ACTA CHEMICA SCANDINAVICA 26 (1972) 3097–3100

3-Chlorotyrosine in Insect Cuticular Proteins

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From enzymatic hydrolysates of insect cuticular proteins an amino acid has been isolated and identified as 3-chlorotyrosine. The conditions for hydrolysis and purification are such that it is improbable that the amino acid is formed as an artifact during the isolation. The possibility that chlorination of tyrosine residues occurs during the natural hardening of the cuticular proteins is discussed in relation to the formation of bromo- and iodotyrosines in other scleroproteins.

Apart from the iodotyrosines present in thyroglobulines halotyrosines are mainly found in some proteins from marine organisms, such as iodo- and bromotyrosines in sponges and horny corals [1,2] and monochloromonobromotyrosine in the opercula of marine gastropods.[3] Chloro- and bromotyrosines can be formed when proteins are hydrolyzed with hydrochloric acid if the acid is not very pure, but this formation of halotyrosines can be prevented by the presence of reducing agents during the hydrolysis.[4] The natural occurrence of 3-chlorotyrosine in a protein has not been reported so far.

While investigating the amino acid composition of the proteins in locust cuticle I noticed the presence of a peak corresponding in position to 3-chlorotyrosine. The peak neither disappeared after purification of the hydrochloric acid used for hydrolysis nor after addition of reducing agents to the acid. Thus the amino acid might be a natural constituent of the proteins and isolation was attempted after enzymatic hydrolysis where the risk for the formation of artifacts should be minimal.

METHODS

2 g of exuviae from fifth instar locusts (*Schistocerca gregaria*) was extracted repeatedly with 500 ml 1 % potassium tetraborate to remove uric acid and other soluble contaminants [5] and was then washed several times with distilled water. The main part of the proteins in the exuviae was brought into solution by reflux with 500 ml 1 M formic acid for 2 h, the supernatant was concentrated in a rotatory evaporator to a volume of approximately 25 ml and was thereafter dialysed overnight against 5 l distilled water. During dialysis a large amount of protein precipitated inside the dialysis bag. The precipitate was suspended in 10 ml 0.05 M potassium borate, pH 8, 50 mg of pronase

(British Drug Houses Ltd.) was added, and the mixture was incubated at 40°C for 48 h. A small amount of undissolved material was removed by centrifugation, the supernatant was concentrated to 3 ml, acidified with formic acid to pH 2.5, and fractionated on a column filled with BioGel P-2. Elution was performed with 0.2 M acetic acid and the UV-absorption at 280 mμ was automatically recorded.

The fractions of interest were pooled, concentrated, and refractionated on a Sephadex G-15 column (2cm² × 50 cm) with 0.2 M acetic acid as eluent, and a final purification was thereafter performed on a column (2 cm² × 50 cm) of cellulose phosphate (Whatman P11). Elution was performed with a linear gradient of sodium chloride as described by Andersen.[6]

Thin layer chromatography was performed on plates precoated with cellulose (MN polygram CEL 300 from Macherey-Nagel & Co.) and the following solvents were used: 1-butanol – acetic acid – water (3:1:1 v/v/v), methanol – water – pyridine (20:5:1 v/v/v), phenol – water (4:1 w/w), and 1 M acetic acid.

RESULTS

Fig. 1a shows the elution pattern obtained when a pronase digest of the cuticular proteins was fractionated on BioGel P-2. The large peak eluted at 110 ml contained material with a UV-spectrum as tyrosine, whereas the

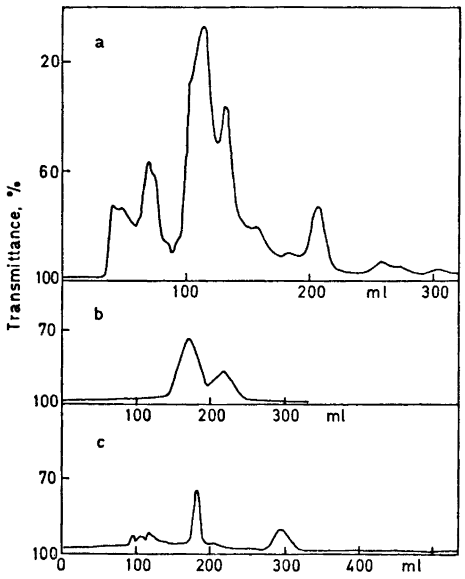

Fig. 1. (a): Pronase digest of proteins obtained from locust casts kins fractionated on a column of BioGel P-2 (2 cm² × 50 cm). Elution was performed with 0.2 M acetic acid at a rate of 20 ml/h, and the transmittance at 280 nm was automatically recorded. (b): The material eluted at 125 – 140 ml in run (a) was concentrated and refractionated on a column of Sephadex G-15 (2 cm² × 50 cm). Elution was performed with 0.2 M acetic acid as described for run (a). (c): The material eluted at 150 – 200 ml in run (b) was concentrated and refractionated on a column of cellulose phosphate (2 cm² × 50 cm). Elution was performed with a sodium chloride gradient established by running 1000 ml 1 M NaCl in 0.2 M acetic acid into a 1000 ml mixing chamber filled with 0.2 M acetic acid. Rate of elution was 33 ml/h.

material eluted at 130 ml had absorption maximum near 280 mμ. The material eluted at 210 ml was according to its UV-spectrum and behaviour on thin layer chromatography identical to the two neutral ketocatechols (3,4-dihydroxyphenylglyoxal and 2-hydroxy-3′,4′-dihydroxyacetophenone) which have been isolated from acid hydrolysates of insect cuticle.[5]

When the material eluted between 125 and 140 ml was further purified on Sephadex G-15 it separated into two components, one of which (at 215 ml in Fig. 1b) was identified as tryptophan and the other (at 170 ml) was further purified on cellulose phosphate as shown in Fig. 1c. The material eluted at 300 ml was identified as arterenone (2-amino-3′,4′-dihydroxyacetophenone), a compound which has also been obtained by acid hydrolysis of insect cuticle.[6] The material eluted at 180 ml gave a UV-spectrum similar to that of 3-chlorotyrosine both in acid and in alkaline solution, and it migrated together with this compound on thin layer chromatograms in four different solvents. As a mixture of the isolated compound and authentic 3-chlorotyrosine could not be separated into its two components by column chromatography on BioGel P-2, Sephadex G-15, or on cellulose phosphate as well as by means of the automatic amino acid anlyzer it was concluded that the compound was 3-chlorotyrosine.

The identity of the compound was confirmed by means of mass spectrometry. The mass spectrum showed the typical chlorine isotopic peak clusters and a molecular ion at *m/e* 215 in agreement with the molecular weight of monochlorotyrosine. Peaks were also present at *m/e* 170, corresponding to the loss of a carboxyl group, and at *m/e* 141, corresponding to the loss of $CH(NH_2)COOH$, which is in agreement with the proposed structure.

To check that chlorotyrosine did not originate from the enzyme used a sample of pronase was incubated without any substrate, and the autodigest was fractionated on BioGel P-2. Two UV-absorbing peaks were obtained and identified as tyrosine and tryptophan, but no chlorotyrosine was present.

Attempts were then made to determine the amount of chlorotyrosine which can be isolated from locust cuticle. Femur cuticle from sexually mature locusts was used for the determinations, and the same isolation procedure as described above was used, while care was taken to keep the losses of the amino acid as small as possible. Two determinations gave a chlorotyrosine content of 0.3 % and 0.4 % of the dry weight of the cuticle, respectively. These values can only be minimum values due to the inevitable losses occurring during the isolation procedure, but they indicate, however, that chlorotyrosine can only be a minor component in the cuticle.

By the methods described here 3-chlorotyrosine has been obtained from hard cuticle of adult locusts and from cuticle of the cockroach, *Periplaneta americana*, indicating that this amino acid could be of general occurrence in insect cuticle.

DISCUSSION

3-Chlorotyrosine is formed when tyrosine is exposed to small amounts of free chlorine, and the formation of chlorotyrosine in insect cuticle can best be explained by assuming that there is an enzyme system present in the cuticle capable of oxidizing chloride ions to free chlorine which would then react spontaneously with the tyrosine residues in the proteins present. Oxidase activity [7] and peroxidase activity [8] have been found in insect cuticle where they are believed to have the function of oxidizing diphenols to reactive intermediates which will react with the proteins to sclerotize the cuticle.

Since the work of Pryor [9] these intermediates have been assumed to be quinones but recent investigations [10] indicate that they are formed by oxidative activation of the aliphatic sidechain of *N*-acetyldopamine by means of a cuticular enzyme. Thereby the sidechain becomes able to link free amino groups and phenolic groups in the cuticular proteins. If the intermediates have an oxidation potential high enough to be able to oxidize some of the chloride ions present to free chlorine this could explain the presence of 3-chlorotyrosine in the proteins. This amino acid should therefore be considered an unavoidable byproduct from the sclerotization of the cuticle, and there is no reason to assume that it has any specific function in the proteins.

It has been reported that radioiodide readily becomes incorporated into scleroproteins of various animals [11,12] and that it afterwards can be recovered as iododerivatives of tyrosine and histidine. Tang and Chaikoff [13] have suggested that also this incorporation is unspecific and due to the action of the oxidative systems present. It is not known whether the hardening of all the structures which readily incorporate radioiodide is due to oxidative reactions, as our knowledge of the formation of scleroproteins is still insufficient. However, when investigating such proteins the readiness with which they bind radioiodide as iodotyrosines might be used as an indication that oxidative reactions are of importance for the formation of the structures.

Acknowledgement. This work was performed in the Department of Zoology, Cambridge, and I would like to express my gratitude to the staff of the department, as well as to cand. scient. G. Schroll, Chemical Laboratory II, University of Copenhagen, who kindly determined the mass spectrum.

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Received January 11, 1972.