ENZYME ACTION AND OFF-FLAVOR IN FROZEN PEAS
II. THE USE OF ENZYMES PREPARED FROM GARDEN PEAS

FRANK A. LEE AND A. C. WAGENKNECHT
New York State Agricultural Experiment Station
Cornell University, Geneva, New York

(Manuscript received May 26, 1958)

Development of off-flavor in raw and underblanched vegetables during frozen storage seems to occur as a result of enzyme action (1). Use of model systems has shown that off-flavors can be produced during frozen storage by adding commercial enzymes employing macerates of enzymatically inert blanched peas (14). Other work of this laboratory (7, 8) showed that changes which take place during the frozen storage of unblanched peas are progressive in character.

Soybean lipoxidase caused deterioration of lipids and lowered flavor scores as determined by chemical analysis and taste panel evaluation, following prolonged storage at $-17.8^\circ$ C. Pancreatic lipase brought about chemical changes but off-flavors could not be determined with certainty because of flavors imparted to the pea slurry by the added enzyme itself. Addition of liver catalase and horseradish peroxidase to blanched peas produced only slight changes in quality during frozen storage.

Quality changes produced by these enzymes were of considerable interest, but it was thought that effects of enzymes from sources other than peas might not necessarily be the same as those produced by native pea enzymes.

This paper deals with the preparations of partially purified enzymes from peas and addition of these enzyme preparations to blanched pea slurries in model systems in order that their effects on quality might be studied following prolonged frozen storage.

MATERIALS AND METHODS

Perfected Freezer peas, grown on Experiment Station plots were harvested, processed, and stored at $-17.8^\circ$ C. (6). Blanched peas were thawed, blended with water, and mixed with enzyme preparations obtained from raw peas as described earlier (14). The ratio of blanched peas to water in the resultant slurry was 2:1. Two series of samples were prepared, one for taste panel evaluation, the other for chemical analysis. Samples of blanched pea slurry without added enzymes were included as controls in each series. A special lipase control consisting of pea catalase, pea lipoxidase, and pea peroxidase was prepared because it was impossible to separate these enzymes from the pea lipase by the procedures employed. Enzyme activity of the samples was checked prior to storage, and again after frozen storage when the residual enzyme activity was determined. Samples were stored at $-17.8$ C. in cellophane bags inside waxed cartons.

Lipoxidase was prepared as follows: Lyophilized raw Perfected Freezer peas were ground to 40 mesh, mixed with 9 times their weight of cold distilled water, stirred for one hour on an ice bath, and centrifuged one-half hour at 17,000 x g. The supernatant liquid was decanted and dialyzed overnight against cold running tap water ($6^\circ$ C.). The dialyzed liquid was taken to 30% saturation at $4^\circ$ C. with solid ammonium sulfate, allowed to stand one hour, and centrifuged as before. The supernatant liquid was then taken to 60% saturation at $4^\circ$ C. with ammonium sulfate, allowed to stand one hour, and centrifuged as before. The precipitate was worked up with a small volume of cold distilled water and

* Approved by the Director of the New York State Agricultural Experiment Station for publication as Journal Paper No. 1118.
dialyzed overnight against cold tap water. The dialyzed material was heated rapidly to 62°C, held at 62°C for 5 minutes, quickly cooled to 6°C on an ice bath, and centrifuged as above. The supernatant liquid was shell-frozen in a dry ice-methyl cellosolve bath and lyophilized. Lipoxidase activity was measured at all stages of the purification procedure by manometric assay (11,13). From 100 g. of lyophilized raw peas, 1.17 g. of lyophilized lipoxidase preparation was obtained having a \( Q_{O_2}^{10} \) of 117.5. This represented a 35-fold increase in lipoxidase activity over that of lyophilized raw peas, and a recovery of 49% of the total activity originally present in the starting material. The pea lipoxidase preparation was added at the level of 0.60 g. of lyophilized powder to 100 g. of blanched pea slurry. A one-gram sample of the resultant mixture caused the uptake of 346 \( \mu l \) \( O_2 \) in 30 minutes in the manometric assay (\( Q_{O_2}^{10} = 7.7 \)).

Peroxidase was prepared from lyophilized raw Perfected Freezer peas according to the method of Kenten and Mann (3). Lyophilized peas were rehydrated by vacuum infiltration with 7 times their weight of cold distilled water, blended for 2 minutes in a Waring blender and the aqueous portion expressed by squeezing through several layers of cheesecloth. The crude extract was then treated in the cold with ethanol-chloroform, ammonium sulfate, and dialyzed according to Kenten and Mann's procedure. Some inert protein was removed by rapidly heating the dialyzed preparation to 59°C, maintaining this temperature for 5 min., and quickly cooling on an ice bath to 17°C, followed by centrifugation for one-half hour at 17,000 \times g. The supernatant liquid thus obtained had a purpurogallin number (P.N.) of 7.0, which represented a 30-fold enrichment of peroxidase activity and a recovery of 37% of the total peroxidase activity initially present in the crude press juice. When 29.4 ml. of pea peroxidase preparation was added to blanched peas and water to make 100 g. of slurry, the resultant mixture had a P.N. of 0.138. Peroxidase activity of all fractions in the purification scheme and of the final mixture was determined by the colorimetric method of Kelin and Hartree (2).

Lipase and catalase were prepared from lyophilized raw Thomas Laxton peas by methods described elsewhere (5,16). The yield of pea lipase preparation was 7.25 g. of lyophilized material from 100 g. of lyophilized peas. This preparation had a \( Q_{CO_2}^{2} \) of 14.6, which represented a five-fold concentration of lipase activity over that of the lyophilized raw peas and a recovery of 41% of the total lipase activity of the starting material. Lipase was determined manometrically using tributyrin as substrate (9,14). Pea lipase preparation was added to blanched pea slurry at the level of 6.75 g. per 100 g. of slurry. A one-gram sample of the resultant mixture caused the liberation of 261 \( \mu l \) \( CO_2 \) in 30 minutes in the manometric assay (\( Q_{CO_2}^{2} = 2.3 \)).

Pea catalase was enriched to Kat. f. 420. It was added at the rate of 13.5 ml. in 100 g. of blanched pea slurry. A 0.1 g. sample of the resultant mixture produced 2.2 ml. \( O_2 \) in the Thompson test (12).

Determinations of acid numbers, peroxide numbers, and chlorophyll were made as reported earlier (4,6,15).

Organoleptic tests were carried out with a qualified panel of 7 members. Scores were based on a 1—10 scale. Ten was the highest value.

RESULTS AND DISCUSSION

Analytical results of this study are summarized in Table I. Samples of blanched peas to which native pea enzymes had been added were subjected to taste panel evaluation for flavor and color after 18 months of storage at —17.8°C. In each case evaluation was based on comparisons with the flavor and color of blanched peas with no added enzymes, which had been stored under similar conditions. As in the previous study (14) in which commercially obtained enzymes were employed, addition of enzymes to blanched peas produced off-flavors ranging in intensity from mild to extremely objectionable. Mild off-flavor was produced by added peroxidase. The more objectionable flavor changes were found in samples to which lipase, lipoxidase, and catalase had been added. The flavor produced in the added catalase was the most objectionable. A mixture containing lipoxidase, catalase, and peroxidase in the amounts present in the pea lipase preparation also produced an objectionable off-flavor when added to the blanched peas.
# TABLE 1

The effect of added pea enzymes on quality of bleached peas

<table>
<thead>
<tr>
<th>Treatment of sample</th>
<th>Acid No.</th>
<th>Peroxide No.</th>
<th>Residual enzyme activity</th>
<th>Percentage of original activity</th>
<th>Chlorophyll destroyed</th>
<th>Taste panel</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Lipase</td>
<td>Lipoxidase</td>
<td>Catalase</td>
<td>Peroxidase</td>
</tr>
<tr>
<td>Lipase</td>
<td>14.3</td>
<td>4.6</td>
<td>129</td>
<td>56</td>
<td>3.5</td>
<td>0.08</td>
</tr>
<tr>
<td>Lipase blank</td>
<td>4.4</td>
<td>1.1</td>
<td>2</td>
<td>0</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>Special lipase control</td>
<td>7.9</td>
<td>5.2</td>
<td>32</td>
<td>65</td>
<td>1.1</td>
<td>0.06</td>
</tr>
<tr>
<td>Lipoxidase</td>
<td>7.4</td>
<td>5.1</td>
<td>0</td>
<td>224</td>
<td>0</td>
<td>0.05</td>
</tr>
<tr>
<td>Lipoxidase blank</td>
<td>5.4</td>
<td>1.8</td>
<td>0</td>
<td>0</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>Special lipoxidase control</td>
<td>5.3</td>
<td>2.0</td>
<td>0</td>
<td>0</td>
<td>0.03</td>
<td>0.03</td>
</tr>
<tr>
<td>Catalase</td>
<td>10.8</td>
<td>4.9</td>
<td>0</td>
<td>0</td>
<td>2.0</td>
<td>0.01</td>
</tr>
<tr>
<td>Catalase blank</td>
<td>6.3</td>
<td>2.0</td>
<td>0</td>
<td>0</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>Peroxidase</td>
<td>6.8</td>
<td>1.1</td>
<td>0</td>
<td>4</td>
<td>0.05</td>
<td>0.13</td>
</tr>
<tr>
<td>Peroxidase blank</td>
<td>4.0</td>
<td>0.9</td>
<td>0</td>
<td>0</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>Original activity prior to frozen storage</td>
<td>261</td>
<td>173</td>
<td>2.2</td>
<td>0.14</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>
Changes in color brought about by the action of added pea enzymes were readily detected by the taste panel, and also evidenced by losses of chlorophyll measured spectrophotometrically. The greatest amount of chlorophyll destruction, 27.5%, was observed in the lipoxidase-treated sample.

Deterioration of lipids was evidenced by increases in titratable acidity and presence of organic peroxides in the ether extracts of lyophilized samples. Increases in acid numbers were quite meager when compared with the very large acid numbers caused by the addition of pancreatic lipase in the earlier experiments with commercial enzymes (14). Catalase-treated blanched peas showed an increase in acid number, as did the lipase-treated peas. Peroxide numbers were of the same general order of magnitude as had been obtained with the commercial enzymes (14). Positive peroxide numbers were obtained by treatment with all four pea enzymes, the lower values from samples treated with peroxidase and catalase, and higher values from samples treated with lipoxidase and lipase. It must be remembered that the lipase used was contaminated with lipoxidase.

Analyses of samples for residual enzyme activity following 18 months of frozen storage indicated that three of the enzymes, lipoxidase, catalase, and peroxidase, had maintained nearly full activity throughout the storage period. The lipase-treated sample contained less than one-half of its original lipase activity prior to storage.

Off-flavors produced in this study were generally similar to those occurring during the frozen storage of raw peas (6). All samples containing added enzymes were tasted prior to freezing. The enzyme preparation, per se, contributed nothing to the taste of the final products.

The color change brought about by lipoxidase action through conversion of chlorophyll to colorless products (14) was again demonstrable when lipoxidase-containing samples were mixed with linoleic acid during determination of residual lipoxidase activity. The pea tissue fragments of such samples showed a marked degree of bleaching when compared with blanched pea control samples.

The enzymatic purity of the various pea enzymes used in this study was ascertained. Pea lipoxidase was found to contain no lipase or catalase activity. The lipase preparation, on the other hand, was found to be a mixture of all 4 enzymes, possessing strong lipoxidase and catalase activity. The heat treatment step employed in preparing pea lipoxidase was found to cause nearly complete inactivation of pea lipase (16). The activity of pea lipase was found to be greatly diminished by such agents as ammonium sulfate and acetone (7). Inasmuch as the purification of the other 3 enzymes involved salt fractionation with ammonium sulfate, and in addition the use of a chloroform-ethanol mixture in preparation of peroxidase, it is logical to assume that only negligible amounts of lipase activity would have remained in the purified preparations of pea lipoxidase, catalase and peroxidase. Analyses of the various samples for residual lipase activity after prolonged frozen storage bore this out. Samples treated with the other 3 enzymes were found to be devoid of lipase activity, with the exception of the special lipase control sample which contained a mixture of lipoxidase, peroxidase, and catalase. Peroxidase activity following 18 months of frozen storage was present in all of the enzyme-treated samples and to a small extent in the blanched pea con-
Regeneration of peroxidase is not uncommon in vegetables held in frozen storage (14).

Of the 4 enzymes used, only pea lipase suffered any appreciable loss of activity during frozen storage, amounting to 52%. It was impossible to determine when this deterioration of lipase had occurred, but the fact that very little increase in acid number was observed in lipase-treated samples may be best explained by the lack of extensive lipase action during storage. In contrast the pancreatic lipase used in the previous study (14) retained 96% of its original activity during 17 months of frozen storage and produced very large increases in titratable acidity.

The finding of positive peroxide numbers, substantial losses of chlorophyll, and lowered flavor scores in lipoxidase-treated pea samples was taken as further substantiation of the role played by lipoxidase in production of off-flavors and other deteriorative quality changes during frozen storage (10,13,14).

The role of lipase is more difficult to assess, primarily because of the relative enzymatic impurity of the pea lipase preparation. Whereas the lipase-treated samples did show a moderate increase in acid number, the catalase-treated sample showed nearly as great an increase. Furthermore, as mentioned, the pea lipase preparation was a fairly good source of peroxidase and a good source of lipoxidase and catalase, both before and after frozen storage.

Treatment of blanched pea slurry with pea lipase must therefore be considered as the addition of a mixture of enzymes, the effect of which was to produce disagreeable off-flavors in the product after 18 months of storage at —17.8°C. The same was true of the special lipase control sample which was found to possess activity of all 4 enzymes after storage, and which also was given a low flavor score by the taste panel.

The action of pea catalase in producing off-flavor when added to blanched peas was certainly much more profound than the corresponding action of liver catalase had been in the previous study (14). The sample containing added pea catalase preparation possessed the most disagreeable off-flavors of any samples in this present study. Furthermore, this catalase preparation also resulted in increased acid number and peroxide number in the ether extracted lipids, following frozen storage. The catalase-treated sample was found to be devoid of any residual lipoxidase or lipase activity and to contain only a small amount of residual peroxidase activity after 18 months' frozen storage. Presence of organic peroxides may have been due to non-enzymatic catalysis (14) but the production of acid which also has been observed when blanched peas were treated with liver catalase, remains unexplained.

In this connection, however, it might be well to consider the work of Keilen and Hartree (3). These authors noted the formation of aldehyde and acid from a system containing xanthine oxidase, alcohol, and catalase. It is within the realm of possibility that a system similar to this could be responsible for the formation of the acid in the slurries containing added pea catalase.

These studies, in which both commercially available enzymes and pea enzymes have been added to blanched pea slurry in model systems, have involved the 4 enzymes which have been thought to be associated with quality changes during frozen storage. The four are lipoxidase, lipase, catalase, and peroxidase. Lowered flavor scores were obtained with all of these enzymes. The
greatest changes in flavor score were associated with the addition of soy-
bean lipoxidase as far as the commercial enzymes used were concerned, and
with preparations of lipoxidase, lipase, and catalase prepared from raw peas.
In the latter the catalase prepared from raw peas produced the most offensive
off-flavor. The results of enzymic action have been demonstrated in chemical
analyses for increases in titratable acidity, development of organic peroxides,
and destruction of chlorophyll, all of which are associated with deterioration
of quality in frozen peas.

Results of these studies do not, however, preclude the involvement of
other enzymes, as yet unidentified. These may be present in raw peas and
may retain their activity through the various fractionation procedures em-
ployed in the partial purification of the 4 above-named enzymes. It seems
likely that off-flavor production in frozen raw vegetables is quite complex in
nature and that the concerted action of several or many enzymes is involved.

SUMMARY

Lipoxidase, lipase, catalase, and peroxidase have been obtained in par-
tially purified form from lyophilized raw peas.

Adding these partially purified preparations of native pea enzymes to
blanched peas resulted in the production of off-flavors following prolonged
frozen storage. The greatest change in flavor score was associated with
added catalase, but lipoxidase and lipase produced pronounced off-flavor.
Peroxidase produced only a mild change in flavor which was not objection-
able.

Changes in green color were brought about by adding all four enzymes.
The greatest lowering of visual color scores was produced by lipoxidase
and lipase.

Deterioration of lipids was evidenced by formation of peroxides and mod-
erate increases in acid number.

LITERATURE CITED

1. Joslyn, M. A. Enzyme activity in frozen vegetable tissue. Advances in Enzymology,
9, 613 (1949).
2. Keilin, D., and Hartree, E. F. Purification of horseradish peroxidase and comparison
of its properties with those of catalase and methaemoglobin. Biochem. J., 49, 88
(1951).
(London) B. 119, 141 (1936).
4. Kenten, R. H., and Mann, P. J. G. A simple method for the preparation of horse-
5. Lee, F. A. Chemical changes taking place in the crude lipids during the storage of
frozen raw vegetables. Food Research, 19, 515 (1954).
tion).
on the development of off-flavor in frozen raw peas. Food Research, 21, 666
(1956).
progressive development of off-flavor in frozen raw vegetables. Food Research,
20, 289 (1955).