHS respectively, were isolated in isomerically pure form. When attached to peptides, the resulting RG1-labeled species tend to have a marginally lower p*I* than that of the RG2-labeled species (see Fig. 2).

Reagents for isoelectric focusing, including anolyte (20 m*M* phosphoric acid, cat. no. 148-5029), catholyte (40 m*M* NaOH, cat. no. 148-5028), mobilizer solution (cat. no. 148-5030) and 3/10 ampholyte solutions (cat. no. 148-503 1) and BioSpin 6 columns were obtained from Bio-Rad (Hercules, CA, USA). Under these conditions, peptides are mobilized past the detector in order of decreasing p*I* values. cIEF was carried out at 25°C in a Beckman eCap neutral coated capillary [Beckman Instruments, Fullerton, CA, USA, cat. no. 477441, 27 cm (20 cm to detector)×50 μm I.D.]. Following a 1.0-min rinse with ampholytes at 20 p.s.i., sample was introduced from a 20–200-μl sample volume, using a second rinse step for 0.3 min, which was also at 20 p.s.i. (1 p.s.i.=6894.76 Pa). After a 5-min focusing step, samples were chemically mobilized [7] past the detector by changing the outlet vial to the salt solution (both steps at 500 V/cm). After the separation was complete, the capillary was rinsed at 20 p.s.i. with ampholyte solution. The total separation time including rinses ranged from 37.8 to 47.8 min. Capillaries were stored at 4°C after use.

2.2. Internal standards

Day-to-day variation in migration times warranted the use of internal standards as reference markers. These markers, RG1–HHHKHG (basic), RG1–VHLTPVEK (intermediate) and RGX–lysine (acidic), were routinely added to mixtures of unknown peptides at concentrations of 100, 50 and 20 p*M*, respectively.

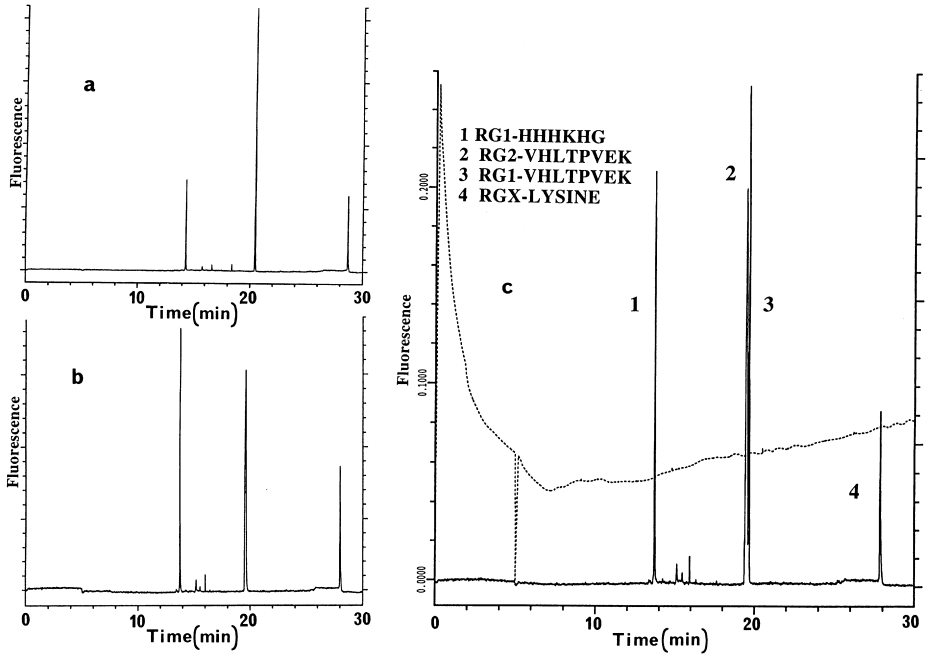

Fig. 2. cIEF separation of a mixture, which was used to determine the ability to reproducibly identify two closely resolving peaks. (a) RG1–VHLTPVEK with internal standards, (b) RG2–VHLTPVEK with internal standards, (c) co-mixture of the RG1/2–VHLTPVEK peaks and internal standards.

RGX–CO_2H was prepared by hydrolysis of RGX–NHS in 0.1 *M* borate solution, pH 8.5, at room temperature overnight, to give a solution of $RGXCO_2H$, which was used at a 200–10 p*M* dilution in ampholytes as an acidic internal standard. Lysine was labeled with RGX–NHS and the product was isolated by RPLC using methods described above. RGX–lysine was used as an alternative acidic internal standard.

2.3. Instrumentation

Capillary electrophoresis was carried out on a P/ACE 2050 instrument equipped with a laser-induced fluorescence (LIF) detection module and used the 488 nm line of an air-cooled argon ion laser (both from Beckman Instruments). Data analysis used the System Gold software package supplied by Beckman.

2.4. Synthesis of focusing peptides

Peptides were conjugated to the appropriate fluorescent dye by either of two methods. Purity and focusability of a new peptide sequence was tested by a solid-phase technique in which the sequence (100 μg of crude peptide) was first attached to SPDP-modified DADPA amino beads (25 μl of ethanol suspension, see Section 2.1). Displacement of the solid-phase thiopyridyl residue with the peptide cysteine sulfhydryl group was carried out in 0.1 *M* phosphate buffer for 1–2 h, followed by overnight reaction of the lysine residue of the appended peptide with the NHS ester of the isomerically pure fluorescent dye in 0.1 *M* sodium borate buffer, pH 8.5. After repeated washing of the solid phase with borate, water and finally ethanol, the solid phase was stored at −20°C suspended in 50% aqueous ethanol (100 μl). The appended fluorescent peptide was released from the solid phase (1.0 μl suspension) using 5 m*M* cysteine in ampholyte solution, diluted extensively with ampholyte solution and analyzed by cIEF as described above.

If the peptide sequence displayed promising peak shape characteristics, the peptide was then labeled and isolated in preparative quantities by a solution phase method. The cysteine residue of the peptide was first protected by conversion to the 2-thiopyridyldisulfide (2-TPDS) derivative with 2,2′-dithiopyridine disulfide in water containing 0.1% trifluoroacetic acid (TFA) using standard methods [8], followed by reaction with the uni-isomeric fluorescent dye NHS ester as described above. The first step was found necessary because peptides with free sulfhydryl groups underwent side reactions during dye labeling. The fluorescent conjugate was isolated by RPLC as an isoelectrically unique species, quantitated by UV spectrophotometry using the absorption coefficient of the dye at the long-wavelength maximum and stored as lyophilized pellets at −20°C. The 2-TPDS fluorescent peptides and the oligonucleotide–peptide conjugates can be stored as lyophilized solids or in solution at −20°C for at least six months.

2.5. Conjugation to nucleic acids

The fluorophore-labeled 2-TPDS peptides were conjugated to oligonucleotides and genomic DNA as follows. 5′-Amino functionalized oligonucleotides and aminated genomic DNAs were converted to the SPDP-derivatives using SPDP–NHS in borate buffer using standard methods. The SPDP derivatized oligonucleotides were quantitatively isolated by RPLC. The SPDP genomic DNAs were isolated by gel filtration and ethanol precipitation. Lyophilized RG–2TPDS peptide (5 nmol) was dissolved in 9.5 μl of 0.1 *M* sodium phosphate, pH 7.5, and 0.5 μl of TCEP (10 m*M* solution in 0.1 *M* phosphate) was added. After 1 h, SPDP–oligonucleotide (10 μg, ~1.0 nmol) in 0.1 *M* phosphate (12 μl) was added, and the mixture was left at room temperature overnight. The mixture was then diluted with water (70 μl) and purified by gel filtration through a BioSpin 6 column prior to quantitation of the conjugate by UV spectrophotometry. Release of peptide after treatment with 5 m*M* cysteine in ampholytes confirmed that conjugation had occurred. A similar procedure was used to conjugate peptides to genomic DNAs, except that the products were isolated by gel filtration and ethanol precipitation.

3. Results and discussion

3.1. pI of labeled peptides

The theoretical p*I* values of the unlabeled peptides were calculated by known methods [3]. The labeling process, entailing the reaction of the lysine epsilon amino group with the dye–NHS ester, and the chemical functional groups of the dye itself significantly alter the overall p*I* of the peptide conjugate relative to the peptide. Attempts to determine the p*I* values of the labeled peptides by IEF on slab gels with size markers were not successful, therefore, we have relied upon the theoretical p*I* of the unlabeled peptides as a general predictor of the p*I* of the fluorescent molecules.

3.2. Reductive release of peptides

Several reducing agents were investigated for reductive release of peptides from conjugates. As a model system, the 2-TPDS derivative of RG1–CHKG C-terminal amide was treated in ampholyte solution (pH 8) with 100, 10 and 0.5 m*M* DTT, 1 m*M* mercaptoethanol, 1 m*M* sodium bisulfite and 5 m*M* cysteine hydrochloride for periods of time ranging from 5–30 min at room temperature. Results indicated that the peptide is rapidly converted to the free thiol form with concentrations of DTT above 0.5 mM, but that the most rapid and side-reaction-free conversion is achieved with 5 m*M* cysteine in ampholyte solution for 5–10 min.

3.3. Detection limits

Using a dilution series (data not shown) of the RG1–VHLTPVEK peptide, we have determined that approximately 1.0 p*M* ($S/N=3$) concentrations of the peptide in ampholyte solution can be detected by isoelectric focusing, under the current conditions. With an approximate injection volume of 0.5 μl, to fill the capillary with sample solution, this corresponds to a mass sensitivity of 0.5 amol of fluorescent peptide.

3.4. Reproducibility

The reproducibility of focusing analyses and, hence, the ability by which very closely resolving peaks could be unambiguously identified, was tested by repeatedly focusing two species, RG1–VHLTPVEK (migration time, $t_m=B1$) and RG2–VHLTPVEK ($t_m=B2$) that are baseline resolved with 0.18 min peak-to-peak separation in a typical run (Fig. 2). Two internal standards, RG1–HHHKHG (basic, $t_m=A$) and RGX–lysine (acidic, $t_m=C$) were included in each analysis, and flanked the unknown peak. For ten replicate injections of two different samples that contained internal standards and either of the two unknowns, we calculated t_m ratios $(B-A)/(C-B)$ for the two data sets. Results are shown in Table 1. Since the 95% confidence limits of the t_m ratio for $B2$ does not overlap the mean of $B1$, we can therefore identify the peaks with a high degree of certainty.

3.5. Multiple analyte detection

A number of different methods were used to generate multiple focusing moieties, including varying the amino acid sequence, chemical modification of the amino and carboxyl termini and conjugation of different dyes. Many different green-fluorescing dyes are now commercially available. Most of these are either based on the fundamental fluorescein or rhodamine structures. The Rhodamine Green dyes also exhibit a diminished fluorescence pH sensitivity [9]. The chemical nature of the green-fluorescing dye used to label a given peptide sequence has a profound effect on the p*I* value, because of the pK_a values of the various ionizable groups. For example, fluorescein dyes confer acidic functionalities to the conjugate because of the carboxylate and phenolate groups, whereas the RhodamineGreen dyes are zwitterionic in nature. In addition, the overall p*I* of a given amino acid sequence can be manipulated by conversion to the N-terminal acetyl, C-terminal amide or both. To demonstrate both of these concepts, a rudimentary 4-mer assay was assembled using either RG2 or 6CF attached to the lysine residue of 2-TPDS–CHKG C-terminal acid and 2-TPDS–CHKG C-terminal amide, to give four un-

Table 1
Migration times (*A*, *B*1, *B*2 and *C*) and migration time differences for the cIEF separation of the analytes shown in Fig. 2

	RG1–HHHKHG	RG2–VHLTPVEK	RGX–lysine			
$n=10$	(*A*)	(*B*2)	(*C*)	*B*2−*A*	*C*−*B*2	(*B*2−*A*)/(*C*−*B*2)
Mean	13.747	19.491	27.777	5.744	8.286	0.693
SD	0.188	0.116	0.178	0.103	0.165	0.008
RSD (%)	1.369	0.594	0.642	1.787	1.986	1.115
SEM	0.060	0.037	0.056	0.032	0.052	0.002
95% confidence interval	13.747±0.119	19.491±0.073	27.777±0.113	5.744±0.065	8.286±0.104	0.6933±0.005
	RG1–HHHKHG	RG1–VHLTPVEK	RGX–lysine			
$n=10$	(*A*)	(*B*1)	(*C*)	*B*1−*A*	*C*−*B*1	(*B*1−*A*)/(*C*−*B*1)
Mean	14.121	20.121	28.350	6.000	8.229	0.729
SD	0.392	0.570	0.688	0.185	0.141	0.013
RSD (%)	2.777	2.833	2.428	3.076	1.712	1.792
SEM	0.139	0.202	0.243	0.065	0.050	0.005

SEM=standard error of the mean.

iquely focusing, releasable peptide species. These moieties were then conjugated to human genomic DNA by methods described above, followed by release of the free thiol peptide with cysteine (Fig. 3).

A mixture of 11 reductively released (5 m*M* cysteine hydrochloride) RG-labeled peptides was assembled containing the sequences CHHKG amide, CHKG amide, CHHKG acid, CHKG acid, VCHLTPVEK acid, CDHK acid (migrates as two species, second species unidentified), CHEEK acid (migrates as two species, second species unidentified), CDEHK acid, VCEK acid, CEEK acid and CDDK acid. The internal standards RG1–HHHKHG, RG1–VHLTPVEK, RGX–lysine and RGX–CO_2H were also included. CDDK acid co-migrates with RGX–CO_2H. Upon storage at 4°C for several days, even in the presence of 1 m*M* cysteine, the mixture was transformed into what we speculate to be a mixture of unsymmetrical peptide disulfides, since the complex mixture can be simplified by the addition of 2 m*M* DTT (Fig. 4).

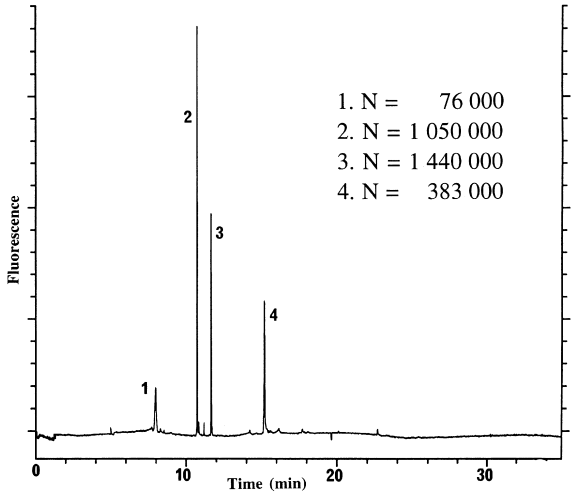

Fig. 3. cIEF separation of RG2- and 6CF-labeled primary peptide sequences released from genomic DNAs with 5 m*M* cysteine. 1=RG2–CHKG amide, 2=RG2–CHKG acid, 3=6CF–CHKG amide, 4=6CF–CHKG acid.

3.6. Assays

A model assay system consisting of a mixture of five synthetic oligonucleotide targets was assembled in order to test the chemistries of the multiple analyte cIEF assay in a sandwich hybridization format. Three probe sequences were designed to detect the pathogenic organisms *Yersinia pestis* and *Bacillus anthraxis* and equine encephalitis virus and two were modeled after human leukemia targets. A 50-ng (~2 pmol) amount of each target was hybridized at 65°C for 15 min) to a mixture of signal and capture probes, followed by a secondary hybridization at 42°C, allowing capture by hybridization onto polystyrene beads. After washing in hybridization buffer, followed by phosphate-buffered saline (PBS), signal

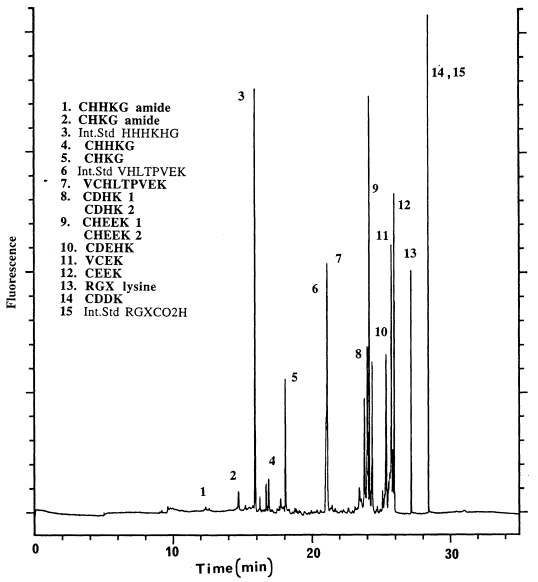

Fig. 4. Separation of RG-labeled cysteine containing peptides by cIEF. The sample had aged at 4°C in 1 mM cysteine in ampholytes for 10 days, and was then feshly reduced with 2 mM DTT prior to reanalysis.

moieties were released by treatment with 5 m*M* cysteine in ampholytes (400 μl) and an aliquot was removed for focusing analysis. Specificity in the hybridization was demonstrated by the background level of signal peptide 5 when only four of the targets were hybridized to a mixture of five signal probes.

4. Conclusions

We have demonstrated all the essential elements of a simultaneous multiple detection assay based on cIEF, including the preparation of dye-labeled peptides that focus as unique species, conjugation of these species to oligonucleotides, controlled chemical release of the species prior to detection and, finally, fluorescence detection. After conjugation with moieties, oligonucleotide probes still maintain hybridization specificity and can be processed under similar assay conditions as conventional hybridization probes. Future work is directed at determining the maximum number of peptide moieties that can be identified in a single cIEF run. A complication has been that the reductively released peptides are not completely stable in ampholyte solution, even when stored in the presence of 5 m*M* cysteine at 4°C. The thiol peptides appear to undergo oxidation to symmetrical disulfides (or unsymmetrical disulfides when mixtures of thiol peptides are present). We speculate that the small secondary peaks observed at the base of some peaks are caused by this process. However, the appearance of secondary species in the electropherogram can be largely reversed by treatment with DTT.

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