Precursors Involved in the Formation of Pink Pigments in Onion Purees

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A preparation of alliinase (alliin alkylsulphenate lyase) from garlic and a system of pyridoxal and Cu^{+2} which simulates the catalytic action of alliinase caused the formation of pigment precursors in the amino acid fraction from onions. The precursors formed were an ether-soluble, ultraviolet-absorbing compound and one or more unidentified carbonyl compounds. Reactions for pigment formation are proposed.

Studies on the formation of pink to red, water-soluble, nitrogenous pigments in onion purees were first reported by Joslyn and Peterson (1958). The horticultural aspects affecting pigment formation have been reported by Yamaguchi *et al.* (1965). Joslyn and Peterson (1960) demonstrated that a carbonyl was necessary for pigment formation. Lukes (1959) showed that an enzyme reaction was necessary for the formation of a colorless ether-soluble precursor which he named color developer. He found that the pigment was formed when pure amino acids were incubated with an extract containing color developer. Shannon *et al.* (1967) hypothesized and their experiments indicated the presence of a second, colorless pigment precursor which was formed by heating mixtures of color developer and amino acids. This compound reacted with carbonyls to form pigments differing according to the kinds of amino acids and carbonyls used. This paper considers the enzyme involved, its substrate, and the product (color developer).

Experimental

Preparation and Activity of Alliinase. Alliinase (alliin alkylsulphenate lyase, E. C. 4.4.1.4) from garlic (*Allium sativum* L.) was prepared in a 0° C. room by a method similar to that given by Stoll and Seebeck (1948). The enzyme catalyzes the cleavage of *S*-aliphatic derivates of cysteine sulfoxide to form thiosulfinate, ammonia, and pyruvic acid. The volatile products of the reaction give odors characteristic of the species.

About 50 grams of peeled garlic cloves chilled to 0° C. were ground with sand and water in a mortar with a pestle. The slurry was filtered through cheesecloth, and the juice was centrifuged to remove particulate materials. The clear supernatant fluid was adjusted with hydrochloric acid to pH 4.0, to precipitate the enzyme. The precipitate was washed twice with acidified water and dissolved in about 50 ml. of 0.2*M* citrate–phosphate buffer (pH 6.8). This enzyme preparation catalyzed the formation of pyruvic acid in juice from boiled onions. The pyruvic acid was determined by a method described previously (Yamaguchi *et al.*, 1965). Possibly there were other enzymes present in this preparation, but none was identified. The preparation remained active for 2 to 3 weeks when stored at −20° C.

The ability of the enzyme preparation to form pigment precursors was determined by incubating 1 ml. of the preparation with 8 ml. of juice from boiled Southport White Globe onions for 10 minutes at 40° C. The mixture then was heated to 100° C. for 10 minutes, both to inactivate the enzyme and to hasten the formation of the final colorless pigment precursor (Shannon, *et al.*, 1967). The mixture was cooled, filtered, and placed in a test tube in a constant temperature bath at 50° C. One milliliter of $3 \times 10^{-3}M$ formaldehyde was added to the mixture for color development. The pigment formed quite rapidly, and the solution was dark red after 1 hour. The pigment did not form in mixtures in which the enzyme preparation was boiled prior to its incubation with the juice from boiled onions. This confirmed Lukes' assertion that an enzymic reaction was necessary for the formation of secondary colorless precursors.

Separation and Purification of Alliinase Substrates and the Primary Pigment Precursor. The naturally occurring substrates of alliinase, the *S*-aliphatic derivatives of L-cysteine sulfoxide, occur in several species of the genus Allium. Garlic contains *S*-allyl-L-cysteine sulfoxide (ACSO) (Stoll and Seebeck, 1948) and also the methyl (MCSO) and propyl (PCSO) derivatives (Fujiwara *et al.*, 1955). Onions contain MCSO, PCSO (Fujiwara *et al.*, 1955), and the *S*-1-propenyl sulfoxide (PECSO) (Spare and Virtanen, 1963). The presence of ACSO in onions also has been suspected (Renis and Henze, 1958). The presence of allyl groups in the volatile compounds from onions was reported by Saghir *et al.* (1964). A sulfur-containing imino acid called cycloalliin has been found in onions and can be formed by the cyclization of PECSO in alkaline medium (Virtanen, 1962). In addition to the common free amino acids and the above-mentioned sulfur-containing amino acids, there are several γ-glutamyl peptides present in onions (Virtanen, 1962).

Since the pigment precursors were formed in an enzyme preparation containing alliinase, the substrates of this enzyme were suspected to be the primary pigment precursors. Amino acids of both onion and garlic were separated and purified by ion exchange techniques as follows. The enzymic degradation of the amino acids was prevented by slicing the onion bulbs or garlic cloves into boiling water. After boiling for 15 minutes, the preparation was cooled and macerated in a Waring Blendor. The proteinaceous material was

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precipitated by adding two volumes of ethanol. The extract was filtered, and the ethanol was removed from the filtrate in a rotary vacuum evaporator. The concentrated extract then was passed through a 2.8 × 20-cm. column of Amberlite IR-120 cation exchange resin saturated with H^+. The resin was washed until the eluate was free of carbohydrates. The adsorbed amino acids and peptides were eluted with 2*N* NH_4OH. Excess ammonia was removed from the eluate by passing through a column of Amberlite IRC-50 resin in the hydrogen form. The eluate from the IRC-50 column was passed through a Dowex-1 anion exchange resin saturated with formate. The acidic amino acids and peptides were adsorbed by this resin, while the neutral and basic compounds passed through. The acidic amino acids and peptides were eluted from the Dowex-1 resin with 2*N* formic acid. The two fractions of amino acids were adjusted to pH 5.5 and tested for the presence of alliinase substrates, for the primary precursor of color developer, and for pigment-forming carbonyls.

Alliinase was added to each of the amino acid fractions, as with the onion juice above. Pyruvate formed only in the neutral plus basic amino acid fractions of both onion and garlic. The pink pigment slowly formed in the enzyme-treated neutral plus basic amino acid fraction, even when formaldehyde was not added to the mixture. Therefore, both color developer and the carbonyl were formed apparently from neutral plus basic amino acid precursors. When formaldehyde was added, the pigment formed more rapidly.

Pigments did not form in any of the other onion or garlic acidic amino acid fractions. However, when a mixture of 8 ml. of the onion neutral plus basic amino acids and 2 ml. of garlic neutral plus basic amino acids was incubated with the enzyme, a blue pigment formed after several hours at 40° C. Therefore, the ether soluble garlic compound which was responsible for blue pigment formation (Yamaguchi *et al.*, 1965; Shannon *et al.*, 1967), also was probably formed enzymically from an amino acid precursor.

Nonenzymic Cleavage of Alliinase Substrates and the Formation of Pigment Precursors. Cleavage of the *S*-aliphatic derivatives of cysteine sulfoxide by alliinase has been shown to be stimulated by pyridoxal phosphate (Kupiecki and Virtanen, 1960). The formation of a Schiff's base with this cofactor is believed to be the mechanism of the catalytic action. Binkley and Boyd (1955) reported the nonenzymic catalytic cleavage of cystathionine and lanthionine at room temperature in the presence of pyridoxal or pyridoxal phosphate and appropriate chelating agents and metal ions. They found that zinc, copper, or manganese activated the cleavage of lanthionine in the presence of pyridoxal, but only copper was effective with cystathionine. Their systems were most active near pH 9.0 with chelating agents such as citrate, oxalate, and pyrophosphate.

Synthetic *S*-ethyl-L-cysteine sulfoxide prepared by the method of Stoll and Seebeck (1949) was catalytically cleaved to form pyruvic acid by a system of pyridoxal, metal ions, and citrate–phosphate buffer. Copper, zinc, and manganese were effective at pH 8.0, but only copper was effective at pH 4.2 (Table I). No pyruvate formed in the absence of pyridoxal or metal ions.

This system offered a means of determining whether the enzymic action responsible for the formation of color developer was similar to that of alliinase. Since color developer was unstable under alkaline conditions, the test was conducted at pH 5.5 with the neutral plus basic amino acid fraction from onions. A 3-ml. portion of the onion neutral plus basic amino acid solution was incubated with 1 ml. of 0.001*M* pyridoxal and 1 ml. of 0.001*M* copper chloride at 40° C. As controls, 3-ml. portions of the onion neutral plus basic amino acids were incubated with pyridoxal or copper alone.

After 11 hours, the system with both pyridoxal and copper was orange-pink, and the system with only pyridoxal was slightly pink. No color formed in the system lacking pyridoxal. In the presence of formaldehyde, the system containing both pyridoxal and copper was red after 3 hours at 40° C.; the system containing pyridoxal alone was pink; and no pigment formed in the system lacking pyridoxal. Since some pigment formed in the absence of formaldehyde, the color developer and the carbonyl were apparently formed nonenzymically in the presence of pyridoxal.

Paper Chromatography of Onion Amino Acids and Color Developer Precursor. To obtain some information regarding the amino acid composition of onions, an extract of Southport White Globe variety was used for this study. The amino acids, free of carbohydrates after the extract had been passed through Amberlite IR-120 and eluted, were separated by two dimensional paper chromatography. Figure 1 shows the tracings after the paper was treated with iodoplatinate reagent for sulfur-containing compounds (Toennies and Kolb, 1951), followed by ninhydrin treatment. Spot 1 is suspected to be the acid peptide γ-L-glutamyl-*S*-L-cysteine glycine; and spot 2, another acid peptide, γ-glytamyl-L-phenylalanine, according to its position in comparison to the R_f values given by Virtanen and Matikkala (1960).

Since the color developer precursor was present in the neutral plus basic amino acid fraction, this fraction was investigated in more detail. The neutral plus basic amino acid extract was applied as a streak to Whatman 3-mm. paper (18 × 22 inches). The chromatogram was developed with the upper phase of 1-butanol–acetic acid–water (4:1:5, v./v.) in the de-

Table I. Nonenzymic Cleavage of *S*-Ethyl-L-cysteine Sulfoxide to Pyruvic Acid by Pyridoxal and Metal Ions[a]

pH	Metal Ion, $2 \times 10^{-4}M$	Pyruvate, μMoles
4.2	Cu^{+2}	1.18
6.2	Cu^{-2}	2.60
8.0	Cu^{+2}	5.27
4.2	Zn^{+2}	0.00
6.2	Zn^{+2}	0.05
8.0	Zn^{+2}	2.68
4.2	Mn^{+2}	0.00
6.2	Mn^{+2}	0.05
8.0	Mn^{+2}	0.36

[a] The reaction mixture contained: $4 \times 10^{-3}M$ *S*-ethyl-L-cysteine sulfoxide, $2 \times 10^{-4}M$ pyridoxal, and $4 \times 10^{-3}M$ citrate–phosphate buffer incubated at 40° C. for 5 hours.

scending direction. The chromatogram was air dried and cut into 2-inch strips. Several strips were treated individually to detect the following compounds. Amino acids were detected with ninhydrin; ultraviolet-absorbing compounds, with a 257-mμ source; sulfur-containing compounds, with iodoplatinate reagent; thiosulfinates, with nitroprusside reagent (Fujiwara *et al.*, 1955); and the unknown compound with Ehrlich's reagent made up in concentrated H_2SO_4. Ehrlich's reagent gives a red color with pyrroles. Color developer was detected by the formation of the pink pigment when the strip was moistened with 1% glycine and $3 \times 10^{-4}M$ formaldehyde and incubated between sealed glass plates at 40° C. One of the strips was sprayed with nitroprusside reagent, another with Ehrlich's reagent, and another with the garlic enzyme preparation. A diagram of the reconstructed chromatogram is shown in Figure 2. At least 14 amino acids or peptides were present in the neutral plus basic amino acid fraction. Four of these were sulfur-containing compounds, two of which overlapped near R_f 0.35. Two thiosulfinates, a compound which gave a red color with Ehrlich's reagent, and the color developer were detected only on the strips treated with the garlic enzyme preparation. One of the thiosulfinates, a compound giving a positive pyrrole test, and color developer were present at R_f 0.32, the same position occupied by the only compound which absorbed 257-mμ radiation.

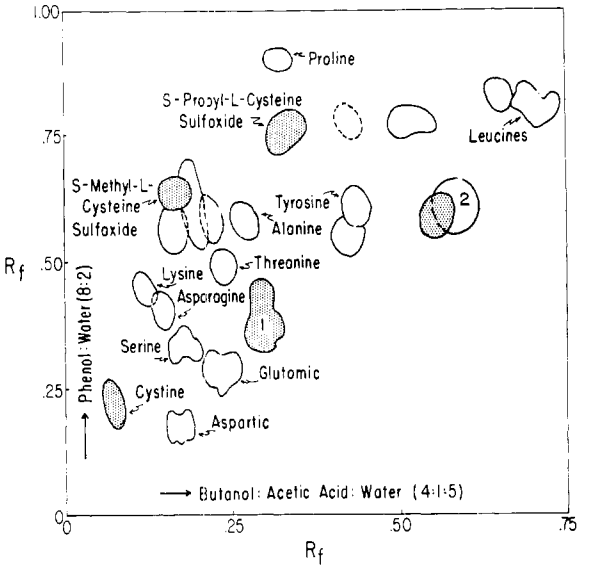

Figure 1. Amino acids of Southport White Globe onion. Tracing of a two-dimensional paper chromatogram

Outlined areas were positive to ninhydrin reagent and stippled areas were positive to iodoplatinate reagent. See text for discussion of areas 1 and 2

The chromatographic behavior of synthetic MCSO and PCSO is compared in Table II with that of the ultraviolet-absorbing compound from the neutral amino acid fraction from onion. The ultraviolet-absorbing compound had the same R_f as did the synthetic PCSO in all three solvents tested. Synthetic PCSO did not absorb 257-mμ radiation, nor did it give rise to a pyrrole-containing compound or color developer when treated with the garlic enzyme. The ultraviolet-absorbing compound was of special interest, not only because it occurred at the same R_f as the primary precursor of color developer (Figure 2), but because color developer previously was shown to absorb radiation at 257 mμ (Shannon *et al.*, 1967).

Additional evidence that PCSO is not the precursor of color developer and that the ultraviolet-absorbing compound probably is the precursor was obtained by chromatography of PCSO and the neutral plus basic amino acids from onion before and after incubation of portions of each with the garlic enzyme preparation. The enzyme in the treated portions was precipitated with ethanol and separated by centrifugation. Equal quantities of the four preparations were chromatographed. The relative quantities of precursors and products detected are given in Table III. A sulfur-containing product at R_f 0.93 was formed from PCSO and also from the neutral plus basic amino acid fraction

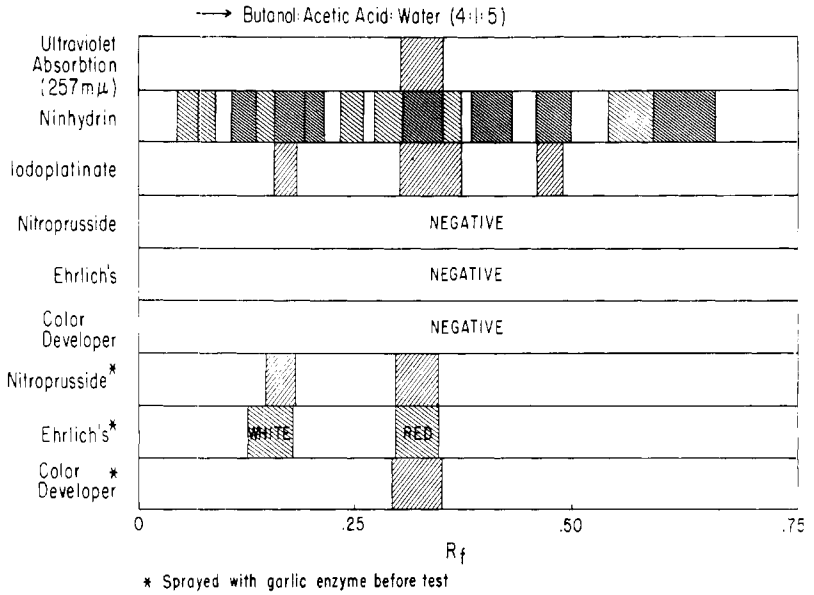

Figure 2. Reconstruction of a streak chromatogram of the neutral and basic amino acid fraction from onion

Crosshatched areas indicate a positive test to the indicator listed at the left of each strip. See text for significance of each test

Table II. R_f Values of *S*-Methyl-L-cysteine Sulfoxide (MCSO), *S*-Propyl-L-cysteine Sulfoxide (PCSO), and an Ultraviolet-Absorbing Compound from Onions

Solvent	R_f on Whatman No. 1 Paper: MCSO	PCSO	UV-absorbing spot from onions[a]
1-Butanol–acetic acid–water (4:1:5, v./v.)	0.14	0.31	0.31
tert-Butyl alcohol–formic acid–water (70:1:30, v./v.)	0.43	0.58	0.58
Phenol–water (8:2, v./v.)	0.62	0.75	0.75

[a] 257-mμ ultraviolet source.

from onion. However, an ultraviolet-absorbing compound also was present at R_f 0.93 on the chromatogram of the treated onion extract. The ultraviolet absorption increased at R_f 0.93 and decreased at R_f 0.31. This ultraviolet-absorbing product at R_f 0.93 was inseparable from the compound which reacted positively to Ehrlich's reagent and from color developer (Shannon, *et al.*, 1967). The authors have concluded that color developer was the high R_f ultraviolet-absorbing compound and also gave the red color with Ehrlich's reagent.

An ultraviolet-absorbing compound also was present on chromatograms of the neutral plus basic amino acid fraction from garlic, but this compound did not give rise to color developer or to a pyrrole-containing compound when treated with the garlic enzyme preparation. However, when the garlic ultraviolet-absorbing compound was eluted from a strip chromatogram and incubated with a mixture of the onion neutral plus basic amino acids and the garlic enzyme, a blue pigment formed. Again, this indicates that an ultraviolet-absorbing amino acid from garlic may be the precursor of the garlic compound responsible for the formation of blue pigments discussed previously (Yamaguchi *et al.*, 1965; Shannon *et al.*, 1967). The garlic ultraviolet-absorbing amino acid overlapped but was not coincident with PCSO on paper chromatograms. However, it was coincident with ACSO, and the double bond in ACSO could be responsible for the observed ultraviolet absorption.

Table III. Chromatographic Comparison of Synthetic *S*-Propyl-L-cysteine Sulfoxide (PCSO) and Onion Amino Acids Following Treatment with Garlic Enzyme

Sample	Relative Intensity of Spot Tests: R_f 0.31[a] UV[b]	s[c]	n[d]	R_f 0.93[a] UV	s	n
Synthetic PCSO only	−	+	++	−	−	−
Synthetic PCSO with garlic enzyme	−	+	+	−	+	−
Onion neutral plus basic amino acids only with garlic enzyme	++	+	++	−	−	−
Onion neutral plus basic amino acids with garlic enzyme	+	+	+	+	+	−

[a] Solvent system: 1-butanol–acetic acid–water (4:1:5, v./v.).
[b] UV = 257-mμ ultraviolet absorption.
[c] s = Iodoplatinate test for sulfur compounds.
[d] n = Ninhydrin test for amino groups.

The identity of the precursor of color developer has not been established, but recent work by Spare and Virtanen (1963) indicates that PECSO travels very close to PCSO on paper chromatograms. Cycloalliin (Virtanen, 1962) is reported to have a higher R_f than PCSO; no such spots were observed on the chromatograms. Since PECSO is unsaturated, it should absorb ultraviolet radiation. PECSO also is a substrate of alliinase, and the products formed are quite unstable. One can assume that a propenyl thiosulfinate would be formed. This compound could be the color developer with a high R_f value and a UV light-absorbing property.

Discussion

Based on the evidence presented, the formation of pink pigments in onion purees can be postulated in the following series of reactions:

$$\text{Unknown } X + \text{alliinase} \xrightarrow[\text{fast}]{\text{very}} \text{color developer}$$

$$\text{Color developer} + \text{free amino acids} \xrightarrow[\text{slow}]{\text{very}} \text{pigment precursor}$$

$$\text{Pigment precursor} + \text{carbonyls} \xrightarrow{\text{slow}} \text{pink pigment}$$

Although conclusive evidence was not obtained, the unknown *X* is presumed to be *S*-1-propenylcysteine sulfoxide.

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