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Chromatography on Sephadex LH20 as an efficient purification step after removal of internucleotide 2,2,2-trichloroethyl protective groups from oligoribonucleotide phosphotriesters[1]

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ABSTRACT

Chromatography on Sephadex LH20, in a linear gradient of methanol in 0.02M TEAB buffer pH 7.5, is proposed as a fast and efficient method for the isolation and purification of protected oligoribonucleotide phosphodiesters obtained by deprotection of internucleotide phosphotriesters, and for the monitoring of the deprotection step itself. Its utility is shown on the example of removal of 2,2,2-trichloroethyl groups from oligoribonucleotide phosphotriester I of sequence CCCAUAA by two methods: /1/ reductive elimination with zinc in the presence of acetylacetone [2] modified as presented here, and /2/ hydrogenolytic dehalogenation over palladium in pyridine [3]. This method of chromatography on Sephadex LH20 is used as a key purification step during the removal of 2,2,2-trichloroethyl groups from I by method /1/ and allows to raise the yield of III during final deprotection step from 5 to 65%.

INTRODUCTION

The removal of internucleotide phosphate protective groups is a crucial step in oligonucleotide synthesis via the phosphotriester approach [4]. Difficulties accompanying the removal of these groups, such as incomplete removal [5,6], internucleotide bond cleavage [7] or side-reactions due to neighbouring group participation [8], were reported. As a result, the deprotection mixtures quite often contain not only a desired protected oligoribonucleotide phosphodiester but also side-products. Usually, at this stage, a necessity of isolation of desired phosphodiester prior to final deprotection arises. Until now chromatography on DEAE-Sephadex was most frequently used for isolation of protected phosphodiesters [9] but the use of other methods such as HPLC [8] or paper chromatography [10] was reported as well.

Here we would like to present these aspects of our studies which show the utility of chromatography on Sephadex LH20 for /i/ fast and efficient isolation of short protected oligoribonucleotide phosphodiesters obtained after removal of internucleotide 2,2,2-trichloroethyl groups by reductive elimination with zinc in the presence of acetylacetone [2] /method 1/ or by hydrogenolysis over palladium in pyridine [3] /method 2/ and for /ii/ monitoring the removal of internucleotide protective groups.

RESULTS AND DISCUSSION

Utility of chromatography on Sephadex LH20 is presented on the example of deprotection of phosphotriester I of sequence CCCAUAA /Fig.1/ chosen as a model, representative substrate.

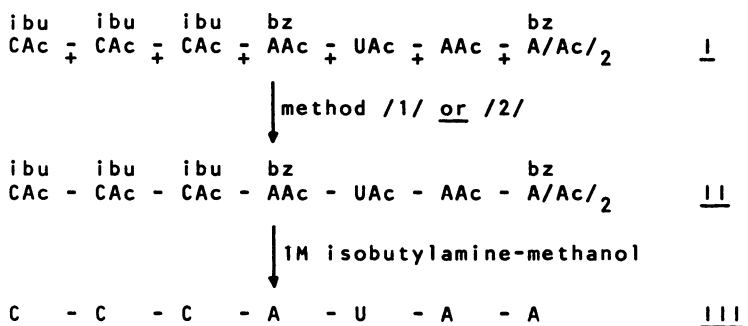
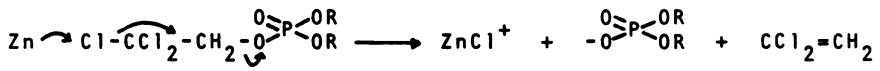


Fig.1 Scheme of the final deprotection of phosphotriester I [3,11]. General abbrevations are as suggested by the IUPAC-IUB /1970/, Biochemistry, 4022. Each internucleotidic phosphate is protected by 2,2,2-trichloroethyl group /±/. Method /1/-reductive elimination with zinc in the presence of acetylacetone, method /2/- hydrogenolytic dehalogenation over palladium.

1. Isolation of phosphodiester II on Sephadex LH20.

Method /1/. The reductive elimination of internucleotide 2,2,2-trichloroethyl groups with zinc powder in the presence of acetylacetone in pyridine led to phosphodiesters in a very high yield /over 90%/ [2]:



However, the formation of zinc cationic species chelated by acetylacetone and bound to oligoribonucleotide created several difficulties. Therefore, an efficient method for isolation of protected phosphodiesters from deprotection mixtures contaminated with zinc was needed. It was essential in our case since the presence of zinc during the second deprotection step /Fig.1/, i.e., removal of base labile 2'-O-acetyl and N-protective groups, led to extensive degradation of the oligoribonucleotide chain /clearly noticable above trimer level/.

We found that purification of the deprotection mixture containing II /Fig.2a/ by extraction with 2% chloroform solution of 8-hydroxyquinoline /to reduce total zinc concentration below 0.5 μg/10.D./, followed by chromatography on Sephadex LH20 in a linear gradient of 0-80% methanol in 0.02M TEAB buffer pH 7.5 led to II with 80% yield /Fig.3d/. Isolated II /purity 95%, see Fig.2c/ when subjected to isobutylamine treatment gave III with 65% yield /Fig.2d/. This was a remarkable improvement of the total deprotection yield since when isolation of II on Sephadex LH20 was omitted III was obtained with 5% yield only /Fig.2b/.

It is difficult to say exactly how Sephadex LH20 operates in this case, especially because the nature of oligoribonucleotide-zinc compounds [12] and other zinc species is not known. Probably, besides both hydrophilic and lipophilic properties of this gel /obtained by hydroxypropylation of Sephadex G25/, its cations retardation properties should be taken under consideration. The latter phenomenon occurs due to a presence of a small amounts of carboxylic functions in the gel structure and may be seen in the media of low ionic strengths [13]. Analysis of the zinc content may partially confirm this, as a side peak /Fig.3d/ contains more zinc /0.9μg/1 O.D./ than the main peak of II /0.2μg/1 O.D./.

At present, zinc-acetylacetone procedure modified as above serves as a very efficient method for removal of internucleotide 2,2,2-trichloroethyl groups.

Method /2/ i.e. hydrogenolysis of 2,2,2-trichloroethyl phosphotriesters over palladium in pyridine leads to phosphodiesters

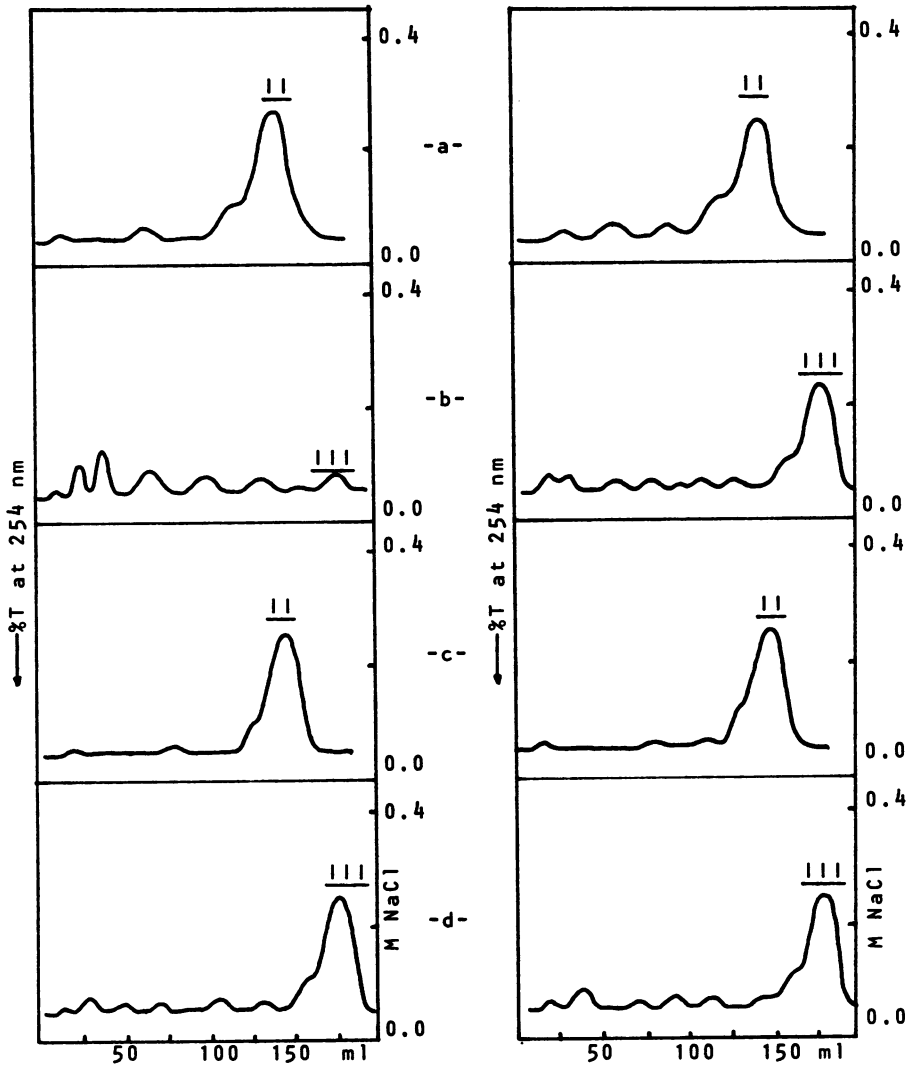


Fig.2 DEAE-Sephadex A25 chromatography to compare the efficiency of two deprotection steps: /i/ removal of 2,2,2-trichloroethyl groups by method /1/-see left column or method /2/-see right column, and /ii/ removal of baselabile groups with 1M isobutylamine in methanol. Elution profiles are representative for the transformations:
-a- I→II,
-b- I→II→III, without isolation of diester II on Sephadex LH20,
-c- purity of II isolated on Sephadex LH20,
-d- II→III, diester II isolated on Sephadex LH20.

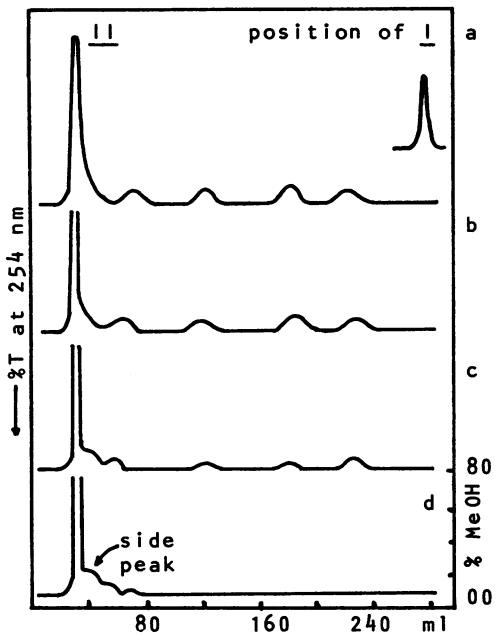
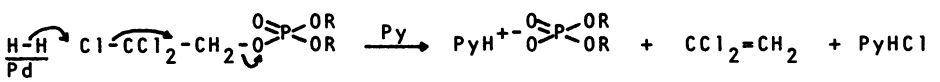


Fig.3 Chromatography on Sephadex LH20 to monitor the progress of deprotection of 2,2,2-trichloroethyl groups from I by reductive elimination with zinc in the presence of acetylacetone in pyridine- /1/.
The elution profiles -a - d- correspond to 0.5,1,2 and 4 hrs. of the deprotection respectively.

free of metal cations and was applied to the synthesis of anticodon loop of $tRNA_i^{Met}$ from yellow lupin [3]:



This method when applied to I led to phosphodiester II which was isolated on Sephadex LH20 /Fig.4c/ with 65% yield /purity 90%,see Fig.2c/. The final deblocking gave III with 45% yield /Fig.2d/. These results indicate that, at least in our case, the chromatography on Sephadex LH20 in TEAB buffer-methanol gradient may compete with the time -consuming DEAE-Sephadex A25 chromatography for isolation of short protected phosphodiesters [14]. The method is fast, reproducible and leads to protected phosphodiesters with sufficient purity /usually over 90%/ for further deblocking.

2. Chromatography on Sephadex LH20 for monitoring the removal of internucleotide phosphotriester groups.

We applied the chromatography on this gel for monitoring the deprotection of I as the reaction was progressing. On the basis

of hydro- and lipophilic properties of the gel, we expected, that separation of the incompletely deprotected heptamers /intermediates still bearing some 2,2,2-trichloroethyl groups/ should be possible. Indeed, the analysis of the deprotection mixture obtained by method /1/ clearly shows /Fig.3a-d/ that Sephadex LH20 eluted with a gradient of 0-80% methanol in 0.02M TEAB buffer pH 7.5 is capable of resolving the mixture containing the desired II, intermediates and phosphotriester I.

In the case of method /2/ we did not observe the elution of peaks attributed to the incompletely deprotected heptamers /intermediates/- at least under conditions studied. When the pyridine supernatant, free of palladium particles, was withdrawn from the deprotection mixture and analyzed on Sephadex LH20, only the presence of II was observed /Fig.4a/. Expecting a strong adsorption of the substrate and intermediates on the palladium, its surface was washed with the 0.02M TEAB buffer pH 7.5 and obtained solution combined with supernatant. Analysis of such mixture /Fig.4b-d/ showed that the main peak of II was accompanied by side-peaks, most probably related to the degradation products of intermedia-

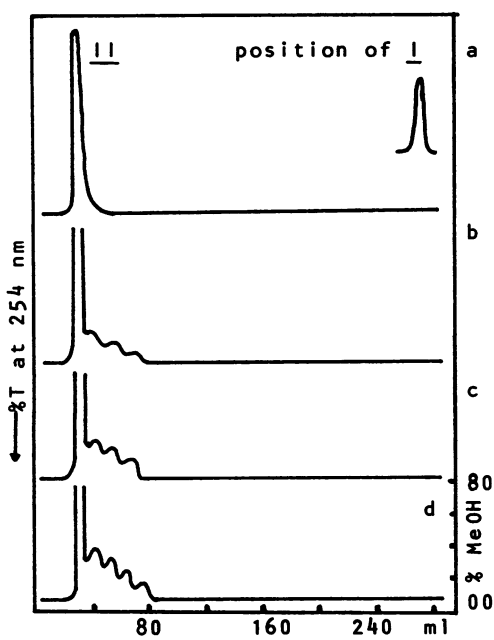


Fig.4 Chromatography on Sephadex LH20 to monitor the progress of deprotection of 2,2,?-trichloroethyl groups from I by hydrogenolytic dehalogenation over palladium in pyridine- method /2/. Profile -a- supernatant without palladium particles after 2 hrs. of deprotection. Profiles -b,c,d- supernatants plus washings of palladium surface after 2,8 and 24 hrs. of deprotection respectively.

tes or the desired phosphodiester itself. As a result, the total yield of II was ca. 20% lower then yield obtained by method /1/. To obtain the optimal yield of II /usually achieved after 8 hrs. of hydrogenolysis/, a ratio of main peak to the side-peaks was monitored by chromatography on Sephadex LH20 /Fig.4b-d/. Separation of side-peaks was sufficient to obtain II with a purity 90%.

CONCLUSION

Recently, de Rooij at al.[15] applied a gel filtration on Sephadex LH60 eluted with tetrahydrofuran-methanol 95:5 for the isolation of oligodeoxyribonucleotide phosphotriesters from the condensation mixtures obtained by phosphotriester method.

Our results indicated that the chromatography on Sephadex LH 20, in a linear gradient of methanol in 0.02M TEAB buffer pH 7.5, may be useful tool for: /i/ a fast isolation and purification of oligoribonucleotide phosphodiesters obtained by the deprotection of corresponding phosphotriesters, /ii/ monitoring of the removal of the internucleotide-bond protective groups [16] and /iii/ purification of oligoribonucleotide phosphodiesters contaminated with zinc cations.

EXPERIMENTAL

General. Palladium oxide, 8-hydroxyquinoline, and TLC plates covered by silica gel F_{254}, cellulose and PEI-cellulose F were purchased from Merck, W.Germany. Sephadex LH20 and DEAE-Sephadex A25 were products of Pharmacia Fine Chemicals, Sweden. Column separations were aided with Uvicord II, LKB, Sweden. Zinc analysis was performed on a Varian-Techtron instrument. Zinc powder was prepared as described previously [2]. 5'-Deprotected [11] oligoribonucleotide phosphotriester I was obtained by removal of monomethoxytrityl group with 2% CF_3COOH in chloroform from corresponding protected heptamer synthesized by phosphotriester method as reported [3].

Protected phosphodiester II. Deprotection mixtures containing II were obtained in an analytical scale by the removal of 2,2,2-trichloroethyl groups from phosphotriester I with the use of:

Method/1/ - reductive elimination with zinc in the presence of acetylacetone, modified as follows. 5'-Deprotected phosphotriester I /1.9 mg, 0.0005 mmole/ was dissolved in anhydrous pyridine /200 µl/ and treated with zinc powder /2.0 mg, 0.03 mmole/ and acetylacetone /3 µl, 0.05 mmole/. The produced suspension was vigorously stirred during 4 hrs. at room temperature. After this time an excess of zinc was centrifugated, supernatant was evaporated with a stream of nitrogen and the residue was dissolved in 0.02M TEAB buffer pH 7.5 /1 ml/. The buffer layer was extracted

with 2% chloroform solution of 8-hydroxyquinoline /4 times per 1 ml/ and was finally washed with chloroform /1 ml/. The aqueous layer /40 O.D. units of crude material/ was concentrated /to 200 µl/ and applied on the Sephadex LH20 column /see below/.

Method /2/ - hydrogenolytic dehalogenation over palladium in pyridine according to [3] with two exceptions: /i/ stirring was replaced by vigorous shaking, /ii/ the amount of palladium was increased to 20 equiv. per one phosphotriester linkage. Crude deprotection mixture /obtained from 1.9 mg of I/ was applied on the Sephadex LH20 column /see below/.

Isolation of phosphodiester II on Sephadex LH20.
Sephadex LH20 was swollen in double-distilled water overnight and then packed into the column /1.2X30 cm/ under conditions described [13]. The gel was then equilibrated with 0.02M TEAB buffer pH 7.5 for 2-3 hrs. The phosphotriester deprotection mixture /40 O.D. in 200 µl of above buffer/ was applied on the column and eluted with a linear gradient of 0-80% methanol in 0.02M TEAB buffer pH 7.5. The observed flow rate was 25-30 ml/hr. Separation was monitored by UV transmission at 254 nm and the main peak was collected and evaporated. Desired II was isolated with 80% 65% yield for method /1/ and /2/ respectively within 2 hrs. time. The gel was regenerated by washing with 0.2M TEAB buffer pH 7.5 until no UV absorbtion was observed and then thoroughly with water, methanol and water again.

Deprotected phosphodiester III. Heptamer CCCAUAA III was obtained by deprotection of II with 1M isobutylamine in methanol /30 hrs.,room temp./ and isolated by DEAE-Sephadex A25 chromatography with use of linear gradient 0-0.4M NaCl in 7M urea, 0.01M Tris-HCl buffer pH 7.5. Yields: 65% and 45% for method /1/ and /2/ respectively.

Analysis. The purity of desired oligoribonucleotides was checked as follows:
- phosphotriester I by silica gel TLC in chloroform-methanol,
- phosphodiester II by chromatography on DEAE-Sephadex A25 column eluted in linear gradient 0-0.4M NaCl in 7M urea, 0.01M Tris-HCl buffer pH 7.5; TLC on PEI-cellulose F in 0.1-0.3M gradient of LiCl in 7M urea, 0.02M Tris-HCl buffer pH 7.5.
- phosphodiester III by TLC on PEI-cellulose F and chromatography on DEAE-Sephadex A25 column /conditions above/ and by 2D TLC on cellulose in the systems /a/ n-butanol:water 86:14 v/v and /b/ isobutyric acid:conc.ammonium hydroxide:water 66:1:33 v/v.

The purity of III and its nucleoside content was checked by enzymatic analysis as presented [3]. 2D TLC on cellulose of the digestion mixtures in systems /a/ and /b/ gave correct ratio of nucleosides. For III hyperchromic effect was found equal 15%.

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