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DISTINCTIVE TEST WITH COPPER(II)–NINHYDRIN REAGENT FOR SMALL α-PEPTIDES SEPARATED BY PAPER CHROMATOGRAPHY

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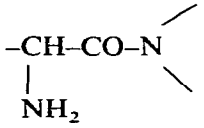
SUMMARY

A Cu^{2+}–ninhydrin reagent was used to distinguish qualitatively small α-peptides and α-amino acid amides from free amino acids on paper after chromatography. With the exception of peptides containing N-terminal L-tryptophan, all the peptides gave a yellow chromophore with a λ_{max} at 395 nm. Protein and non-protein amino acids and polyamines gave different chromophores which varied from one compound to another. Peptides with N-terminal L-proline gave a very faint yellow chromophore or no coloured product at all, while glutathione, a γ-glutamyl peptide, gave a red colour. Polypeptides and proteins did not produce a yellow chromophore. Evidence is provided for a reaction sequence in which peptides first react with Cu^{2+} to form a complex which then reacts with ninhydrin to give the yellow chromophore. These results and also studies with several peptides having N- and C-terminal substitutions suggest that the minimum structural requirement around the α-peptide linkage for the formation of the yellow chromophore with the Cu^{2+}–ninhydrin reagent is as follows:



INTRODUCTION

Until recently, ninhydrin was essentially the only colour reagent used to detect amino acids and peptides on paper. Fluorescamine was introduced a few years ago as a reagent for the qualitative detection and quantitative determination of primary amines, amino acids, peptides and proteins[1,2]. This reagent has been successfully employed to assay the peptide hormones vasopressin and oxytocin[3]. It is also known that *o*-phthalaldehyde reacts with amino acids and peptides in the presence of 2-

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mercaptoethanol to yield highly fluorescent products[4,5]. However, the above reagents are not useful for a general procedure to distinguish small α-peptides from amino acids.

We report here the use of a Cu^{2+}–ninhydrin reagent in an unambiguous qualitative differentiation on paper chromatograms of small α-peptides (with the exception of those containing N-terminal L-tryptophan) from amino acids.

MATERIALS AND METHODS

Most of the peptides, peptide and amino acid derivatives and polyamines were purchased from Sigma (St. Louis, MO, U.S.A.). Hexaglycine, gramicidin-A, -S and thiostrepton were gifts from Professor L. K. Ramachandran (Biochemistry Department, Osmania University, Hyderabad). Poly(L-lysine) (MW 500,000), poly-(L-aspartic acid) (14,000) and poly(L-serine) (11,000) were kindly donated by Professor D. Balasubramaniam (School of Chemistry, University of Hyderabad). Lutropin-releasing hormone (LHRH) and substance-P were gifts from Dr. E. Vijayan (School of Life Sciences, University of Hyderabad). All amino acids were obtained from Calbiochem (Los Angeles, CA, U.S.A.). *cis*-4-Hydroxy-L-proline[6] and *sym*-homospermidine[7] were available in the laboratory. Polyglycine (Cat. No. 901288) was from Schwarz/Mann (Orangeburg, NY, U.S.A.). Ninhydrin of specially purified grade was from Pierce (Rockford, IL, U.S.A.). Cadmium acetate, cupric nitrate, zinc acetate and nickel chloride were of AnalaR grade (BDH, Bombay, India). All the solvents (methanol, isopropanol and acetone) were of analytical grade.

Reagents

Salts of different metals were each dissolved in a mixture of water (3 ml) and glacial acetic acid (1 ml) and the solution was made up to 30 ml with acetone, giving a final metal salt concentration of 25 mmol/l. A 1 % w/v ninhydrin solution in aqueous acetone (95 %) was employed.

Metal–ninhydrin solutions were prepared by dissolving appropriate quantities of the metal salt (final, 25 mmol/l) and ninhydrin (final, 1 % w/v) in a mixture of water (3 ml) and glacial acetic acid (1 ml) and the solution was made up to 30 ml with acetone.

Development of chromatograms

Descending chromatograms (Whatman No. 1 paper, chromatography grade, 20-h run) of the peptides or amino acids were developed with a single phase solvent system, isopropanol–water (4:1). The chromatogram was then dried in air at room temperature for a minimum of 3 h.

Spraying methods

(A) Metal–ninhydrin. The dried chromatogram was uniformly sprayed with the "metal–ninhydrin solution", then air dried and heated at 65°C for 30 min.

(B) Ninhydrin followed by metal. The dried chromatogram was uniformly sprayed with "ninhydrin solution", then air dried and heated at 65°C for 30 min. The paper was sprayed with the "metal salt solution" and finally air dried.

(C) Metal followed by ninhydrin. The chromatogram was uniformly sprayed

with the "metal salt solution" and air dried for 10 min. The paper was then sprayed with ninhydrin solution, air dried and heated at 65°C for 30 min.

The coloured spots and spots of similar areas from an uncoloured portion of the chromatogram (to serve as blanks) were cut out and eluted with 4 ml of methanol–water (80:20). The absorption spectra were recorded on a double beam spectrophotometer (Shimadzu, Model UV 200S).

Unless otherwise stated, 0.01 μmole of each amino compound was used.

RESULTS AND DISCUSSION

Effect of metal ions in the metal–ninhydrin solution

Initial experiments were done with two dipeptides (Gly-L-Leu and L-Leu-Gly) and two tripeptides (Gly-Gly-L-Leu and L-Leu-Gly-Gly). Fig. 1 shows the spectra of the coloured products of the peptides obtained with the combined metal–ninhydrin reagent (spray method A). Of the four metal ions used (Cu^{2+}, Cd^{2+}, Zn^{2+} and Ni^{2+}), only Cu^{2+} gave a single yellow chromophore with all the four peptides. The λ_{max} of the chromophore was around 395 nm (Gly-L-Leu, 392 nm; L-Leu-Gly, 396 nm; Gly-Gly-L-Leu, 392 nm; L-Leu-Gly-Gly, 398 nm). Cd^{2+} gave a yellow chromophore with Gly-L-Leu and Gly-Gly-L-Leu (λ_{max}, 390 nm) and a red chromophore with L-Leu-Gly and L-Leu-Gly-Gly. The spectrum of the red chromophore (Fig. 1) showed absorption maxima at 390 nm and 505 nm. Both Zn^{2+} and Ni^{2+} produced a yellow chromophore with Gly-L-Leu and Gly-Gly-L-Leu (λ_{max}, 386 nm) and an orange colour with the other two peptides. This orange chromophore had absorption

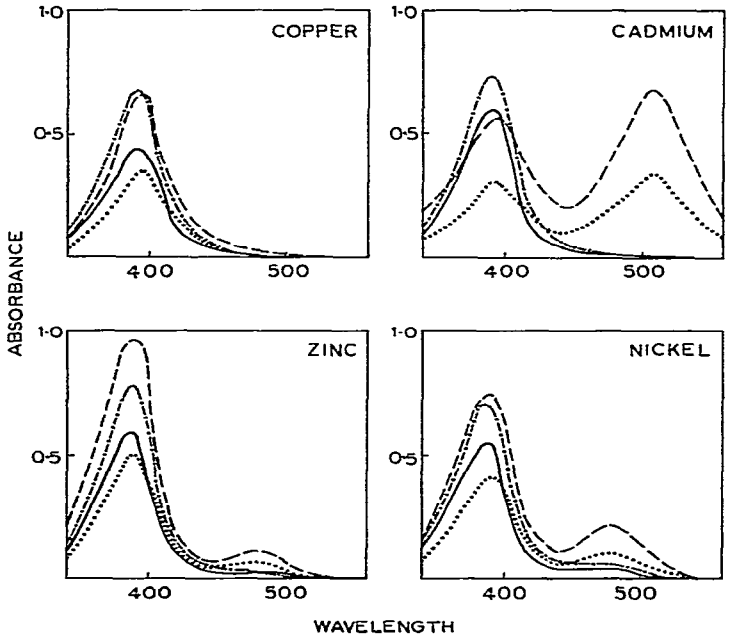


Fig. 1. Absorption spectra of the chromophores obtained by the reaction between peptides and the combined metal–ninhydrin reagents. ———, Gly-L-Leu (0.05 μmole); — — —, L-Leu-Gly (0.1 μmole); —·—·—, Gly-Gly-L-Leu (0.08 μmole); ·····, L-Leu-Gly-Gly (0.05 μmole).

maxima at 390 nm and 480 nm. Since the Cu^{2+}–ninhydrin reagent gave a single yellow chromophore with all the four peptides tested, further studies were carried out with this reagent.

The use of a Cd^{2+}–ninhydrin reagent was suggested for determination of dipeptides as yellow chromophores (λ_{max}, 390 nm) based on a study of peptides containing N-terminal glycine[8]. However, it was found during the present study that this reagent gives a yellow chromophore only with peptides having glycine as the N-terminal amino acid (seventeen dipeptides and eight tripeptides were tested), while in the case of others (thirteen dipeptides and four tripeptides) a red chromophore results (λ_{max}, 390 nm and 505 nm).

Studies with the Cu^{2+}–ninhydrin reagent

(a) Simple peptides, oligopeptides, cyclic peptides, peptide derivatives, polypeptides and proteins. All 157 compounds were tested (Table I). Most of the smaller peptides gave an yellow chromophore (λ_{max} 395 $\pm$ 4 nm) with the exception of peptides containing N-terminal tryptophan which gave a reddish to purple colour.

It is interesting that, while Gly-L-Trp and L-Val-L-Trp gave a yellow colour, L-Trp-L-Leu gave a red colour. Therefore, six other dipeptides with L-tryptophan as the N-terminal residue (Table I) were tested for their reaction with the Cu^{2+}–ninhydrin reagent. All gave a reddish colour. This reagent, thus, may be of use in identifying peptides with L-tryptophan as the N-terminal residue, but obviously more peptides of this type should be tested.

Among the L-proline-containing peptides tested, only Gly-L-Pro produced a visible yellow colour, while peptides with L-proline as the N-terminal residue either gave a faint yellow colour or did not give a coloured product at all. Gly-L-Hyp gave a yellow colour but L-Hyp-Gly did not give a coloured product. The yellow chromophore obtained with Gly-L-Pro had a single λ_{max} at 395 nm. It is to be noted that this chromophore is different from the yellow chromophore which results from the reaction between L-Pro or L-Hyp and ninhydrin which has a λ_{max} at 440 nm.

Glutathione, a γ-glutamyl tripeptide, gave a red colour while peptides with an α-Glu linkage gave an yellow colour. Peptides with a modified carboxyl group such as L-Asp-L-Phe methyl ester, tri-L-Tyr methyl ester and glycyl-L-leucinamide produced an yellow chromophore. Peptides with modified amino groups such as N-CBZ-Gly-L-Leu, N-CBZ-Gly-L-Pro and N-CBZ-Gly-Gly-L-Leu (Table I) gave no colour. Carnosine (β-Ala-L-His) gave a red chromophore.

Oligopeptides such as tetra-L-Ala, penta-L-Ala, hexa-Gly, tuftsin and methionine enkephalin produced a yellow colour. On the other hand, thyrotropin-releasing hormone (TRF) and lutropin-releasing hormone (LHRH) did not give coloured products. Cyclic peptides such as gramicidin-A, -S and thiostrepton also did not produce any colour with this reagent. It is interesting that none of the polypeptides and proteins examined (Table I) produced any colour, except for poly-L-lysine. The latter produced a purple colour which can be attributed to the participation of its ε-amino groups in the reaction.

(b) Protein amino acids and derivatives. All the protein amino acids and the amides Gln and Asn were tested for their reaction with the Cu^{2+}–ninhydrin reagent on paper. Each amino acid produced a different shade of colour but none gave a yellow colour. The spectra of the coloured products of Gly, L-Leu, L-Phe, L-Asp and

TABLE I

COLOURS OBTAINED BY REACTION OF Cu^{2+}–NINHYDRIN REAGENT AND AMINO COMPOUNDS

Compounds are classified according to the colour of the chromophore formed. All compounds were tested at a level of 0.01 μmole.

I. *Compounds yielding yellow chromophores*
Amino acids and derivatives
L-Leucinamide
Dipeptides
Gly-X: X = Gly; L-Leu; L-Ala; L-Ser; L-Met; L-Phe; L-Trp; L-Thr; L-Tyr; L-Glu; L-Ile; L-His; L-Pro; L-Hyp; D-Leu; γ-aminobutyric acid
L-Ala-X: X = Gly; L-Leu; L-Val; L-Ala; L-Met; L-Asp; L-Ser; L-Pro
L-Leu-X: X = Gly; L-Leu; L-Phe; L-Met
L-Met-X: X = Gly; L-Ala; L-Val
L-Phe-X: X = L-Leu; L-Met; L-Pro
L-Tyr-X: X = L-Leu; L-Lys
L-Glu-X: X = L-Ala; L-Val; L-Tyr; L-Glu
L-Val-L-Trp; L-Asp-Gly; L-His-L-Leu; L-Lys-L-Phe
L-Asp-L-Asp; L-Ser-Gly
Tripeptides
Gly-Gly-X: X = Gly; L-Leu; D-Leu; L-Ile; L-Phe; L-Val; L-Ala
Gly-L-Leu-X: X = Gly; L-Tyr
Gly-L-Phe-X: X = L-Phe; L-Ala
Gly-L-Pro-L-Val; Gly-L-Ala-L-Ala; Gly-L-His-Gly;
L-Leu-Gly-Gly; L-Tyr-Gly-Gly;
L-Glu-L-Val-L-Phe; L-Phe-Gly-Gly; D-Ala-Gly-Gly;
Gly-DL-Leu-DL-Ala; tri-L-Leu; tri-L-Ala; tri-L-Tyr;
tri-L-Phe; tri-L-Ser
Tetra and higher peptides
Tetra-L-Ala; penta-L-Ala; hexa-Gly;
L-Thr-L-Lys-L-Pro-L-Arg (tuftsin);
L-Tyr-Gly-Gly-L-Phe-L-Met (Met-enkephalin)
Peptide derivatives
Gly-L-Leu-NH_2; L-Asp-L-Phe methyl ester;
tri-L-Tyr methyl ester

II. *Compounds yielding reddish, purple and similar chromophores*
Amino acids and derivatives
Gly; L-Ala; L-Leu; L-Val; L-Phe;
L-Tyr; L-Ile; L-Lys;
L-Arg; L-Glu; L-Gln;
L-Asp; L-Thr; L-Ser; L-Pro;
L-Orn; L-citrulline; L-2,4-diaminobutyric acid;
L-α,β-diaminopropionic acid; β-Ala;
L-His; L-Cys
Dipeptides
L-Trp-X: X = Gly; L-Phe; L-Ala; L-Tyr; L-Trp; L-Glu; L-Leu
Non-α-peptides
γ-L-Glu-L-Cys-Gly; β-Ala-L-His
Polypeptide
Poly-L-Lys

(Continued on p. 312)

TABLE I *(continued)*

III. *Compounds yielding grey chromophores*
Amino acids and derivatives
cis-L-Hyp; L-Asn; L-Trp

IV. *Compounds yielding no visible colour*
Amino acid derivatives and polyamines
Sarcosine; L-cystathionine; taurine; N-carbamoyl-L-α-Ala; benzoyl-L-Phe; pyroglutamic acid; cadaverine; putrescine; *sym*-homospermidine
Dipeptides
L-Pro-X: X = Gly; L-Leu; L-Ala; L-Trp
L-Hyp-Gly
Tripeptides
L-Pro-Gly-Gly
Peptide derivatives
N-CBZ-Gly-X: X = L-Leu; L-Pro
N-CBZ-Gly-Gly-X: X = Gly; L-Leu; L-Val; L-Ala; L-Ile; L-Met; L-Ser; L-Pro
N-CBZ-Ala-Gly-Gly; N-CBZ-Ile-Gly-Gly
Oligopeptides, polypeptides and proteins
Gramicidin-A; gramicidin-S; thiostrepton; substance-P; LHRH; poly-Gly; poly-L-Asp; poly-L-Ser; bovine serum albumin; carboxypeptidase-A; chymotrypsin; trypsin; pepsin and leucine aminopeptidase

L-Trp are shown in Fig. 2. The chromophore of each amino acid had its own characteristic absorption spectrum and all the chromophores showed two or more absorption maxima. Asparagine gave a greyish colour whereas glutamine gave a red colour. Proline gave a reddish colour while *trans*- and *cis*-4-hydroxy-L-proline gave greyish colours both with a λ_{max} of 460 nm. On the other hand, L-leucinamide, an α-amino acid amide, and glycyl-γ-aminobutyric acid gave a yellow colour. Pyroglutamic acid and other amino acid derivatives such as N-carbamoyl-L-α-alanine and benzoyl-L-phenylalanine gave no colour.

(c) Non-protein amino acids and polyamines. It has been reported that polyamines and certain non-protein amino acids such as β-Ala, γ-aminobutyric acid and taurine behave like peptides on Cu–Sephadex columns[9]. They are not retarded on the column as are protein amino acids. We, therefore, studied the reaction of polyamines and non-protein amino acids with the Cu^{2+}–ninhydrin reagent (Table I). All the non-protein amino acids gave different chromophores but none gave a yellow chromophore. Polyamines such as putrescine and *sym*-homospermidine did not give a coloured product.

Order of addition of reagents

Metal ions have been widely used in the preservation of ninhydrin-stained amino acid chromatograms. Kawerau and Wieland[10] reported a method for the preservation of chromatograms using certain metal ions. When ninhydrin-stained amino acid chromatograms were sprayed with a cupric salt solution, all amino acids

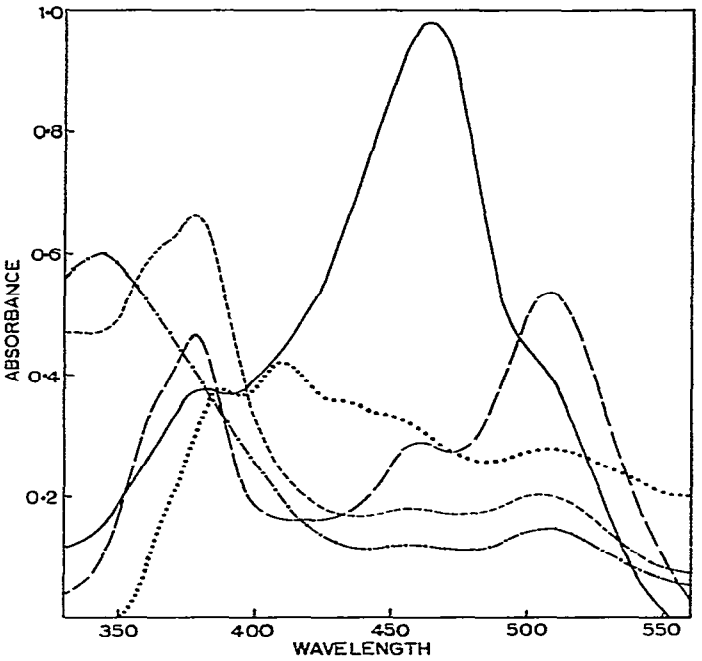


Fig. 2. Absorption spectra of chromophores obtained by the reaction between amino acids and the combined Cu^{2+}–ninhydrin reagent. ———, Gly (0.5 μmole); – – –, L-Leu (1.0 μmole); – – – – –, L-Phe (0.5 μmole); —·—·—·—, L-Trp (0.5 μmole); ·····, L-Asp (1.0 μmole).

produced a reddish colour except for L-Pro and *trans*-4-hydroxy-L-proline. Our earlier study[8] showed that the dipeptides Gly-Gly and Gly-L-Leu also gave a red colour when the chromatogram was first sprayed with ninhydrin, heated at 65°C for 30 min and then sprayed with cupric nitrate solution. However, during the present study it was found that both Gly-Gly and Gly-L-Leu gave a yellow colour with the combined Cu^{2+}–ninhydrin reagent. We, therefore, investigated the individual effect of cupric ions and of ninhydrin on peptides and amino acids by changing the order of spraying (see Materials and Methods). The results of these experiments with three amino acids and three peptides are given in Table II.

TABLE II

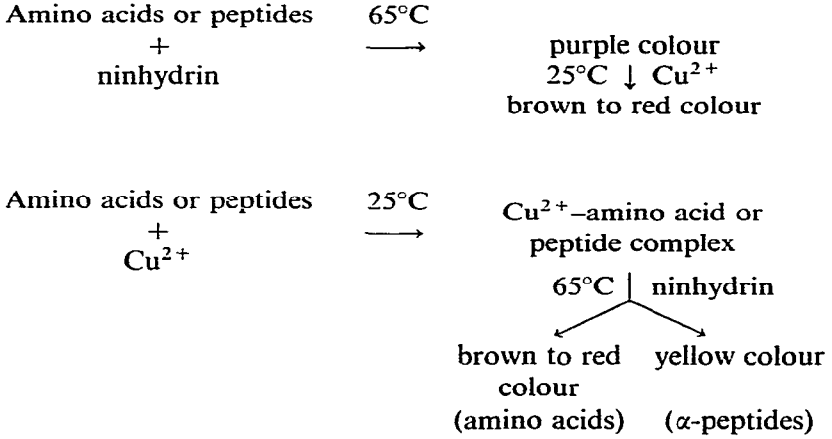
EFFECT OF Cu^{2+} AND NINHYDRIN AND THEIR ORDER OF ADDITION

Amino acid or peptide	*Colour produced*		
	With ninhydrin	*Ninhydrin followed by* Cu^{2+}	Cu^{2+} *followed by ninhydrin*
Gly	Purple	Red	Orange
L-Leu	Purple	Red	Pink
L-Phe	Purple	Red	Pink
Gly-L-Leu	Purple	Red	Yellow
L-Leu-Gly	Purple	Purple	Yellow
Gly-Gly-L-Leu	Purple	Red	Yellow

When a paper containing amino acid and peptide spots was first sprayed with ninhydrin and then with cupric salt solution, both amino acids and peptides gave colours ranging from brown to pink and it was not possible to distinguish peptides from amino acids from the colours of the spots obtained. However, when the paper was first sprayed with cupric salt solution and then with ninhydrin reagent, only amino acids gave colours ranging from brown to pink but *all* the peptides gave a yellow colour. The same result was obtained when the paper containing the spots was sprayed with the combined Cu^{2+}–ninhydrin reagent. This experiment clearly shows that the final products of the reactions between a peptide–ninhydrin complex and Cu^{2+} and between a peptide–Cu^{2+} complex and ninhydrin are not identical.

Small peptides are known to form deep blue complexes with Cu^{2+} (for a review, see ref. 11). However, in the present study, the peptide spots on paper after spraying with cupric salt solution were either colourless or lightly blue since the quantity of the peptides used was very low (0.01 μmole).

The results of this experiment can be summarized as follows:



Thus, it is clear that, when the combined Cu^{2+}–ninhydrin reagent is used, Cu^{2+} reacts rapidly at room temperature with the peptide to yield a complex which then reacts with ninhydrin at 65°C to give a yellow color.

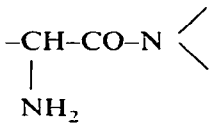
The complex of Gly-Gly with Cu^{2+} was prepared and crystallized according to the procedure of Manyak *et al.*[12]. An aliquot of an aqueous solution of this complex (0.01 μmole) was spotted on chromatographic paper and then sprayed with 1% ninhydrin in aqueous acetone (95%). The paper was heated for 30 min at 65°C. A yellow chromophore with a single λ_{max} at 390 nm was obtained. This experiment clearly shows that the yellow chromophore is the product of the reaction between the copper(II) complex of Gly-Gly and ninhydrin.

Ninhydrin reagent has long been used for qualitative and quantitative analysis of amino acids. However, amino acids cannot be qualitatively distinguished from small peptides using this reagent since both amino acids and peptides give a purple colour with ninhydrin on paper. A Cd^{2+}–ninhydrin reagent was recommended by Atfield and Morris[13] for the detection of amino acids and peptides on paper after

electrophoresis. This reagent was later successfully employed by Ganapathy and Radhakrishnan[8] to quantitate dipeptides having N-terminal glycine. Most of the amino acids gave a red colour with this reagent, while dipeptides containing N-terminal glycine gave a yellow colour. However, the present study shows that peptides having N-terminal residues other than glycine give a red colour with Cd^{2+}–ninhydrin. Thus the use of Cd^{2+}–ninhydrin is limited and it cannot be employed as a general reagent to differentiate small peptides from amino acids.

Minimum structural requirements

From the structure of the compounds employed in this study which are substituted at the N- and C-terminus and α- or other peptide bond, it emerges that the minimum structural requirement around the α-peptide linkage to give a yellow chromophore with the Cu^{2+}–ninhydrin reagent is as follows:



It would appear that the method could be used with confidence to distinguish amino acids and linear peptides containing up to six amino acid residues. Larger linear peptides and cyclic peptides cannot easily be distinguished from amino acids.

The Cu^{2+}–ninhydrin reagent described can conveniently be used to qualitatively differentiate simple α-peptides from amino acids on paper after chromatography or electrophoresis. Work is in progress to determine whether this method can be adapted for quantitation of small α-peptides. Preliminary studies already indicate that reliable linearity between concentration of peptide and absorbance (range, 0.02–0.12 μmole) is obtained for a number of peptides using the 390 nm absorbance, and among several metal ions tested the most sensitive was Zn^{2+} which gives a uniformly higher reading at 390 nm than Cu^{2+}. However, the latter has the advantage of giving a single yellow chromophore.

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