SOLID PHASE EDMAN DEGRADATION. HIGH YIELD ATTACHMENT OF TRYPTIC PROTEIN FRAGMENTS TO AMINATED SUPPORTS

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1. Introduction

The solid phase Edman degradation demands a reliable procedure for the attachment of chemically or enzymatically obtainable protein fragments. The reliability of the attachment method depends on the specificity of the activation reaction to which the peptide has to be subjected. C-terminal activation is preferred and over-derivatization of the peptide side chains clearly gives rise to complications during the degradation process.

The only procedure that warrants a reasonably specific C-terminal activation consists of the generation of homoserine lactone residues by treatment of purified fragments from a cyanogen bromide degradation with trifluoroacetic acid. The (weak) activation as occurring in the lactone ring prevents cross-linking and hydrolysis to a great extent and appears to be sufficient to undergo a rapid aminolysis with a polymer substituted with a primary amine [1]. The application of this elegant method, which results in sufficiently stable peptide-support adducts, is hampered by the relative scarcity of methionyl residues in protein, which moreover should not be clustered. Another straightforward method [2] utilises *p*-phenyl-di-isothiocyanate as a bifunctional reagent, for linking-up an amino group in the support with a side chain amino group of a peptide. Here, however, a great excess must be used to prevent cross-linking or even polymerization of peptides. In an attempt to combine the merits of both procedures we investigated *p*-isothiocyanatobenzoyl-DL-homoserine lactone (IBHL) as a bifunctional reagent. The method, which is especially suited for tryptic protein fragments, met all requirements and has successfully been applied in our sequence work.

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2. Experimental

2.1. *Materials and methods*

p-Isothiocyanatobenzoyl-DL-homoserine lactone (IBHL) was prepared from DL-homoserine lactone by acylation with *p*-nitrobenzoylchloride, hydrogenation of the nitro-group with hydrogen and palladium and subsequent treatment of the resulting amine with thiophosgene in aqueous hydrochloric acid. Crystalline IBHL precipitated during the last reaction and appeared to be analytically pure after drying. Details of the synthesis will be published elsewhere [3].

For attachment experiments, horse heart cytochrome *c* was used; for primary modification and sequence studies melittin (Serva, Heidelberg) was chosen as the model and the technique was applied to several tryptic peptides of the βB_p chain of β-crystallin [4]. One example will be given. Further reagents, apart from *N*-methylmorpholine and phenylisothiocyanate which were of 'sequenal grade' (Pierce), were of 'pro-analyse' quality and were commercial products.

The support, controlled pore glass (Corning CPG-10, 200–400 mesh, 75 Å mean pore diameter) was aminated with *N*-(2-aminoethyl)-3-aminopropyltrimethoxysilane, which was obtained from Pierce. The substitution of the support was carried out according to the directions of Wachter [5] with the application of the variations of Bridgen [6]. Spectrophotometric determination of the amount of primary amino groups was carried out with sodium 2,4,6-trinitrobenzene sulfonate: 170 neq/mg were present. Automatic sequential degradation was performed with the Sequemat 10K (purchased from LKB). Amino acid analyses were performed with the Beckman Multichrom automatic amino acid analyzer.

2.2. *Derivatization and attachment of peptides using N-(p-isothiocyanato-benzoyl)-DL-homoserine lactone (IBHL) (Scheme 1, step I)*

Melittin was dissolved in a mixture of *N*-methylmorpholine and water (1:1, pH 10.7) and in the same mixture containing sufficient trifluoroacetic acid to give pH 9.5. Into two series of test tubes 100 μl samples of the solutions were pipetted, each sample comprising about 100 nmol of the peptide. Varying amounts of IBHL dissolved in 100 μl of dimethylformamide were added (molar ratio increasing from 1–10). After 1 h at room temperature the mixtures were evaporated in vacuo and subjected to paper chromatography (Whatmann 3 MM) using *n*-butanol, acetic acid, pyridine, water (75:15:60:50 by vol) as the solvent system. Spots were detected by u.v. fluorescence, and with the ninhydrin and chlorine/tolidine tests.

For attachment of peptides to the aminated support a known tryptic hexapeptide of the βB_p chain of β-crystallin and melittin were treated with IBHL at pH 10.7 as described, but an intermediate concentration of IBHL corresponding with 2 equivalents was chosen for each amino group present in the peptides to ensure full modification. The evaporated peptide derivatives comprising 100–200 nmol were dissolved in 1 ml trifluoroacetic acid (100%) (step II), and again evaporated in vacuo. The residues were coupled to 100 mg of aminated glass (step III) using the conditions of Horn and Laursen [1], with the exception that coupling was performed at pH 9.7 (*N*-methylmorpholine:water = 1:1, acidified with TFA to pH 9.7). For experiments with cytochrome *c*, 0.1% (v/v) aqueous triethylamine was used for derivatization of the amino groups. Coupling to the glass support was carried out in a similar manner.

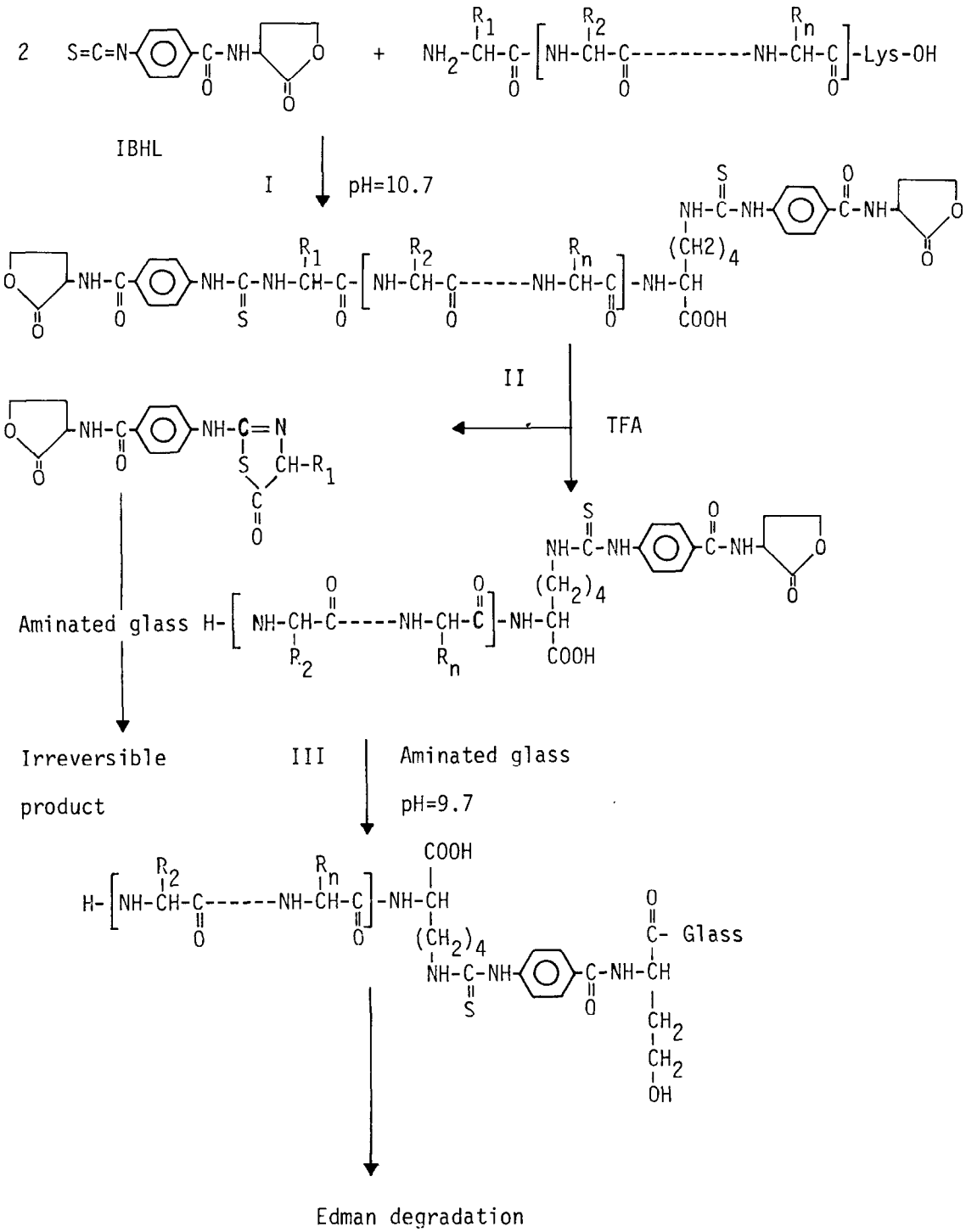
Coupling efficiency was determined by amino acid analysis of hydrolysates prepared from the peptide glass adducts containing the βB_p hexapeptide and melittin. The amount of cytochrome *c* retained by the support was determined by spectrophotometric examination (410 nm) of the supernatant after centrifugation.

2.3. *Automatic sequencing*

The dry support was mixed with glass beads and subjected to degradation as described by Laursen [7]. Liberated thiazalinones were converted to phenylthiohydantoins by incubation in 20% trifluoroacetic acid at 80°C for 10 min. The samples were purified by filtration through a column of Dowex-50 (X-2, 200–400 mesh) in methanol. The phenylthiohydantoins were identified by thin-layer chromatography and gas chromatography as described by Van der Ouderaa et al. [8].

3. Results

N-(*p*-isothiocyanatobenzoyl)-DL-homoserine lactone (IBHL) reacts readily with the amino groups of the three compounds, which were chosen as models. The reaction proceeded repidly at pH 10.7 and did not leave unaltered starting material when equi-molecular amounts were reacted for one hour. At pH 9.5 the reaction had a much slower course. Even in the presence of an excess of the reagent (2.5 equivalents per amino group) the reaction did not reach completion in the same time. The subsequent reaction with 2-aminoethyl-3-aminopropyl–glass gave excellent results (100% coupling) with the hexapeptide from the βB_p chain and with cytochrome *c* (95%). With melittin the incorporation was lower, but still satisfactory (60%). The Edman degradation of the adducts of the hexapeptide and of melittin had the expected course. The amino acid sequence of the former proved to be Glu–Thr–Gly–Val–Glu–Lys. C-terminal lysine was not eluted from the column. The same holds for the lysyl residue in position 7 of melittin, but this point of fixation could be passed without decrease in yield of the liberated thiazalinones, indicating multiple



Scheme 1. Derivatization and attachment of tryptic peptides bearing C-terminal lysine to an aminated (glass) support. The part between brackets was found to be suited for solid phase Edman degradation.

fixation (positions 21 and/or 23) of the hexacosapeptide amide. From this model 20 residues were removed stepwise. The average yield per cycle amounted to 90%.

4. Discussion

The here advocated reagent for solid phase Edman degradation unifies – in our hands – the advantages of the proposed methods of Horn and Laursen [1] and Laursen et al. [2]. It offers a panacea for the general scarcity of methionine in proteins, which restricts application of the first technique, and it eliminates some drawbacks of the second. The reagent is thought for rapid derivatization of tryptic protein fragments terminated by lysine. Since tryptic attack of peptide bonds can be restricted to lysine by blocking of arginine residues [9], the method seems to gain in general applicability. The risk of cross-linking of peptides by a bifunctional reagent bearing two groups of equal reactivity (as in 1,4-di-iso-thiocyanatobenzene) is greatly suppressed, thus rendering application of large excesses of the bifunctional reagent unnecessary. Moreover, in general derivatives of homoserine lactone are stable compounds, which can be reactivated by a simple treatment with an anhydrous acid. This allows, if desired, and extensive purification, prior to sequencing. A drawback of the second method [2] which cannot be eliminated is the inherent unavailability of the N-terminus, which remains to be determined in a separate experiment. However, the concomitant loss of peptide molecules, fixed 'wrongly' by aminolysis of the substituent at the first amino acyl residue by the support during the first acid treatment is completely eliminated by our approach. The included pretreatment with trifluoroacetic acid removes this residue, leaving the remainder of the peptide as a monovalent compound, bearing only one activated substituent at the ϵ-amino-function of C-terminal lysine.

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