ENZYMIC NATURE OF THE CAROTENE-DESTROYING SYSTEM OF ALFALFA*

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Rapid destruction of carotene occurs in alfalfa during the field curing process. The loss under good curing conditions may be 45 to 80 per cent of the carotene originally present (1–4). Hauge and Aitkenhead (5) and Hauge (6) presented evidence which indicates the presence of a carotene-destroying enzyme system in alfalfa. The belief that the destruction of carotene during field curing may be largely enzymic in character is supported by later investigations (7–10). This view has been based chiefly on the observation that autoclaving or blanching of alfalfa prevents the destruction.

The present investigation was conducted to determine the time-temperature relationships involved in the inactivation of the carotene-destroying system by heat, and to obtain additional data concerning the enzymic nature of the system.

EXPERIMENTAL

To insure a series of samples that would be uniform with respect to maturity and conditions of growth, fresh field-grown alfalfa was brought to the laboratory, the leaves were removed, thoroughly mixed, and 5 gm. portions of the leaf tissue were placed in 1 ounce bottles. The bottles were stoppered and stored at −15° until used. Dry matter was determined at the time of storage.

Inactivation of System by Heat Treatment of Plant Tissue—5 gm. portions of frozen tissue were spread on a paper and defrosted at room temperature for 7 minutes. Each defrosted sample was placed in a 1 inch cube made of wire screen. This was dipped in water at a definite temperature for a definite period of time, removed, and cooled by plunging into cold water. Each sample was transferred to a bottle, stoppered, and incubated at 37° for 24 hours. Carotene was determined by a modification of the method of Moore and Ely (9). Measurements were made with a Beckman spectrophotometer at 4360 A.

The results of this experiment are presented in Figs. 1 and 2, from which it is seen that heat treatments prior to incubation greatly affect the preser-

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**Fig. 1.** The relation of heat treatment to enzyme inactivation as measured by the preservation of carotene in alfalfa leaves (initial value, 413 γ per gm. of dry matter).

**Fig. 2.** Rate of inactivation of the enzymes in alfalfa leaves at different temperatures (initial value, 413 γ per gm. of dry matter).
vation of carotene. As seen in Fig. 1, the maximum inactivation of the
carotene-destroying system at a given temperature was obtained in 2
minutes, since heating the tissue for 4 minutes did not result in greater
preservation of carotene. The amount of inactivation increased rapidly
between 65-80°.

From Fig. 2 it is apparent that inactivation is a function of both tem-
perature and time. The carotene content of the leaves before heat treat-
ment was 413 γ per gm. of dry matter. 89 per cent of the carotene was
retained after the leaves were heated at 100° for 3 seconds and subsequently
incubated, while 93 per cent was retained after treatment at 90-100° for
10 seconds. Only 55 per cent was retained after heat treatment at 70°
for 10 seconds, followed by incubation.

Inactivation of System by Heat Treatment of Plant Extracts—Waugh et al.
(9) showed that aqueous extracts of alfalfa destroyed carotene when used
according to the method of Reiser and Fraps (11) for measuring the lip-
oxidase activity of legume seeds. The effect of heat on the activity of an
alfalfa extract was studied by this technique.

An aqueous extract was prepared with a Waring blender by dispersing
5 gm. of whole alfalfa tissue grown in the greenhouse in 200 ml. of water.
Portions of the extract were heated in a water bath for 5 minutes at various
temperatures. The carotene-destroying activity of each heated extract was
measured by an adaptation of the method of Reiser and Fraps.

Into a 25 x 250 mm. tube were pipetted 2 ml. of the alfalfa extract, 2
ml. of phosphate buffer of pH 6.5, and 2 ml. of water. The contents of the
tube were mixed and 1 ml. of a carotene solution was added. The carotene
solution contained 90 γ of carotene and 0.6 mg. of Wesson oil per ml. of
acetone. The tube was placed in an incubator at 37° for 1 hour. 50
ml. of heptane (petroleum ether, b.p. 90-100°) were pipetted into the tube.
About 15 ml. of 95 per cent ethanol were added to facilitate extraction of
the carotene from the aqueous phase by the heptane. The tube was
stoppered, shaken vigorously, and the contents were transferred to a
separatory funnel. Water was added to facilitate the separation of the
heptane extract, after which the aqueous phase was drawn off. The
heptane solution was shaken with 10 to 15 ml. of a 25 per cent solution of
KOH in methanol to remove chlorophyll. The heptane was washed with
water until free from alkali and was dried with anhydrous Na₂SO₄. Caro-
tene was measured with a Beckman spectrophotometer, as previously
described. A blank determination was made by substituting an equal
volume of water for the alfalfa extract. Carotene-destroying activity
was expressed as per cent destruction of the carotene.

The results of this experiment, presented in Fig. 3, show that aqueous
extracts of alfalfa contain a carotene-destroying system which is inactivated
by heat. Maximum inactivation was obtained at about 70°, somewhat
lower than the temperature of maximum inactivation of the system in the intact leaf.

Since inactivation by heat is one of the characteristics of enzyme systems, the heat lability of the carotene-destroying system strongly suggests the enzymic nature of this system.

_Destruction of Carotene in Blanched Alfalfa Leaves by Added Extracts_—If the material which is water-soluble and which shows activity by the Reiser-Fraps method is the same material that causes destruction of carotene in alfalfa, it should cause destruction of carotene when added to alfalfa leaves in which the carotene-destroying system has been inactivated by heat.

Alfalfa juice was obtained by defrosting frozen alfalfa leaves and expelling the juice in a hydraulic press. 30 ml. of the juice were blended with 5 gm. of blanched alfalfa leaves in a small Waring blender cup. The mixture was incubated for 8 hours at 37°. A control was prepared by blending 30 ml. of water with blanched leaves and incubating for 8 hours at 37°. Carotene was determined as previously indicated.

Since a lipoxidase has been reported by Sumner (12) to be present in alfalfa, and since soy bean lipoxidase is known to bleach carotene solutions if unsaturated fat is present (13), an experiment was conducted to determine the effect of soy bean lipoxidase upon blanched alfalfa leaves. 0.1 gm. of defatted soy bean meal was ground with 30 ml. of H₂O in a mortar. The
resulting suspension was added to 5 gm. of blanched leaves in a small Waring blendor cup and treated as described above.

The results of these experiments, as presented in Table I, show that both alfalfa juice and soy bean lipoxidase are capable of destroying carotene in blanched leaves. It can therefore be concluded that the carotene-destroying system of the leaf is soluble in water and that its solution can destroy carotene in comminuted plant tissue as well as in carotene solutions.

Agents Affecting Activity of Aqueous Extracts—Although the preceding experiment indicates that destruction of carotene in alfalfa may be due to a lipoxidase, it is possible that other substances may be present in the extract which are responsible for the destruction. A series of experiments was conducted to elucidate the enzymic nature of the system further.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Carotene</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Loss</td>
</tr>
<tr>
<td></td>
<td>γ per gm. dry weight</td>
</tr>
<tr>
<td>Initial (no incubation)</td>
<td>512</td>
</tr>
<tr>
<td>Blanched leaves, H₂O</td>
<td>482</td>
</tr>
<tr>
<td>&quot;           alfalfa juice</td>
<td>289</td>
</tr>
<tr>
<td>&quot;           soy bean lipoxidase</td>
<td>317</td>
</tr>
</tbody>
</table>

An extract was prepared by dispersing 5 gm. of defrosted alfalfa leaves in 200 ml. of water with a Waring blendor. The extract was filtered. 3 ml. aliquots were placed in 25 × 250 mm. tubes and the weight or volume of the various reagents as shown in Table II was added. The tubes were allowed to stand at room temperature for 1 hour, after which the carotene-destroying activity was measured both in the absence and in the presence of added oil by the adaptation of the method of Reiser and Fraps. The carotene solutions employed were: (1) 90 γ of carotene per ml. of acetone and (2) 90 γ of carotene and 0.6 mg. of Wesson oil per ml. of acetone.

In the final experiment of this series, 50 ml. of the extract were half saturated with (NH₄)₂SO₄ and centrifuged. Carotene-destroying activity was determined on 3 ml. of the supernatant liquid. The precipitate was dispersed in 50 ml. of the phosphate buffer (pH 6.5) and the carotene-destroying activity was determined on a 3 ml. aliquot. The results of these experiments are presented in Table II.

The protein coagulants, heat and ethanol, and the protein precipitants,
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CuSO₄ and Pb(OAc)₂, caused partial or complete loss of carotene-destroying activity. Likewise formaldehyde and pancreatin, which modify the structure of proteins, reduced the activity of the system. Inactivation by NaCN and thiourea, which is characteristic of many oxidizing enzymes, is in agreement with the observation of Silker et al. (10) that the addition of these substances to fresh ground alfalfa tends to preserve the carotene during dehydration. NaF, which is a strong inhibitor for hydrolytic enzymes, had relatively little effect on the system.

In contrast to CuSO₄ and Pb(OAc)₂, Na₂SO₄ and NaCl had no inhibitory action at the lower concentration studied. At the higher concentration these salts, along with (NH₄)₂SO₄, caused a slight reduction of activity.

The active principle can be salted-out by half saturating the extract with (NH₄)₂SO₄. The extract after salting-out had little activity.

The results of these experiments, together with the heat inactivation studies already discussed, clearly indicate the enzymic nature of the carotene-destroying system. A lipoxidase is shown to be present in the extract by the fact that greater destruction of carotene occurred when oil

<table>
<thead>
<tr>
<th>Treatment of extract</th>
<th>Carotene destroyed</th>
<th>Original activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg. per 3 ml.</td>
<td>No oil added</td>
</tr>
<tr>
<td>None</td>
<td>10</td>
<td>36.8</td>
</tr>
<tr>
<td>Na₂SO₄</td>
<td></td>
<td>38.8</td>
</tr>
<tr>
<td>NaCl</td>
<td></td>
<td>29.0</td>
</tr>
<tr>
<td>(NH₄)₂SO₄</td>
<td></td>
<td>38.3</td>
</tr>
<tr>
<td>NaF</td>
<td></td>
<td>26.3</td>
</tr>
<tr>
<td>NaCN</td>
<td></td>
<td>15.5</td>
</tr>
<tr>
<td>Thiourea</td>
<td></td>
<td>2.8</td>
</tr>
<tr>
<td>CuSO₄·5H₂O</td>
<td></td>
<td>11.0</td>
</tr>
<tr>
<td>Pb(OAc)₂</td>
<td></td>
<td>12.9</td>
</tr>
<tr>
<td>Pancreatin</td>
<td></td>
<td>5.0</td>
</tr>
<tr>
<td>Ethanol, 95%</td>
<td>(1 ml.)</td>
<td>11.2</td>
</tr>
<tr>
<td></td>
<td>(2 &quot; &quot; )</td>
<td>5.9</td>
</tr>
<tr>
<td>Formaldehyde, 5%</td>
<td>(1 &quot; &quot; )</td>
<td>15.6</td>
</tr>
<tr>
<td>(NH₄)₂SO₄, ppt.</td>
<td>Half saturation</td>
<td>42.1</td>
</tr>
<tr>
<td>filtrate</td>
<td></td>
<td>10.1</td>
</tr>
</tbody>
</table>
was added than when oil was not added. The destruction of carotene in the absence of added oil may also be due to lipoxidase activity if it is assumed that the small amount of oil which might have been dispersed in the extract from the leaves was sufficient for the reaction. If not, another enzyme is indicated. However, the effect of a given treatment in the presence and in the absence of added oil was similar when compared as percentage of the original activity. This suggests that the destruction of carotene is due to one enzyme.

**DISCUSSION**

The data on the relation of time and temperature to carotene preservation may be of importance to the engineer in the development of equipment for the production of alfalfa hay and leaf meal of high carotene content. To be of practical value, the heat treatment must be of short duration. This period of treatment may be less than 10 seconds at 90–100° if the heat transfer is efficient. However, preliminary experiments in this laboratory showed that inactivation could not be obtained by subjecting the plant tissue for short periods of time to a stream of air heated to 200°. The evaporation of water prevented the leaves from reaching the temperature necessary for inactivation of the enzyme. Therefore, in the design of equipment, consideration should be given to methods for the effective transfer of heat to the tissue.

**SUMMARY**

Evidence is presented for the enzymic nature of the carotene-destroying system of alfalfa. The enzyme involved appears to be a lipoxidase.

The enzyme was partially or completely inactivated by heat, ethanol, CuSO₄, Pb(OAc)₂, formaldehyde, pancreatin, NaCN, thiourea, and NaF, but not by Na₂SO₄ and NaCl. The enzyme was salted-out of solution by half saturation with (NH₄)₂SO₄.

The inactivation of the enzyme by heat was a function of both temperature and time. Maximum inactivation occurred in the plant tissue above 80° and in plant extracts above 70°. Virtually complete inactivation of the enzyme in the plant tissue was obtained at 90–100° in less than 10 seconds.

The practical application of this information is indicated.

**BIBLIOGRAPHY**

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