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## alylprolylarginine: a ninhydrin negative tripeptide

Ninhydrin is the most popular location reagent for amino acids and peptides, nce its first application to paper chromatography[1]. For peptides, however, the ensitivity of the reagent is known to decrease as the molecular weight of the peptide ncreases. A further disadvantage is that ninhydrin produces no colour with cyclic eptides or the peptides whose terminal $\alpha$-amino groups are acetylated or similarly ubstituted. In this communication, we wish to present a new example showing an dditional limitation of the use of ninhydrin for the location of peptides on filter paper.

In the course of our investigations[2,3] on the primary structure of stem bromelain EC 3.4.4.24), we isolated a glycoundecapeptide from the peptic digest of the parent lycoprotein. The amino acid sequence now determined is: Ala–Arg–Val–Pro–Arg–sn–Asn(sugar)–Glu–Ser–Ser–Met, where sugar stands for the carbohydrate moiety resent. When the tryptic digest of the glycopeptide was subjected to paper chromatoraphy (pyridine–acetic acid–*n*-butanol–water, 10:3:15:12, by volume) or paper lectrophoresis with pyridine acetate buffers at pH 3.5 and 6.5 (ref. 4), the occurrence f a peptide fragment that did not produce a ninhydrin colour at all was demonstrated; n the other hand, it gave positive stainings with both Sakaguchi reagent and the eptide reagent, *tert.*-butyl hypochlorite–*o*-tolidine–potassium iodide[5,6]. The fragment ave an amino acid composition of valine, proline and arginine in 1:1:1 ratio. The ubtractive method for Edman degradation[7] indicated valine to be the amino group erminus, while carboxypeptidase B-DFP (EC 3.4.2.2, Worthington Biochemical Corp., Freehold, N.J., U.S.A.) revealed only arginine: thus the fragment must have been a tripeptide, Val–Pro–Arg. Chemical synthesis of L-Val–L-Pro–L-Arg was then kindly performed for us by Dr. S. SAKAKIBARA of the Institute for Protein Research, Osaka University, Osaka, Japan. The product, upon chromatography and electrophoresis on paper, was found to behave in exactly the same way as that shown by the solated tripeptide, giving practically no colour development on spraying with an 0.2% ninhydrin solution in water-saturated *n*-butanol, by dipping in 0.2% ninhydrin solution in acetone, or by using ninhydrin–cadmium reagent[8]. Faintly positive colour development was noted only when a spot test was made directly on the filter paper with as much sample as 0.1 $\mu$mole per spot.

To eliminate the possibility of an interaction between the tripeptide and the filter paper in the ninhydrin reaction, the colour development was carried out in solution, instead of on paper. The synthesized tripeptide was chromatographed on Hitachi spheric resin No. 2610 (0.9 × 50 cm) using a Hitachi Model KLA-3B amino acid analyzer with the solvent system for acidic and neutral amino acids. The ninhydrin colour produced for the tripeptide, as recorded at 570 m$\mu$, was compared with that for norleucine which was used as an internal standard. Several other di- and tripeptides were also analyzed in the same way. As shown in Fig. 1, Val–Pro–Arg gives an extraordinarily low value, indicating that poor ninhydrin colour production is an intrinsic property of this tripeptide. The poor colour production was not due to the amino terminal valyl residue, because longer and shorter valyl peptides were found to give reasonably high colour values (Fig. 1).

Further experiments were carried out to see whether the negative result of

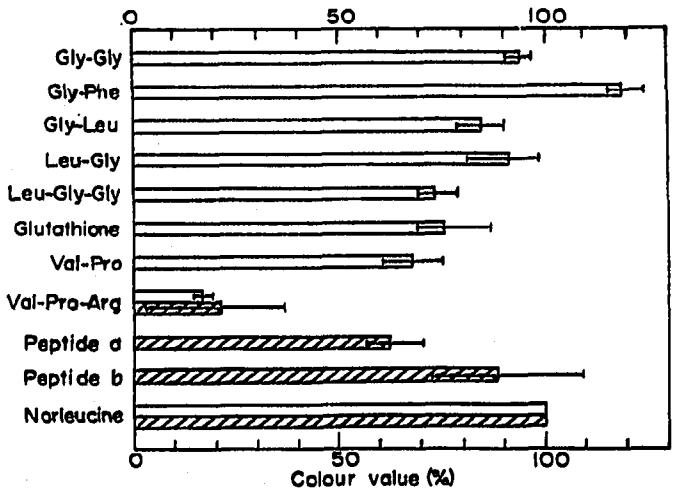


Fig. 1. The ninhydrin colour values of the peptides in solution. Clear bars are the values obtained by the amino acid analyzer (see text); hatched bars are those by the method of YEMM AND COCKING[9]. The value for norleucine was taken as 100%. Peptide a = Val–Pro–Arg–Asn–Asn-(sugar)–Glu–Ser–Ser–Met; peptide b = Ala–Arg–Val–Pro–Arg–Asn–Asn(sugar)–Glu–Ser–Ser–Met. Both peptides were isolated from the proteolytic digest of stem bromelain.

ninhydrin test on filter paper was due to poor reactivity of the tripeptide, or due to the fact that the product was colourless, even though it was formed. Val–Pro–Arg and Leu–Gly–Gly were spotted on filter paper and sprayed with ninhydrin reagent. After heating the paper, only the leucyl peptide stained. Both materials were then eluted from the paper with water and the eluates were hydrolysed in 6 *N* HCl at 105° for 20 h. The amino acid analysis of the hydrolysates showed that leucine was almost completely lost from the leucyl peptide, but there was a good recovery of glycine; while for the valyl peptide, valine as well as proline and arginine remained almost intact. The results indicate that ninhydrin hardly reacts with the α-amino group of Val–Pro–Arg under the conditions employed.

Very poor colour production by ninhydrin has also been observed with the tripeptide, Ile–Pro–Pro, which was obtained by tryptic hydrolysis of a decapeptide isolated from the venom of *Agkistrodon halys blomhoffii* (private communication from Dr. H. KATO, Institute for Protein Research, Osaka University). By analogy, it seems that its being a tripeptide with a bulky aliphatic side chain group on the amino group terminus, followed by a prolyl residue, is significant in reducing the reactivity toward the ninhydrin reagent on filter paper.

*Department of Biochemistry,*
*Nagoya City University,*
*School of Medicine,*
*Nagoya (Japan)*

KASHIKO KITO
TAKASHI MURACHI

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## Détermination de l'acide aminé N-terminal d'une protéine par application de la méthode d'Edman au matériel fixé sur gel de polyacrylamide

Au cours de notre travail portant sur la séparation des deux isoenzymes du rypsinogène de mouton[1], nous avons été amené à mettre au point une technique de létermination du résidu N-terminal des protéines fixées sur gel de polyacrylamide en ıtilisant non pas la réaction avec le fluorodinitrobenzène (FDNB) comme l'a décrite ATSIMPOOLAS[2] mais bien celle au phénylisothiocyanate (PITC) selon la méthode bien onnue d'EDMAN. C'est cette technique que nous allons décrire en détail.

Sur des gels à 15% d'acrylamide à pH 2.5 préparés selon CHOULES ET ZIMM[3] sont lacés 200 à 300 $\mu$g de protéine afin de charger ceux-ci au maximum mais sans avoir outefois de recouvrement des bandes. L'électrophorèse s'effectue pendant 4 h sous ne différence de potentiel de 80 V. Comme la réaction avec le PITC se réalise en nilieu organique basique le pH des gels a été modifié progressivement par agitation endant 15 min dans un tampon phosphate 0.5 *M* pH 8.7, puis 30 min dans le même ampon auquel on ajoute progressivement un volume égal de pyridine. Ce processus pour effet d'éviter autant que possible un éclatement des gels lors de la réaction avec e phénylisothiocyanate en milieu organique. A ce moment les gels sont à nouveau gités 1 h en présence d'un mélange composé d'un volume de tampon phosphate et l'un volume de solution de PITC à 5% dans de la pyridine. Ils sont alors placés dans ne solution à 2.5% de PITC dans de la pyridine pendant une nuit. Leur aspect et leur onformation changent, ils durcissent et perdent leur transparence.

Le lendemain les gels sont lavés au benzène, ensuite recouverts de 100 ml de benzène et de 10 ml d'eau; le mélange est agité et remplacé jusqu'à ce que le support reprenne sa forme initiale et soit complètement décoloré. Les tranches de gels correspondant aux dérivés phénylthiocarbamylés des protéines sont alors découpées par comparaison avec des gels colorés au FDNB ou au bleu de Coomassie[4] et dont l'électrophorèse a été réalisée en parallèle. Une vingtaine de tranches sont réunies, séchées sous vide et broyées afin d'être réduites en poudre; ce matériel est extrait 5 fois au benzène puis séché afin d'éliminer les traces résiduelles de solvant organique. L'hydrolyse ménagée libérant le PTH-acide aminé correspondant à l'extrémité N-terminale de la protéine est effectuée sur cette poudre à l'aide d'acide chlorhydrique 1 *N* à 100° pendant 90 min dans un tube à bouchon rodé. Après refroidissement on extrait 5 fois la solution acide par de l'acétate d'éthyle. Les phases organiques réunies contenant les PTH-acides aminés, sauf ceux de l'arginine et de l'histidine, sont alors lavées 3 fois par agitation en présence d'une quantité minimum d'eau. L'identification des PTH-acides aminés se réalise par chromatographie sur papier selon la méthode de SJOQUIST[5]. Elle