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Note

Some new by-products in primary structure analysis with dimethylaminoazobenzene isothiocyanate as an aid in the thin-layer chromatographic identification of residues

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4-N,N-Dimethylaminoazobenzene 4'-isothiocyanate (DABITC) is a sensitive chromophoric reagent for protein sequence analysis[1]. For use in N-terminal analysis it can be used as the only coupling reagent[2]. However, the low coupling yield with DABITC makes the use of a second coupling reagent, phenyl isothiocyanate (PITC), necessary in sequencing procedures[3]. Both high-performance liquid chromatography[4,5] and thin-layer chromatography (TLC)[3,6] have been employed to identify dimethylaminoazobenzene thiohydantoins (DABTH).

Manual sequencing with DABITC and PITC and identification of the residues with TLC is an inexpensive method, as it does not require much instrumentation. A serious drawback, however, is the difficulty in identifying some of the DABTH derivatives. The hydrophobic residues are clustered and leucine and isoleucine co-chromatograph on polyamide thin layers[3]. Leucine and isoleucine can be separated on silica gel, but this procedure reduces the sensitivity of the method[6]. In addressing the problem, Von Bahr-Lindström *et al.*[7] published some characteristic TLC by-product patterns that are very useful in identifying several residues, including isoleucine and leucine.

We have found some additional by-product spots on polyamide thin layers that make the identification of especially histidine and lysine much easier. In this paper we describe the chromatographic patterns that can be obtained for proline, alanine, lysine and histidine. A reason for the formation of the by-products is discussed.

EXPERIMENTAL

Chemicals

Heptane, ethyl acetate, trifluoroacetic acid (TFA) and PITC (Fluka, for sequential analysis) were used without further purification. Pyridine (Fluka, puriss. p.a.) was redistilled twice. *n*-Butyl acetate (Merck, analytical-reagent grade) was redistilled and DABITC (Fluka) was recrystallized from boiling acetone.

The peptides used were L-lysyl-L-serine and L-histidyl-L-aspartic acid (Sigma). Peptides were also obtained during primary structure determination of carbonic anhydrase from tiger shark.[8]

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Amino acid sequence analysis

The sequencing protocol described by Chang *et al.*[3] was used, except for the conversion reaction, where 50 μl of 50% (v/v) TFA was used. DABTH derivatives were separated on 3 × 3 cm polyamide sheets (F 1700, Schleicher & Schüll) in a two-solvent system. For the first dimension acetic acid–water (1:2, v/v) and for the second dimension toluene–*n*-hexane–acetic acid (2:1:1, v/v) were used as solvents as described by Chang *et al.*[3].

RESULTS AND DISCUSSION

In addition to the by-product patterns reported by Von Bahr-Lindström *et al.*[7], we have found several new by-products during TLC identification of the DABTH derivatives. The chromatographic patterns shown in Fig. 1 are characteristic of the respective amino acids.

For alanine we always obtain a red spot, which appears above and to the right of the red spot for DABTH-alanine. Proline gives a red spot to the right of the red DABTH-proline spot. When lysine is the N-terminal amino acid, there is a blue spot above and to the right of the point of sample application, whereas an N-terminal histidine gives a blue spot to the right of the sample application point.

The two latter spots have proved most useful for identification of the respective amino acids. Histidine residues may be difficult to identify for two reasons. First,

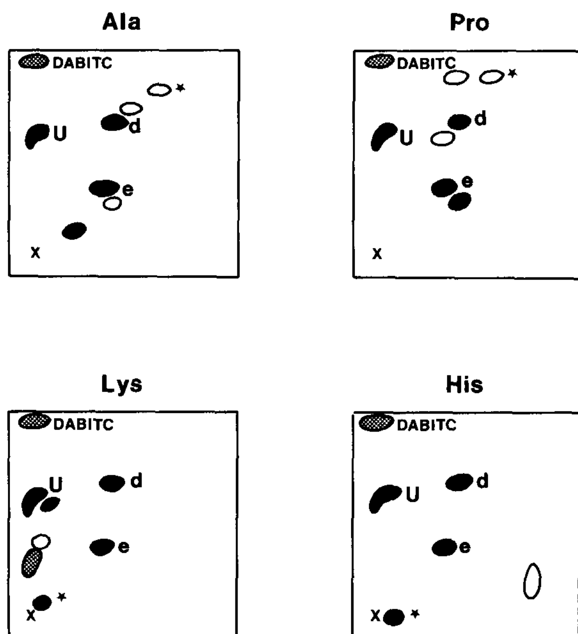

Fig. 1. Chromatographic patterns obtained in the DABITC degradation of alanine, proline, lysine and histidine. Red spots are marked as open, blue as filled and purple as hatched areas. The spots marked d and e are the markers DABTC-diethylamine and DABTC-ethanolamine, U is a thiourea product formed by the coupling of PITC with hydrolysed DABITC[3]. The new by-product spots are labelled with asterisks, and the other spots were described by Chang *et al.*[3] and Von Bahr-Lindström *et al.*[7].

DABTH-histidine is difficult to distinguish from DABTH-arginine and second there is often a reddish streak on the right edge of the TLC plate that might obscure or be mistaken for DABTH-histidine. The new by-product spot for histidine makes the identification of histidine residues much more reliable. With lysine the previously reported chromatographic pattern might be obscured by a bluish streak from the point of sample application. The newly discovered spot, which we always obtain in high yield, is easily detected.

If the dimetylaminoazobenzenethiazoline (DABTZ) derivatives are extracted with newly distilled *n*-butyl acetate, the by-product spots have a low intensity compared with the DABTH spots, but with time the by-products increase at the expense of the DABTH derivatives. If the *n*-butyl acetate is redistilled, the relative amount of by-products decreases. When the volume of *n*-butyl acetate used is decreased, the relative amount of by-products also decreases. The smaller the amount of peptide being sequenced, the higher is the ratio of by-products to DABTH derivatives. Apparently the formation of by-products depends on the purity and amount of the *n*-butyl acetate used. Taken together, this suggests that the by-products are formed in a constant amount by reaction of an impurity in the *n*-butyl acetate with the DABTZ derivative, the DABTH derivative or an intermediate.

Our conclusion is that in addition to the by-products reported by Von Bahr-Lindström *et al.*[7], the by-product spots of especially histidine and lysine residues are very useful in their identification on polyamide TLC plates.

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