PROCESS MONITORING IN SOLID PHASE PEPTIDE SYNTHESIS, AMINO GROUP BLOCKING EFFECT OF IMPURE METHYLENE CHLORIDE

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1. Introduction

A major difficulty encountered in the solid phase synthesis of larger peptides is the lack of a reliable control of cleavage of the α-amino protecting group and coupling. A method by which this in principle can be performed has been described [1, 2]. The determination is carried out by potentiometric titration with perchloric acid, dissolved in glacial acetic acid, on the resin bound peptide suspended in a mixture of methylene chloride and glacial acetic acid 1/1 (v/v). By repeated titrations of an alanine resin, decreasing values were, however, found.

Experiments are described below in which it is shown that the decrease in the titration values are caused by one or more impurities present in the methylene chloride used.

2. Experimental

2.1. *Preparation of resin*

The resin used was Bio-Rad, Bio-Beads S-X2, 200–400 mesh. Before the chloromethylation the resin was suspended in methylene chloride for removal of smaller particles.

Two different samples, A and B, of copolystyrene – 2% divinyl-benzene were chloromethylated [3]. The degree of substitution was determined to be 1.52 and 1.83 meq Cl/g chloromethylated resin, respectively. Boc–L-alanine was esterified to the resin according to Merrifield [4] although only 24 hr of reaction was applied. The degree of substitution was determined by amino acid analysis and by Volhard titration.

In order to control that efficient washing out of the non-covalently bound alanine had taken place and that a sufficient amount of alanine was available for coupling, Boc–leucine was coupled to the alanyl resin. Amino acid analysis was performed after hydrolysis in a mixture of 6 N hydrochloric acid and glacial acetic acid 1/1 (v/v) for 96 hr at 110° in evacuated sealed ampoules. The amino acid analysis gave the following values for the 2 resins; A: alanine 0.42, leucine 0.40; B: alanine 0.69, leucine 0.69. The values were calculated as mmole per g Boc–leucyl–alanyl resin. The Boc–alanine substitution was determined for the 2 resins to be 0.44 and 0.75 mmole Boc–alanine per g Boc–alanyl resin, respectively.

The Volhard titration was carried out after cleavage of the Boc-group from the Boc–alanyl resins with 1 N HCl in glacial acetic acid and subsequent washing with triethylamine. The degrees of substitution were determined to be A: 0.54 and B: 0.74 mmole Boc–alanine per g Boc–alanyl resin.

2.2. *Titration*

For the titrations Radiometer pH-meter 28, Titrator TTT11, and autoburette ABU 11 were used. The titrations were performed in the reactor system previously described [2] on the whole batch of resin. A glass electrode Radiometer G202C and a calomel electrode with a double salt bridge Radio-

meter K701 were used. The secondary salt bridge consists of saturated aqueous LiCl and glacial acetic acid 1/9 (v/v). The automatic peptide synthesizer [5, 6] was used in the experiments, but the titration was activated manually. After the titration, which lasted for approx. 30 min with a delayed shut off time of 5 min, the synthesizer was manually reactivated to carry out the remaining operations. The following cycle was performed:

Step	Reagents and operations	Time (min)
1	CH_2Cl_2 wash 38 ml (1 time)	2.5
2	1 × Et_3N 5 ml + 2 × CH_2Cl_2 76 ml (1 time)	10
3	CH_2Cl_2 wash 38 ml (6 times)	2.5
4	abs. EtOH wash 38 ml (3 times)	2.5
5	HOAc wash 38 ml (3 times)	2.5
6	2 × HOAc 76 ml + 2 × CH_2Cl_2 76 ml, titration	30 approx.
7	CH_2Cl_2 wash 38 ml (3 times)	2.5

The coding of the synthesizer was the following:

S162S1V0S142S162S162S162S162S162S162
T162T162T162U162U162U162U162U1U1S1S1*2S162
S162S162 RUB OUT

0 (zero): Drain triethylamine metering vessel
1 : Drain metering vessel
2 : Drain reactor
4 : Agitation 10 min
6 : Agitation 2.5 min
S : Methylene chloride
T : Glacial acetic acid
V: Triethylamine
* : Agitation during titration
RUB OUT: Stop

The titrations were carried out with approx. 0.05 N $HClO_4$ in HOAc. The minimum volume titrant to be added from the autoburette was approx. 40 μl. The titrant was prepared by dissolving 72% aqueous perchloric acid in glacial acetic acid and standardized against Tris with an SD of 0.0005 N.

Methylene chloride purum was obtained from May and Baker. Purification prior to use was performed by distillation over K_2CO_3 (temp 39–40°). Methylene chloride from Merck was pro analysi grade,

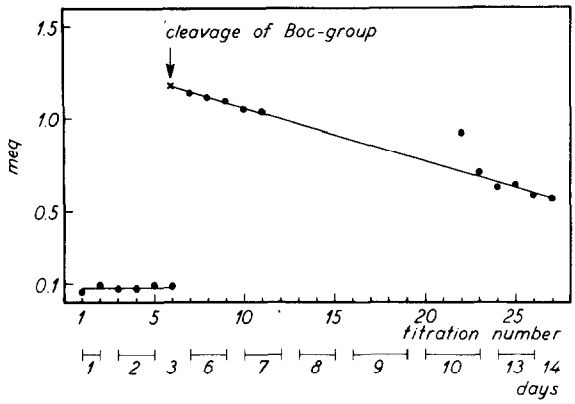

Fig. 1. Experiment with methylene chloride from May and Baker, distilled purum. Titrations of resin A before and after cleavage of the Boc-group are performed. Cycle number 12–21 were simulated titrations with omission of perchloric acid. X: Extrapolated initial value, 1.173 meq.

and not further purified. Glacial acetic acid was May and Baker pronalys grade. Absolute ethanol was obtained from the Danish Distillers Ltd., Pharmacopoeia Nordica grade. Triethylamine puriss was obtained from Fluka.

3. Results

From figs. 1 and 2 it is seen that constant values were obtained by titrations of Boc protected alanyl resin A in amounts of 2.50 and 2.23 g, respectively. Average values of 0.080 meq SD 0.015 and 0.054 meq SD 0.005 were found. These values represent titratable groups as they exceed the value obtained by titration of the solvent. This latter value is mainly due to the inertia of the titration system and in the described experiments was approx. 0.5 ml, corresponding to 0.025 meq.

In fig. 1 are presented the results of an experiment with May and Baker methylene chloride. Titrations were performed before and after cleavage of the Boc-group followed by 10 simulated titrations. Finally 6 titration cycles were carried out. In the simulated titrations addition of perchloric acid was omitted and stirring was carried out for 30 min in the glacial acetic acid–methylene chloride mixture. It is seen that the decrease in the titration values

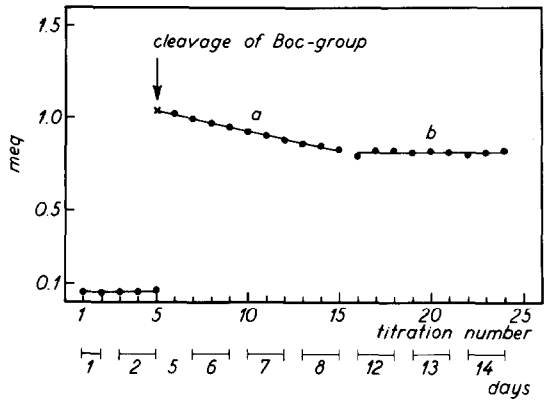

Fig. 2. Experiment with methylene chloride from a: May and Baker, distilled purum and b: Merck, pro analysi. Titration of resin A before and after cleavage of the Boc-group were performed demonstrating the blocking effect of impurities present in methylene chloride a. X: Extrapolated initial value, 1.036 meq.

are demonstrated irrespective of addition of perchloric acid. An initial value of 1.173 meq, calculated by extrapolation, and an average decrease per cycle of 0.029 meq was found. An anomalous high value was observed for the first titration after the simulated titrations, most likely due to an accumulation of triethylamine. In the first 4 titrations after the simulated titrations the initial addition of the titrant took place considerably faster than normally, possibly due to a conformational change of the resin.

In fig. 2 are presented the results of an experiment with resin A in which first methylene chloride from May and Baker and subsequently methylene chloride from Merck was used. The initial value calculated by extrapolation was 1.036 meq and an average decrease per titration cycle was 0.022 meq SD 0.004. After replacing the May and Baker methylene chloride with the Merck product, constant values were obtained with an average of 0.807 meq SD 0.011.

In an experiment with resin B using May and Baker methylene chloride an average decrease of 0.023 meq per cycle was found. DCC coupling with Boc–glycine was performed after 9 titration cycles. After cleavage of the Boc-group a further decrease in the titration value of 0.033 meq was found. Amino acid analysis showed, calculated per g dried crude material, 0.630 mmole alanine and 0.513 mmole glycine. The ratio glycine/alanine was 0.82. The initial titration value of the alanyl resin was calculated to 1.353 meq by extrapolation and of the glycyl–alanyl resin corrected for the decrease in the titration value 1.108 + 0.033 = 1.141 meq. The titration value before cleavage of the Boc-group of glycine was determined to 0.117 meq. Thus the ratio of the titration values (1.141–0.117)/(1.353–0.117) = 0.83 was found to be in good agreement with the result of the amino acid analysis.

4. Discussion and conclusion

The experiments show that impurities present in methylene chloride are able to block irreversibly amino groups under the conditions of the solid phase synthesis. They also show that the amino groups are not deblocked by the procedure used for coupling of Boc–glycine and cleavage of the Boc-group. Irreversible blocking of α-amino groups under the conditions of solid phase peptide synthesis must be considered a serious problem due to the repetitive procedure applied. It is therefore of the utmost importance that solvents and/or reagents freed of deleterious impurities are used in the synthetic procedure. The present experiments also demonstrate the value of a method for monitoring of the synthesis.

The 2 methylene chloride products were subjected to gas chromatographic analysis and certain differences in the chromatograms were found. Both products, however, contained some contaminating substances. The nature of the active substance or substances have, however, not yet been determined.

Good agreement was found between the values for substitution calculated from the titrations with perchloric acid and the values obtained by amino acid analysis. So by using the calculated initial values from figs. 1 and 2, with subtraction of the values obtained before cleavage of the Boc-group, substitution degrees of 0.44 and 0.44 mmole per g Boc–alanyl resin were found, respectively.

Bayer et al. [7] demonstrated decreasing Volhard titration values during the synthetic procedure in the synthesis of ferredoxin, indicating a decrease in the number of free amino groups. In those experiments an irreversible blocking of the amino groups may as well have been the reason for the decreasing values.

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