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SEPARATION OF HISTIDYL-PEPTIDES BY THIN-LAYER CHROMATOGRAPHY AND MICROSPECTROFLUOROMETRIC CHARACTERIZATION OF THEIR *o*-PHTHALALDEHYDE-INDUCED FLUORESCENCE*

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SUMMARY

o-Phthalaldehyde forms fluorescent condensation products with a number of biogenic compounds both in solution and on silica gel. Among these compounds are histamine, histidine and peptides with histidine in the N-terminal position; this latter group includes biologically important peptides such as glucagon, secretin and "vasoactive intestinal peptide". With *o*-phthalaldehyde, peptides and polypeptides with N-terminal histidine can be detected in submicrogram amounts. The *o*-phthalaldehyde-induced fluorescence of all the histidyl-peptides has maximum excitation in the range 410–420 mμ. Excitation at 365 mμ gives maximum emission at about 450 mμ, while excitation at 405 mμ gives maximum emission at 480–490 mμ. R_F values in five solvent systems are given for a number of dipeptides and polypeptides with N-terminal histidine.

INTRODUCTION

o-Phthalaldehyde (OPT) has been widely used as a reagent in the fluorometric[1], chromatographic[2–4] and fluorescence microscopic[5–8] demonstration of histamine. Apart from histamine, other compounds, such as histidine[9], arginine[10], agmatine[10], spermidine[11], glutathione[12], 5-hydroxy- and 5-methoxyindolamines[13], react with OPT, forming intensely fluorescent condensation products. The chemical reactions involved in the formation of these fluorophores are unknown. It is assumed that for histamine the initial reaction step is condensation of the amino nitrogen with one of the OPT aldehyde groups, resulting in a Schiff base. Conceivably, this condensation product is subsequently transformed by secondary reactions[1]. It could be predicted that also histidyl-peptides (*i.e.*, peptides with N-terminal histidine) should react with OPT, giving rise to fluorophores similar to those resulting from the condensation of OPT with histamine. In previous studies it was shown that glucagon, secretin, and "vasoactive intestinal peptide" (VIP), which have histidine in the N-terminal position[14], give strong OPT-induced fluorescence under certain experimental conditions[15,16]. In the present study other histidyl-peptides besides

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glucagon, secretin and VIP were found to give strong OPT-induced fluorescence on silica gel thin layers, permitting the chromatographic detection of submicrogram amounts of such compounds. Peptides with histidine in the C-terminal position gave no or very weak OPT-induced fluorescence.

EXPERIMENTAL

Compounds

Stock solutions of the following compounds were prepared (usually 1 mg/ml): histamine dihydrochloride (Fluka), tryptamine hydrochloride (Regis), 5-hydroxytryptamine creatinine sulphate (Fluka), 5-methoxytryptamine hydrochloride (Aldrich), adrenaline (Rhone-Poulenc), noradrenaline hydrochloride, 3-hydroxytyramine hydrochloride, DL-3,4-dihydroxyphenylalanine (Fluka), L-histidine (Merck), L-carnosine (Nutritional Biochemicals Corp.), L-alanyl-L-histidine, L-glycyl-L-histidine (Sigma), L-histidyl-L-alanine, L-histidyl-L-serine, L-histidyl-L-lysine, L-histidyl-L-leucine, L-histidyl-L-phenylalanine, L-histidyl-L-tyrosine, L-glycyl-L-histidyl-L-glycine, L-tyrosyl-L-alanine, L-tyrosyl-L-glutamic acid, L-tyrosyl-L-glycine, L-tyrosyl-L-tyrosine, L-phenylalanine-L-tyrosine, L-valyl-L-tyrosyl-L-valine (Miles-Yeda), L-tryptophyl-L-phenylalanine, L-tryptophyl-L-tryptophan, L-arginyl-L-aspartic acid, bradykinin triacetate (Sigma) and angiotensin II monoacetate hydrate (Miles-Yeda). Porcine glucagon was provided by Dr. H. EGE, Insulin Laboratory, Novo, Copenhagen, Denmark. Porcine secretin and "vasoactive intestinal peptide" (VIP) were supplied by Professors J. E. JORPES and V. MUTT, Karolinska Institute, Stockholm, Sweden. Amines, amino acids and the small peptides were dissolved in water, the polypeptides in 0.01 *N* acetic acid.

Sensitivity and specificity of OPT

For this study small silica gel thin layers were prepared by coating histological cover slips (24 × 32 mm) with approximately 100 μ of Kieselgel H. The solutions of amines, amino acids or peptides were spotted onto these thin layers in volumes of 1 μl. After spotting the polypeptides, equal amounts of 0.01 *N* NaOH were usually also applied to the spots in order to neutralize the pH. OPT was applied in drops from a 1 % xylene solution. The sensitivity and specificity of OPT as a chromatographic detection reagent was evaluated by establishing the smallest visible amount of the compounds when examining the thin layers in UV light (Sterisol UV-lamp, Original Hanau, equipped with a UG 1 filter). In a series of experiments the thin layers were exposed to formaldehyde gas (generated from paraformaldehyde) at 80° for 45 min either before or after treatment with OPT.

Microspectrofluorometric analysis

The spectral properties of the fluorophores were analyzed with a modified Leitz microspectrograph (for details, see ref. 17). The exciting light was passed through an optical system consisting entirely of quartz components. The thin layers were placed upside-down with the silica gel facing the quartz bright-field dry condenser. The thin layer outside the fluorescent spots was used to obtain blank spectra. All spectra were corrected for blanks and instrumental errors as previously described[17]. All values given for excitation and emission maxima are the means of at least four separate recordings.

TLC of histidyl-peptides

Glass plates (5 × 20 cm or 20 × 20 cm) were coated with 250 μ of silica gel (Kieselgel H, Merck, Darmstadt) and dried at room temperature. 1 μl volume of the stock solutions of histamine, histidine and histidyl-peptides were applied to the plate with a capillary approximately 2 cm from the base. The time of development varied with the solvent system used but never exceeded 3 h for a height of 12 cm. After chromatography the plates were dried in an oven at 60–80° for a few min, sprayed with 1 % OPT in xylene or acetone and then dried again. Fluorescent spots were outlined upon examination in UV light. All R_F values given refer to the distance from starting point to the front line of each spot.

RESULTS

Sensitivity and specificity of OPT as a detection reagent

All the histidyl-peptides tested gave strong bluish fluorescence upon OPT treatment. The reaction was unspecific in that also tryptophyl-peptides, indolamines and catecholamines in submicrogram amounts gave fairly intense OPT-induced fluorescence. Dipeptides with histidine in the C-terminal position gave no or very low fluorescence at concentrations below 1 μg per spot. Arginyl-peptides (L-arginyl-L-aspartic acid and bradykinin) gave fluorescence of moderate intensity only. Tyrosyl-peptides failed to give detectable OPT-induced fluorescence. As a chromatographic detection reagent OPT has approximately the same sensitivity (on an equimolar basis) for histidyl-peptides as for histidine and histamine. The minimum detectable amount varied from 0.1 to 0.3 μg per cm^2 for the various histidyl-dipeptides (Table I). For the three polypeptides the minimum detectable amount was 0.3–1.0 μg. The

TABLE I

FLUORESCENCE CHARACTERISTICS OF OPT-INDUCED FLUOROPHORES OF HISTAMINE, HISTIDINE AND HISTIDINE-CONTAINING PEPTIDES

The values given for maximum emission are those recorded with the 365-mμ lines (in parentheses) and with the 405-mμ lines, respectively.

Compound	*Minimum detectable amount (μg)*	*Excitation/emission max. (mμ)*
Histamine dihydrochloride	0.1	390/(480)500
L-Histidine	0.1	390/(470)490
L-Histidyl-L-alanine	0.1	415/(460)490
L-Histidyl-L-serine	0.1	415/(460)490
L-Histidyl-L-lysine	0.3	415/(450)480
L-Histidyl-L-leucine	0.3	410/(460)490
L-Histidyl-L-phenylalanine	0.3	420/(440)490
L-Histidyl-L-tyrosine	0.3	410/(460)490
Glucagon	0.3–1	415/(460)490
Secretin	0.3–1	415/(460)490
VIP	0.3–1	410/(450)490
L-Alanyl-L-histidine	> 1	
L-Carnosine	> 1	
L-Glycyl-L-histidine	> 1	
L-Glycyl-L-histidyl-L-glycine	> 1	

fluorescence yield of the polypeptides was much lower if the pH of the spot was not adjusted by means of NaOH.

Effect of formaldehyde treatment

Glucagon, secretin and VIP gave strong fluorescence with OPT also after pretreatment with formaldehyde gas. No or only very weak fluorescence resulted from the formaldehyde treatment alone. No histidyl-dipeptide tested gave formaldehyde-induced fluorescence and without exception formaldehyde pretreatment prevented the subsequent OPT-induced fluorescence. Exposure to formaldehyde after OPT did not overtly affect the OPT-induced fluorescence of any of the histidyl-peptides.

Microspectrofluorometric characterization

The spectral properties of the various histidyl-peptide fluorophores resulting from the OPT treatment were fairly similar (Table I). It may be noted that the spectral characteristics of the OPT-induced fluorescence of the histidyl-peptides were rather similar to those of histamine and histidine. The spectral properties of the OPT-induced fluorescence did not change upon subsequent formaldehyde treatment.

Solvent systems

The R_F values obtained with some of the solvent systems tested are listed in Table II.

TABLE II

R_F VALUES OF HISTAMINE, HISTIDINE AND HISTIDYL-PEPTIDES

Solvent systems: A = 0.8% NaCl; B = ethyl acetate–acetic acid–water (15:15:10); C = *n*-butanol–pyridine–water (1:1:1); D = 0.5 *M* phosphate buffer, pH 8; E = 0.5 *M* phosphate buffer, pH 5.

	Solvent system				
	A	*B*	*C*	*D*	*E*
Histamine	0.67	0.48	0.07	0.24	0.81
L-Histidine	0.61	0.60	0.62	0.73	0.90
L-Histidyl-L-alanine	0.45	0.57	0.87	0.57	0.82
L-Histidyl-L-leucine	0.40	0.75	—	0.36	0.52
L-Histidyl-L-lysine	0.21	0.41	0.21	0.31	0.74
L-Histidyl-L-phenylalanine	0.31	0.78	0.81	0.43	0.56
L-Histidyl-L-serine	0.60	0.59	0.76	0.72	0.92
L-Histidyl-L-tyrosine	0.50	0.75	0.85	0.70	0.88
Glucagon	0	0.56	—	0	0
Secretin	0	0.48	—	0	0
VIP	0	0.39	—	0	0

DISCUSSION

It appears that all peptides, and conceivably also proteins, with N-terminal histidine are capable of giving strong fluorescence with OPT. C-Terminal histidine peptides gave no or very weak OPT-induced fluorescence. The spectral properties of the fluorescence of the various OPT-induced histidyl-peptide fluorophores were found to be similar, thus supporting the view that the fluorophores are chemically

similar. The nature of the fluorophores and the mechanism of the reaction between OPT and the histidyl-peptides is unknown although it can be assumed that initially a Schiff base is formed from the histidine α-amino nitrogen and one of the OPT aldehydes[1]. Since the OPT-peptide fluorophores were unaffected by subsequent exposure to formaldehyde it may be concluded that the fluorophores are substituted (non-reacting) Schiff bases, incapable of transaldimination. Formaldehyde treatment, which did not give fluorescence with any of the histidyl-peptides, prevented the subsequent OPT-induced fluorescence as could be predicted since formaldehyde and OPT probably compete for the same amino groups. Glucagon, secretin and VIP, however, were notable exceptions to this rule, giving the same fluorescence intensity with OPT after formaldehyde pretreatment as with OPT alone. One possible explanation for this discrepancy is that with histidyl-dipeptides the Schiff base formed from formaldehyde and the α-amino nitrogen of the histidine is rapidly transformed into a non-reacting derivative incapable of engaging in transaldimination reactions, whereas for some as yet unknown reason this transformation of the Schiff base into a non-reacting derivative does not occur — or proceeds more slowly — with the polypeptides.

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