

DETECTION, ACTIVITY AND RESISTANCE TO THERMAL INACTIVATION OF
PEROXIDASE IN THE BLUE CRAB, (CALLINECTES SAPIDUS)

by

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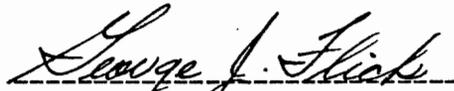
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INTRODUCTION

Peroxidase is a member of a large group of enzymes called the oxidoreductases and is considered to have an empirical relationship to off-flavors and off-colors in raw and unblanched vegetables (Joslyn and Bedford, 1940; Joslyn, 1949; Joslyn, 1966; Bedford and Joslyn, 1939; Weaver and Hautala, 1970). The enzyme is an iron-porphyrin organic catalyst which occurs naturally in nearly all plants and animals and recently its presence in microorganisms has been suggested by Reed (1975). Theorell (1940) was one of the first to crystallize peroxidase. He was able to separate horseradish peroxidase into two enzymes, called peroxidase 1 and peroxidase 2. Sumner and Somers (1947) stated that there was some doubt in the 1930's as to the occurrence of peroxidase in animal tissue, however, this doubt was dispelled by the separation and isolation of peroxidase from leukocytes by Agner (1943).

Peroxidase is found primarily in the roots and sprouts of higher plants (Tauber, 1949). Reed (1975) stated that unlike many of the digestive enzymes, peroxidase is usually intracellular, as are the other oxidoreductases like polyphenyloxidase, lipoxygenase, ascorbic acid oxidase, glucose oxidase and many others. The work of Scandalios (1969) showed the enzyme is tissue- and organ-specific. The most documented sources of peroxidase in plants are horseradish roots and the sap of the fig tree (Sumner and Somers, 1947; Tauber, 1949; and Sumner and Howell, 1936).

Little work has been done with peroxidase in animal tissue and

seafood products, particularly in the blue crab, Callinectes sapidus. Loss of flavor and the development of off-flavors due to oxidative and enzymatic rancidity in crab meat has been a problem for many years. The use of antioxidants has not previously been proven very effective with seafood products (Sweet, 1973). Also, additives are usually ineffective in preventing enzyme-induced discoloration or off-flavors in foods (Furia, 1968). Therefore, a study of peroxidase was undertaken to determine if the enzyme was present, to obtain a better understanding of the chemical and physical properties of the enzyme in crab meat and to establish an optimum time-temperature relationship for pasteurization to help maintain the quality of crab meat during storage. Presently, crab meat is pasteurized at 185 F for 1 min and can be stored up to four months under refrigerated conditions (32-36 F). However, with an extended storage period, off-flavors may develop. By inactivating peroxidase in crab meat and preventing or retarding its regeneration, the shelflife of the product could possibly be extended or improved while maintaining good quality.

REVIEW OF LITERATURE

A. Classes of Peroxidase:

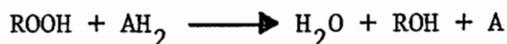
Reed (1975) reported that there are basically three classes of peroxidases: ferriprotoporphyrin peroxidases, verdoperoxidases, and flavoprotein peroxidases. The first group, ferriprotoporphyrin (hematin) peroxidase, is brown colored in nature and includes the peroxidases from higher plants (horseradish root, turnip root and radish root), animals (tryptophan pyrrolase and thyroid iodine peroxidase), and microorganisms (yeast cytochrome C peroxidase). The prosthetic group for the ferriprotoporphyrin peroxidases is ferriprotoporphyrin III.

The second group, verdoperoxidase, is green colored in nature and is considered very important in foods. It is primarily found in milk (lactoperoxidase) and in animal tissues, as reported by Karlson (1965). Reed (1975) reported the prosthetic group contains an iron porphyrin group other than ferriprotoporphyrin III, which in the past was known as green hematin (Karlson, 1965).

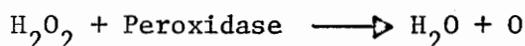
The third group, flavoprotein peroxidase, as described by Reed (1975) has been purified from several streptococci, such as Streptococcus faecalis, and from several animal tissues. The prosthetic group is flavin-adenine-dinucleotide (FAD).

B. Mechanism of Peroxidase:

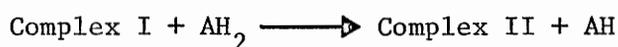
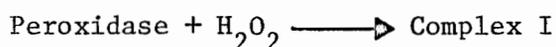
Reed (1975) defined peroxidase as an enzyme catalyzing the general reaction:



A simpler version was given by Dutcher et al. (1951):



Reed stated that his is the true peroxidatic reaction and generally the reaction considered of most importance. In his reaction, ROOH can be HOOH or some other organic peroxide. The hydrogen donor complexes and two univalent oxidation steps are involved. In the following equation, AH₂ stands for the hydrogen donor and A for the oxidized donor:



Studies show that the rate peroxidase activity is lost seems to follow first order kinetics (Lu and Whitaker, 1974). The last step in the above chain mechanism appears to be rate limiting (Reed, 1975).

Peroxidase is not specific in its action and catalyzes the oxidation of a large number of phenols and aromatic rings which occur naturally in plant tissue (Sumner and Somers, 1947; Danner et al., 1973). Dutcher et al. (1951) reported the products of peroxidase catalyzed reactions as H₂O plus an oxidized substrate. Lehninger (1975) stated that fatty acid peroxidase in germinating plant seeds reacts only with fatty acids which contain from 13 to 18 carbon atoms. The carboxyl carbon is lost as CO₂ and the α-carbon atom is oxidized to an aldehyde at the expense of H₂O₂, thus releasing H₂O. The H₂O₂ required is furnished by the direct oxidation of reduced flavoprotein by molecular oxygen. Since fatty acid peroxidase only attacks fatty acids of specific chain lengths, there can not be complete oxidation

of long chain fatty acids. Sullivan (1946) suggested that a possible metabolic role for peroxidase would be the oxidation of toxic components to less harmful products.

The hydrogen donor for peroxidase is not specific with respect to organic compounds (Reed, 1975). For some peroxidases the number of different hydrogen donors is small. For example, horseradish peroxidase uses only hydrogen peroxide, methyl-hydrogen peroxide and ethyl-hydrogen peroxide. Peroxidase also oxidizes iodides, setting free the iodine (Sumner and Somers, 1947). This principle was used by Murphy (1970) as a means of detecting peroxidase bands following gel electrophoresis. In the presence of starch, the iodine forms a deep blue-black complex, permitting the use of starch-iodide as a stain.

Animal peroxidase as reported by Price and Schweigert (1971) and milk peroxidase, which is less specific than plant peroxidase, as reported by Reed (1975) and Thurlow (1925), are capable of oxidizing nitrites and tryptophan. Nitric oxide is capable of forming complexes (Price and Schweigert, 1971) with peroxidase as well as with hemoglobin and myoglobin. Myoglobin is the major pigment in meats. Information is not available on the effect of peroxidase in meat curing. Reed (1975) further reported that milk peroxidase is also able to utilize persulfate, although plant peroxidase is unable to do so.

Since peroxidase acts upon many different substrates, numerous by-products are formed (Sumner and Somers, 1947). Some of the substrates and products are given in Table 1. A comparison of peroxidase specificity toward various substrates in different vegetables (Joslyn,

Table 1

Action of Peroxidase-H₂O₂

Substrate	Product
Pyrogallol	Purpurogallin
Guaiacol	Tetraguaiacoquinone
Hydroquinone	Quinhydorone
Benzidine	p-Quinone di-imide
o-Phenylene diamine	Phenazine
Leucomalachite green	Malachite green
Catechol	o-Quinone
p-Cresol	Milky precipitate
o-Cresol	Green solution
m-Cresol	Flesh-colored solution
Tyrosine	Yellow solution
Adrenaline	Reddish solution
Bilirubin	Biliverdin

1949) is given in Table 2.

C. Effects of Temperature upon Peroxidase Activity:

Peroxidase appears to be the most heat stable enzyme in plants (Reed, 1975; Sumner and Somers, 1947; Tauber, 1949; Zoueil and Esselen, 1959; Joslyn, 1949). Since peroxidase is very resistant to thermal inactivation, it is widely used as an index of blanching and other heat treatments. Reed (1975) stated that it has been generally accepted that if peroxidase is destroyed, then it is quite likely that all other enzyme systems will have been inactivated.

Peroxidase can regenerate in foods if the heat treatment is not sufficient to irreversibly denature the enzyme (Reed, 1975). Research by Schwimmer (1944) on the effect of time and temperature on turnip juice showed that the shorter the time of exposure to heat, the greater the portion of enzyme subsequently regenerated. Farkas et al. (1956) investigated an optimum high temperature short time (HTST) process for preventing enzyme regeneration. They reported 6 min at 121.1 C (250 F) is needed to inactivate peroxidase in green peas. This gave a "z" value (negative reciprocal slope of the logarithmic heat inactivation curve) of 48 F. Since a "z" of 18-20 F is commonly assumed in calculating processes for low acid foods, the heat resistance of peroxidase above 123.4 C (255 F) is an important quality consideration. Samples heated at 130 C (266 F) for 36 sec reduced the activity to 6% after one to two days storage. After five days of storage, the activity had increased to 10% of the original preparation.

Nebesky et al. (1950) and Zoueil and Esselen (1959) reported the

Table 2

Comparison of Peroxidase Specificity in Vegetables

Substrate	Specificity			Comparison of Peroxidase Indicators in Unblanched, Blanched, and Overblanched Tissues								
	Horseradish	Peas	Spinach	Asparagus			Peas			Spinach		
				U	100(2) ^a	100(5)	U	100(2)	100(5)	U	100(2)	100(5)
Compounds with a Free OH												
o-Cresol	5+	+	-	-	-	-	2+	-	-	-	-	-
m-Cresol	±	-	-	-	-	-	±	-	-	-	-	-
p-Cresol	-	-	-	-	-	-	-	-	-	-	-	-
Guaiacol	5+	5+	5+	+	-	-	+	+	-	5+	-	-
Compounds with a Free NH ₂												
Aniline	3+	-	-	-	-	-	±	-	-	-	-	-
o-Toluidine	3+	-	-	-	-	-	-	-	-	-	-	-
Compounds with 2 or More OH												
Catechol	5+	5+	5+	+	+	-	5+	3+	+	5+	-	-
Chlorogenic Acid	+	+	+	+	+	±	+	+	-	+	-	-
Gallic Acid	2+	4+	5+	5+	2+	-	5+	2+	±	5+	-	-
Guaiacum	3+	3+	4+	3+	+	-	3+	+	-	4+	-	-
Hydroquinone	4+	3+	3+	2+	2+	+	3+	3+	+	3+	±	-
Pyrogallol	5+	5+	5+	5+	3+	+	5+	3+	3+	5+	±	±
Compounds with 2 NH ₂ Groups												
Benzidine	+	+	2+	+	±	-	+	+	-	2+	-	-
o-Phenylenediamine	5+	3+	5+	+	+	+	3+	3+	+	5+	+	+

^a Indicates temperature of blanching water in C and time of blanching in min.

U=Unblanched.

destruction time for peroxidase in certain products varies according to the substrate used. For example, when using guaiacol as a substrate, peroxidase has a greater thermal resistance than peroxidase measured with other substrates. Consequently, it is extensively used as the preferable substrate in measuring peroxidase activity and destruction. It also produces more distinct color differences which are easily recognized and provide greater accuracy in conducting enzyme assays.

Peroxidase has distinctive absorption spectra due to the heme-prosthetic group (Lehninger, 1975). Peroxidase exhibits transient changes in spectra on mixing with hydrogen peroxide, its substrate, which reflect the formation and decomposition of its enzyme-substrate complex. Theorell (1940) stated that peroxidase has absorption bands at 640, 583, 548 and 498 nm. Peroxidase 1 absorbs at 583 and 548 nm while peroxidase 2 absorbs at 640 and 498 nm.

D. Effect of pH upon Peroxidase Activity:

Oxidative activity of peroxidase is affected by pH (Eriksson and Vallentin, 1973; Lu and Whitaker, 1974). Acidification of peroxidase causes a pronounced change in the protein from the native state to the reversible denatured state. A pH of 2.4 at 25 C with low chloride concentrations causes total detachment of the heme. Once the heme-protein interaction is disturbed, there is a loss of protein stability. A transfer of the protein from the reversible denatured state to the irreversible denatured state is influenced by the pH and protein concentration. Due to the presence of various isozymes, the less heat resistant peroxidases easily form aggregates with the

exposed heme-groups which are then able to catalyze lipid oxidation but unable to catalyze H_2O_2 breakdown. The more heat resistant peroxidases do not aggregate to the same extent and account for some residual peroxidase activity. It was therefore concluded that the increased lipid oxidative activity of peroxidase aggregates was due to increased heme exposure as a result of temperature and pH changes and to an increased number of active sites due to heme migration. Hayaishi (1974) stated that the heme groups in animal peroxidases are more tightly bound to the protein than are the hemes in plant peroxidases.

Peroxidase has various pH optima which are substrate dependent (Reed, 1975). Haard (1973) showed that peroxidase activity extracted at low ionic strength is pH invariant with ripening and senescence of banana fruit. Increasing the concentration of $CaCl_2$ from 0.18 to 1.4 M resulted in a gradual decline in peroxidase activity recovered in the supernatant fluid. Bruemmer et al. (1976) suggested that the loss of peroxidase activity in pasteurized orange juice is pH dependent.

E. Regeneration of Peroxidase:

Heat treatment applied to a particular food product for a longer time duration than required to process the food is needed to prevent the regeneration of peroxidase activity (Reed, 1975; Sumner and Somers, 1947; Joslyn, 1949; Tauber, 1949; Esselen and Anderson, 1956). Any regeneration which does occur is probably due to the enzyme not being completely or irreversibly inactivated by heat. Several factors

affecting the apparent regeneration of peroxidase activity are the method used for detecting the activity, the severity of the heat treatment combined with the time treated, and the temperature during storage of the inactivated enzyme prior to regeneration (Reed, 1975).

Woods (1901) reported the first observation of regeneration of peroxidase activity. He observed that the peroxidase activity of tobacco leaves reappeared some time after heat inactivation.

In 1924 Gallagher showed that peroxidase from the mangold root gave positive tests for aldehyde and iron. He observed that iron added to aqueous solutions of the aldehydes gave rise to peroxidase-like activity. Those results indicate that heating peroxidase produces a zymogen which is an aldehyde and a regeneration enzyme produced by catalysis with iron. This was later supported by Lehninger (1975).

Schwimmer (1944), working with turnip and cabbage juices, first separated peroxidase into two parts in aqueous solution. One part was a denatured protein which precipitated during centrifugation. The second was the hemin group that was originally attached to the protein and remained in solution. When the two parts were mixed under proper conditions, the protein reverted and recombined with the hemin group. The source of the protein used determined which peroxidase would be reformed. Both the precipitate formed on heating and the supernatant liquid contained factors essential for the reappearance of peroxidase.

Zoueil and Esselen (1959) reported that the longer the heat

treatment at a particular temperature the greater the denaturation of peroxidase, and the less likely it became for the undenatured enzyme to regenerate during the first few days of storage. Pinsent (1962) worked with peas that were blanched just sufficiently to inactivate the original peroxidase activity and reported that regeneration usually occurred in a few hours when the product was held at room temperature. It may, however, take several months if the product is stored at -18°C .

Sumner and Gjessing (1943) reported that peroxidase can be inactivated by an excess of H_2O_2 ; however, activity is restored if the level of H_2O_2 is neutralized with catalase. Peterson and Strong (1953) demonstrated that catalase activates H_2O_2 and decomposes it to water and oxygen. Other inhibitors as demonstrated by Sumner and Gjessing (1943) include hydrocyanic acid, hydrogen sulfide, sodium azide, nitric oxide, hydroxylamine and sodium dithionite. Sumner and Howell (1936) found that fig peroxidase is readily destroyed by acids and reducing agents. Thiourea is also an inhibitor and, therefore, is used to keep fruits and vegetables from darkening upon exposure to air.

F. Tests for Peroxidase Activity:

Sumner and Gjessing (1943) found peroxidase activity to be proportional to the amount of enzyme used provided excess substrate is present and the hydrogen peroxide concentration is low. In earlier methods as reported by Reed (1975), Maier et al. (1955), and Jacobs (1951), the activity was expressed in terms of Purpurogallin Zahl (P.Z.). This is the number of mg purpurogallin produced per mg enzyme

in five min at 20 C in a system containing 5 g pyrogallol and 50 mg H_2O in a total volume of 2 liters. This technique was based on the Willstatter Method (Willstatter and Stoll, 1918) and involves the conversion of pyrogallol to purpurogallin. One peroxidase unit as defined by Sumner and Gjessing (1943) is that amount of peroxidase that will form 100 mg purpurogallin.

Willstatter and Weber (1926) measured the color produced from pyrogallol colorimetrically as it is converted to purpurogallin. As described by Tauber (1949) this method is convenient for plant peroxidase estimation.

A modification of the Willstatter Method was devised by Sumner and Gjessing (1943). A small volume reaction mixture was used containing phosphate buffer and a relatively high concentration of H_2O_2 . After the addition of sulfuric acid to stop the enzyme action, the purpurogallin formed was extracted with ether, the solution filtered, and the absorbance of the ether solution spectrophotometrically determined.

Tests utilizing substrates other than purpurogallol have been studied. Joslyn (1955) used an ascorbic acid oxidation method for the determination of peroxidase activity in frozen vegetables. Wood and Lopez (1963) compared peroxidase activity in vegetables, particularly green beans, sweet green peas, broccoli and spinach, obtained using the indophenol and o-phenylenediamine methods. Ponting and Joslyn (1948) used guaiacol as the substrate for determining peroxidase activity in apple tissue and expressed the activity in $\Delta \log T$ units/min.

Masure and Campbell (1944) devised a test for the rapid estimation of peroxidase in vegetables using guaiacol as the substrate. This test has been widely used in industry and processing research for more than 30 years for determining adequacy of blanch and by the USDA Agricultural Marketing Service to establish grades in frozen vegetables.

G. Effects of Peroxidase in Food Processing:

Active enzyme systems can spoil fruits and vegetables at sub-zero temperatures; as low as -18 C as reported by Reed (1975) and Tauber (1949), and low moisture levels, as low as 12.5% water (Reed, 1975; Acker, 1962). The development of off-flavors in canned fruits and vegetables is associated with the regeneration of peroxidase activity (Nebesky et al., 1950; Esselen and Anderson, 1956; Guyer and Holmquist, 1954; Farkas et al., 1956). Tressler (1947) stated that apple, pear, plum, peach and apricot juice will turn brown due to the action of peroxidase, however, this is usually secondary in oxidative discoloration of fruit products (Balls and Hale, 1935; Ponting and Joslyn, 1948). Joslyn and Ponting (1951) showed polyphenolase was the main enzyme involved in browning of fruit.

Vegetables and some fruits are blanched during the process of canning and freezing (Tauber, 1949; Nebesky et al., 1950; Bruemmer et al., 1976; Esselen and Anderson, 1956; Bedford and Joslyn, 1939; Farkas et al., 1956; Wagenknecht and Lee, 1958; Lopez et al., 1959). If enzymes are not destroyed, their activity continues causing off-odors, off-flavors and color changes. These findings were supported

by Nebesky et al. (1950) using canned acid foods such as fruits and pickles, and the work of Esselen and Anderson (1956) on low acid foods. As Bruemmer et al. (1976) noted with orange juice, the significant negative correlation between peroxidase activities and flavor scores of high and low-yield orange juice suggests the high possibility of using peroxidase activity as an "index of adverse flavor of high yield juice."

Bedford and Joslyn (1939) studied the activity of catalase and peroxidase in string beans blanched under various conditions in relation to flavor retention, when stored at 17 C. The qualitative peroxidase test was found to be the best index of adequate blanching. Inactivation of peroxidase corresponded more closely to the inactivation of off-flavor producing agents than did catalase inactivation. Joslyn and Bedford (1940) using gum guaiac as the substrate found that the flavor retention in blanched asparagus is closely related to inactivation of peroxidase activity. Blanching in water for 4 min at 92 C or 3 min at 100 C was satisfactory for retention of flavor.

Campbell (1940) used the peroxidase test to determine the adequacy of blanching cut corn for freezing. The test consisted of adding 10 drops of 3% H_2O_2 and 10 drops of an alcoholic solution of gum guaiac to 5 cc of filtered extract. His results indicate that more peroxidase is inactivated when the blanching time is increased from 30 sec to 45 sec. These results can be seen in Table 3.

Contrary to the work of Campbell (1940), Wagenknecht and Lee

Table 3

Comparison of Blanching, Peroxidase Activity^a
and the Quality of Cut Corn
Held at -15 C for 11 Months

<u>Scalding Treatment</u>	<u>Peroxidase</u>	<u>Quality</u>
None	Strongly positive	Poor color; sour odor; bitter flavor
15 sec flowing steam	Strongly positive	Fair color; good odor; slight bitter flavor
30 sec flowing steam	Slightly positive	Good color; good odor; good flavor
45 sec flowing steam	Negative	Good in all respects
60 sec flowing steam	Negative	Good in all respects

^a Peroxidase activity was determined using gum guaiac as the substrate.

(1958) found that although there was regeneration of peroxidase activity in some samples of peas after prolonged storage following blanching, there was no detectable off-flavor. When pure horseradish peroxidase, in varying concentrations equaling 25% and 50% of the original amount in peas, was added to pea slurries, off-flavors developed.

The work of Bedford and Joslyn (1939) showed that complete inactivation of peroxidase is not necessary to prevent off-colors and off-flavors in foods even for storage of 3 to 7 years at 17 C, provided that most of the initial activity is destroyed. Cruess et al. (1944) reported that some residual peroxidase activity may be detected in dehydrated potatoes without the keeping quality being impaired. Kiermeyer (1951) demonstrated that peroxidase activity in kohlrabi is stimulated 8-fold by freezing. Maier et al. (1955) found that peroxidase was inactivated reversibly by freezing which he concluded was due to an increase in intramolecular hydrogen bonding.

Nebesky et al. (1950) reported that an increase in the concentration of peroxidase increased its resistance to heat inactivation. The varying concentration of peroxidase in a given product is due to the maturity, variety, rate of respiration and previous storage conditions of the product. The same authors showed that the addition of certain additives to foods affected the thermal stability of peroxidase. Sugar increased the resistance of apple and pear peroxidase to inactivation by heat, while vinegar decreased the resistance of

cucumber peroxidase to inactivation by heat. The addition of 2% salt had no effect on the thermal stability of cucumber peroxidase.

H. Peroxidase Activity Related to Microorganisms:

Certain microorganisms can cause an exogenous increase in peroxidase activity which has an effect on the quality of foods. Cherry et al. (1972) have indicated that peanuts which exhibit the growth of the weakly pathogenic and saprophytic organism Aspergillus parasiticus show a change in the "standard" peroxidase gel-electrophoretic patterns. Not much is reported as to what biochemical changes take place in peanuts due to the Aspergillus species, however, it is known that the saprophyte converts seed storage material to nutrients for its own development. In relation to this, there is a stimulation of peroxidase activity to oxidize organic substrates. Wood (1971) reported cucumber leaves show increased peroxidase activity in the presence of the W strain of cucumber mosaic virus.. Increased peroxidase activity can have a negative effect on food flavor and quality.

I. Isozymes:

Much of the difficulty in understanding peroxidase is due to the presence of multiple isozymes in various products. McCune (1961) obtained six peroxidase active fractions in corn leaf sheaths. Kon and Whitaker (1965) showed the presence of three different peroxidases in fig latex, which varied with respect to electrophoretic properties and heat stability. LaBerge et al. (1973) reported that the number of peroxidase isozymes detected in extracts of barley kernels was variable, depending on the hydrogen donors used in detecting the

isozymes. Zymogram patterns showed differences in electrophoretic mobility of peroxidase isozymes from different barley cultivars. The extracts from mature barley kernels contained 14 cationic peroxidase isozymes that could be separated by disc electrophoresis at acid pH.

Rucker and Radola (1971) reported the basic similarity of peroxidase isozyme patterns from different tissues and organs of tobacco tissue cultures using thin-layer isoelectric focusing. For some, large quantitative differences were observed, however, all isoenzymes were present in the enzyme patterns of all samples studied. Their results showed that all the peroxidases from different tobacco tissue cultures have a similar molecular size.

The work of Cherry and Ory (1973) with various peanut cultivars suggested that conditions in the mature peanut may be conducive to the synthesis and storage of isoperoxidases formed during earlier stages of development, and may vary with seeds of cultivars grown in different geographical regions. Also, at maturity, all seeds in a sample may not have reached the same level of development and isoperoxidase synthesis.

Gardner et al. (1969) showed that peroxidase activity varied with respect to anatomical parts in corn. Actual analysis of activity demonstrated that endoderm and pericarp had slight activity, the germ more, and the aleurone layer at least three times as much activity as germ. This supports the work of Joslyn and Bedford (1940) in which the peroxidase activity in asparagus was greater in the more

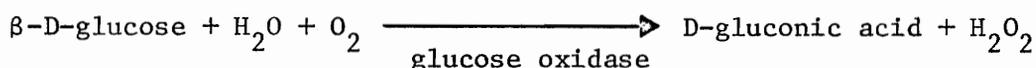
physiologically active tissue. In working with citrus fruits, Davis (1942) reported that the greatest peroxidase activity was in the inner seed coat.

The various isozymes of peroxidase have been shown by Maier et al. (1955) to have different heat constants. Yamamoto et al. (1962) in doing kinetic studies of peroxidase in corn found two enzymes or enzyme groups each having different heat stabilities. Kobrehel and Gautier (1974) reported that the variability in the isozymes in wheat has been related to genetic character. Scandalios (1969) reported developmental shifts in peroxidase isozymes during the course of differentiation of the maize sporophyte. The synthesis of specific peroxidase in a product appears to be genetically controlled through maturation.

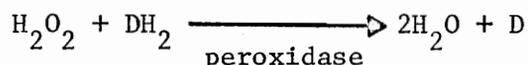
J. Peroxidase in Meats and Seafood:

In the past, many test methods used to determine the presence of peroxidase activity in skeletal muscles have failed (Lundquist and Josefsson, 1971) to detect peroxidases in these tissues. This would seem unusual since it has been shown that peroxidase activity is associated with hemoprotein (Uglow, 1969). Lundquist and Josefsson (1971) explained this phenomena by the fact that earlier methods for the quantitative determination of mammalian peroxidase were quite insensitive probably due to the high level of H_2O_2 used for analysis that seemed to inactivate peroxidase activity in tissues containing small amount of peroxidase. They therefore proposed a method by which the continuous supply of H_2O_2 formed during the glucose oxidase

reaction in the body could be used for peroxidase analysis:



H_2O_2 along with peroxidase would thus oxidize o-dianisidine, the hydrogen donor, to a brown-yellow dye (D):



The production of this brown-yellow dye would be proportional to the peroxidase activity present. This method was found to be 10 times more sensitive than any colorimetric method used for determination of low levels of tissue peroxidase.

Peroxidase tests have been used to some extent to determine if canned meat products have received a heat treatment sufficient to inactivate enzyme systems in the product (Livshits, 1968). One such method consisted of adding 10 drops of a 1% alkaline solution of guaiacium plus 5 drops of 1% H_2O_2 to 10 drops of a filtered aqueous extract which came from 10 g of meat in 20 ml of H_2O . If the test solution turned blue, it indicated that the product did not receive a sufficient heat treatment, otherwise no color changes would occur.

Peroxidase appears to be an inherent part of haemolymph in the blue crab. Uglow (1969) was able to show by electrophoresis and the addition of a 5% KCN solution that one fraction of haemolymph gave a positive peroxidase reaction with the o-dianisidine H_2O_2 staining system. This method as devised by Manwell and Baker (1963) has been proposed as a suitable method for the detection of hemocyanin.

Manwell and Baker (1963) showed how hemocyanins in the blue crab

(Callinectes sapidus), the burrowing sand crab (Emerita talpoida) and the fiddler crab (Uca pugilator) could be identified by using their tendency to peroxidize dianisidine and other dyes. By use of starch gel electrophoresis they were able to show how hemocyanin generally produced several zones. Hemocyanins can be classified as electrophoretically "fast" in that they yield broad diffuse bands, and the electrophoretically "slow" hemocyanins that form dark sharp bands. Most crustaceans studied were shown to possess at least one band of each.

Horn et al. (1969) showed that the "fast" hemocyanin of Manwell and Baker (1963) stained for protein, copper and peroxidase and the "slow" hemocyanin of Manwell and Baker (1963) is a copper-bearing protein which stained for peroxidase. Even when present in large quantities, "slow" hemocyanin was the slowest reacting of all hemocyanin subfractions. This particular protein was rarely found in male blue crabs. Only trace amounts were observed in the few males possessing this protein. On the otherhand, concentrations of "slow" hemocyanin was found in most, but not all, female blue crabs.

Horn et al. (1969) reported that the two hemocyanins, "fast" and "slow", are not interconvertible. Each protein maintained its identity during electrophoresis in acrylamide and starch gel electrophoresis in different buffers of varying pH. They further revealed that species specificity of C. sapidus is limited to the characteristic mobility of "fast" hemocyanin.

Ghiretti (1956) reported evidence for the ability of hemocyanin

to decompose H_2O_2 catalytically, however, he stated that this reaction was solely dependent on the Cu present and was in no way related to any catalase or peroxidase present. This is contrary to studies of Godbillon and Frenz (1972) where peroxidase activity was detected in the serum of Carcinus maenas. Two active fractions of peroxidase were found upon electrophoresis on cellulose acetate; hemocyanin and a glycoprotein fraction. The peroxidase activity could not be attributed to the presence of Cu in the molecule.

Lu and Whitaker (1974) showed that hematin added to horseradish peroxidase decreased the rate of peroxidase inactivation. One M hematin added to 0.1 M peroxidase was more rate limiting than 10 M hematin. Other experiments run with 5, 10, 50, 100, and 200-fold molar ratios of added hamatin to peroxidase gave unexplainable results. After heating for 3 min at 76 C, the enzyme solutions with 5 and 10-fold molar increases of hematin retained 1.7 and 1.2 times more activity than enzyme solutions without added hematin. But with 50, 100, and 200-fold molar increases of hematin, the retention of activity was the same as the control.

Lu and Whitaker (1974) also showed that the rate peroxidase activity was regained did not appear to be affected by added hematin or the extent of inactivation of the enzyme. There did, however, appear to be an effect of added hematin on the amount of activity regained. Also, following partial heat inactivation and incubation at 35 C, the enzyme activity was higher than the activity of the unheated sample.

Peroxidase, therefore, is present in the blue crab (Callinectes sapidus). Although no information is reported regarding the effect of peroxidase on the quality of blue crab meat, due to past reports concerning the deleterious effects of peroxidase on the quality of fruits and vegetables, peroxidase may be responsible for the development of off-flavors and off-colors in crab meat.

MATERIALS AND METHODS

A. Test Organism:

Live blue crabs (Callinectes sapidus) used in this study were supplied by Graham and Rollins, Inc., Hampton, Virginia. Pasteurized lump (backfin), flake and claw meat samples processed at P. K. Hunt and Son, Hampton, Virginia, were purchased from a seafood market also located in Hampton, Virginia.

B. Chemicals and Reagents:

All chemicals and reagents were purchased commercially at the highest purity available (National Formulary, United States Pharmacopeia or manufacturer's standards which equal or exceed these).

Horseradish peroxidase Type II, o-dianisidine (3-3'-dimethoxy benzidine; fast blue B base), potassium phosphate (monobasic), and glucose oxidase were obtained from Sigma Chemical Company, St. Louis, Missouri.

Hydrogen peroxide (30%), calcium chloride, ammonium chloride, dextrose (α -D-glucose), THAM (Tris (hydroxymethyl) aminomethane), glycine (aminoacetic acid), bromphenol blue, phosphoric acid (ortho 85%), acetic acid (99.7%), hydrogen chloride (36.5-38%) and sodium citrate were all obtained from Fisher Scientific Company, Fairlawn, New Jersey.

Methyl alcohol (anhydrous) and potassium iodide (free flowing) were obtained from Mallinckrodt Chemical Works, St. Louis, Missouri.

Ethanol (95%) was obtained from U. S. Industrial Chemical Company, Newark, New Jersey.

Triton x-100 (alkylaryl polyether alcohol) was obtained from J. T. Baker Chemical Company, Phillipsburg, New Jersey.

Acrylamide, bis-acrylamide (N,N'-methylene-bis-acrylamide), riboflavin, coomassie brilliant blue G-250 (xylene brilliant cyanine G; acid blue 90) and 2,3',6-trichloroindophenol (leuco-TIP) were obtained from Eastman Organic Chemicals, Rochester, New York.

TEMED (N,N,N',N'-tetramethylenediamine) and ammonium persulfate were obtained from Bio-Rad Laboratories, Richmond, California.

C. Sample Extraction

Ten g of meat were removed ("picked") from a crab, rapidly weighed, placed into a blender with 20 ml of cold triple distilled deionized water and homogenized for 30 sec at high speed. The homogenate was filtered through a Whatman No. 42 filter paper. Since only a crude extract was needed for the qualitative studies, this crude extraction was considered adequate.

D. Enzyme Assay:

A modified Nagle and Haard (1975) procedure was used to determine peroxidase activity in all samples. The substrate consisted of 0.1 ml of H_2O_2 (30%) in 100 ml of 0.01 M potassium phosphate (pH 6.0). A 1% (w/v) solution of o-dianisidine in methyl alcohol was used as the hydrogen donor during the enzymatic reactions since the oxidized dye is chromogenic. Consequently, the rate of the reaction could be spectrophotometrically determined by measuring the rate of oxidized dye formation at maximum wavelength absorption.

The enzyme assays were performed at 25 C in a Perkin-Elmer double

beam Coleman model 124 spectrophotometer equipped with a Perkin-Elmer model 56 recorder (Coleman Instruments Division, Maywood, Illinois). The total volume of reaction mixture in each cuvette was 3 ml (2.8 ml of 0.01 M phosphate buffer containing H_2O_2 , 0.1 ml of o-dianisidine solution and 0.1 ml of enzyme or extract). The reactions were initiated by the addition of 0.1 ml of the crude extract. The rates of the reactions were measured as the initial change in absorbance (1 cm light path) at a fixed wavelength of 460 nm per unit of time and expressed as $\Delta OD_{460}/min.$

Two other methods which have been proven sensitive to determine peroxidase activity were tested for their relative sensitivity to peroxidase in the blue crab. One of these was the method tested by Nickel and Cunningham (1969) using leuco 2,3',6-trichloroindophenol (leuco-TIP) which is oxidized by H_2O_2 in the presence of peroxidase. The rate of blue color formation is proportional to enzyme concentration. The other method was that tested by Lundquist and Josefsson (1971) using glucose oxidase to catalyze the reaction of α -D-glucose plus H_2O and O_2 to D-gluconic acid and H_2O_2 . The H_2O_2 formed would then react with peroxidase in the extract to oxidize o-dianisidine.

E. Influence of pH:

The influence of pH on enzymatic activity was determined by changing the pH of the phosphate buffer by the addition of 1.0 N HCL or 1.0 M NaOH and measuring $\Delta OD_{460}/min.$ Increments of 1.0 pH unit were tested over the pH range from 3 to 10.

F. Chemical Analysis:

The percent nitrogen of raw and pasteurized blue crab extracts was determined using the macro-Kjeldahl method (AOAC, 1975). Peroxidase activity could then be based on $\Delta OD_{460}/\text{min}/\text{mg N}$.

G. Gel-electrophoresis:

Polyacrylamide disc gel electrophoresis was performed by combining the procedures given by Nagle and Haard (1975) and Lee *et al.* (1976). The whole gel consisted of two portions; stacking gel (upper gel) and separating gel (lower gel). The solutions for both gels were all made to a final volume of 100 ml. For a run of 13 gels, the separating gel was made by mixing 3.0 ml of a solution A (30 g acrylamide plus 0.8 g bis-acrylamide), 3.0 ml of solution B (24 ml 1.0 N HCL, 18.15 g THAM (pH 8.9) and 0.4 ml TEMED) and 6.0 ml of solution C (1.0 mg riboflavin).

Following polymerization of the separating gel, the stacking gels of 1 cm in length were formed on top of the separating gels. The stacking gels were prepared by mixing 2.0 ml of solution D (5.0 g acrylamide plus 1.25 g bis-acrylamide), 1.0 ml solution E (12.8 ml 1.0 M H_3PO_4 , 2.85 g THAM and 0.1 ml TEMED) and 1.0 ml of solution F (2.0 mg riboflavin). After complete polymerization of the gels, they were placed in an electrophoresis unit and immersed in a bath buffer (pH 8.3) containing 0.025 M THAM and 0.192 M glycine. A predetermined amount of the prepared sample (0.1 ml) was applied to the top of each gel. Five milliliters of bromphenol blue (0.05%) was placed in the upper buffer to serve as a tracking dye. Electrophoresis was carried

out using a current of 1.0 mA per gel until the bromphenol blue marker dye passed through the stacking gel (about 1 hr). The current was then changed to 2.0 mA per gel until the marker dye was approximately 1.0 cm from the bottom of the gel (about 3 hr). After electrophoresis, the gels were removed from the tubes and stained overnight in test tubes at room temperature with an iodide stain (0.02 M potassium iodide, 0.125 M phosphate citrate buffer and 5×10^{-4} M H_2O_2) (Murphy, 1970). The gels were rinsed and placed in a destainer (Isco Model 422 rapid destainer tank) filled with 7% acetic acid and destained until distinct blue-black bands could be seen. After complete destaining, the gels were photographed and drawn.

H. Thermal Enzyme Inactivation Study:

Two milliliter samples of the crude crab homogenate were placed in 10 inch long $\frac{1}{4}$ inch diameter glass tubes and subjected to different heating temperatures (80, 110, 150 and 200 F) for varying time periods (1, 2, 8, 20 and 30 min) to determine percent enzyme activity remaining after heating. The tubes were placed in a hot oil bath (Haake Model FS-2). Following the desired heating time, the tubes were removed and transferred to an ice-water (32 F) in order to cool the homogenate. The homogenate was then centrifuged at 20,384 x g for 1.0 min and the supernatant fluid tested for enzyme activity.

I. Heat Penetration Curve:

One pound of commercially hand picked crab meat was placed in each of 6 cans (301 x 401) which contained one copper-constantan thermocouple inserted into the geometric center of the can (cold

point). The thermocouples were attached to a temperature recorder (Honeywell 24 point recorder) which monitored the temperature of each thermocouple every min. The cans were filled with the crab meat leaving 1/8 inch headspace and sealed with a commercial can seamer. The canned crab meat was then placed in a hot water bath at 195 F (Napco Model 220) until an internal can temperature of 185 F was obtained. The cans were removed and allowed to cool in an ice-water bath (32 F) until the internal can temperature was 60 F.

RESULTS

A. Comparative Sensitivity of Different Assays to Peroxidase:

The comparison of relative sensitivity of each assay procedure tested against a pure horseradish peroxidase standard is illustrated in Figure 1. The Nickel and Cunningham (1969) assay proved to be the most sensitive followed by the modified Nagle and Haard (1975) method. The method used by Lundquist and Josefsson (1971) was found to be least sensitive.

A comparison of the relative sensitivity of each assay tested toward peroxidase present in a mixed raw blue crab extract is given in Figure 2. The modified Nagle and Haard (1975) procedure proved the most sensitive. The Nickel and Cunningham (1969) procedure, even though it was very sensitive to a pure standard, exhibited essentially no change in optical density with the crude crab extract. The Lundquist and Josefsson (1971) procedure indicated only minor changes in optical density with the crude crab extract.

B. Influence of pH;

The percent peroxidase activity at different pH levels was based upon standard horseradish peroxidase equaling 100% activity at pH 4.0. As the pH of the phosphate buffer increased from pH of 4.0, the percent activity decreased until pH 9.0, then increased to pH 10.0 (Figure 3). Peroxidase activity, using the modified Nagle and Haard procedure, was measured at pH 6.0 which according to Figure 3 was not in the range of maximum activity. This was considered important since at maximum activity the reaction would be too fast to give reliable

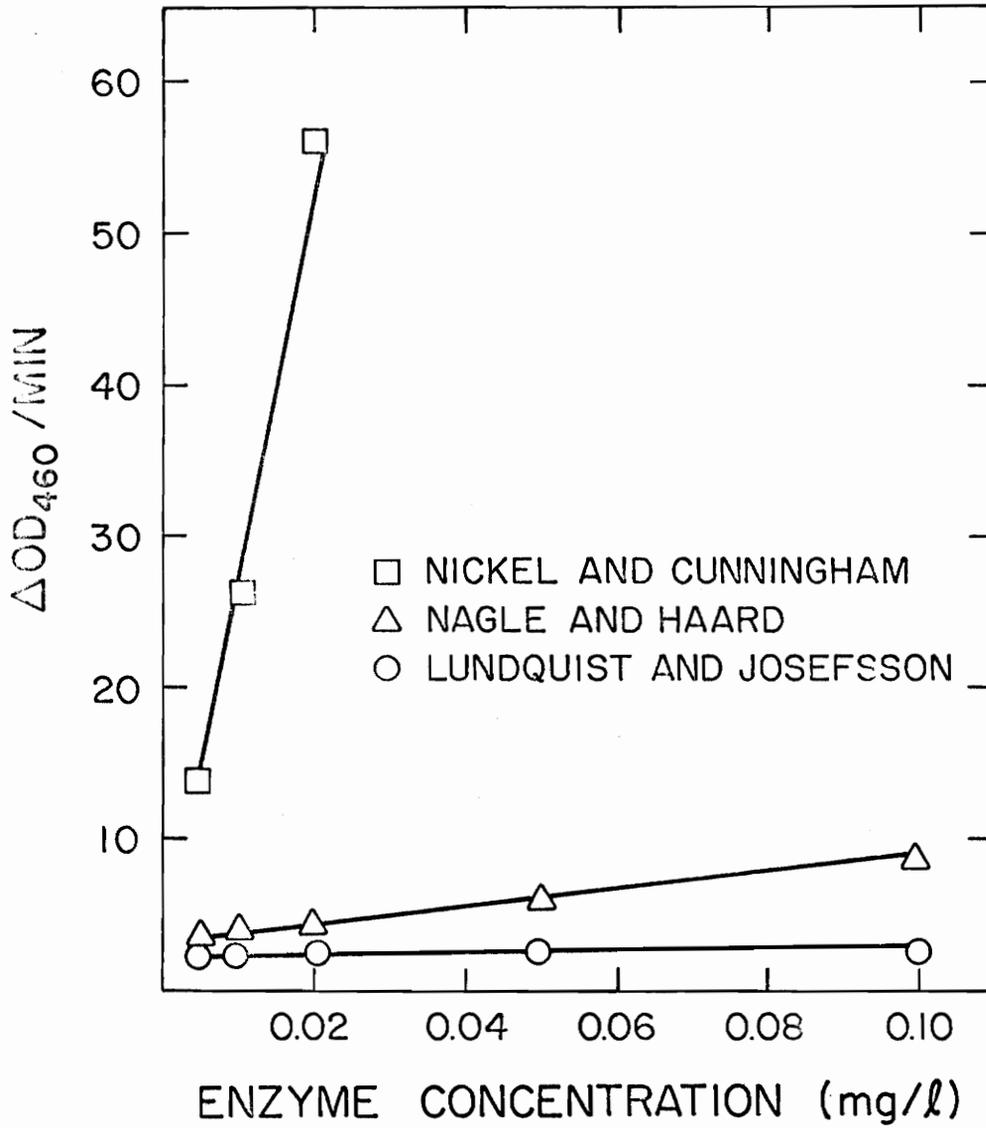


Figure 1: Comparative sensitivity of three assays for the determination of peroxidase activity in a pure horseradish peroxidase standard.

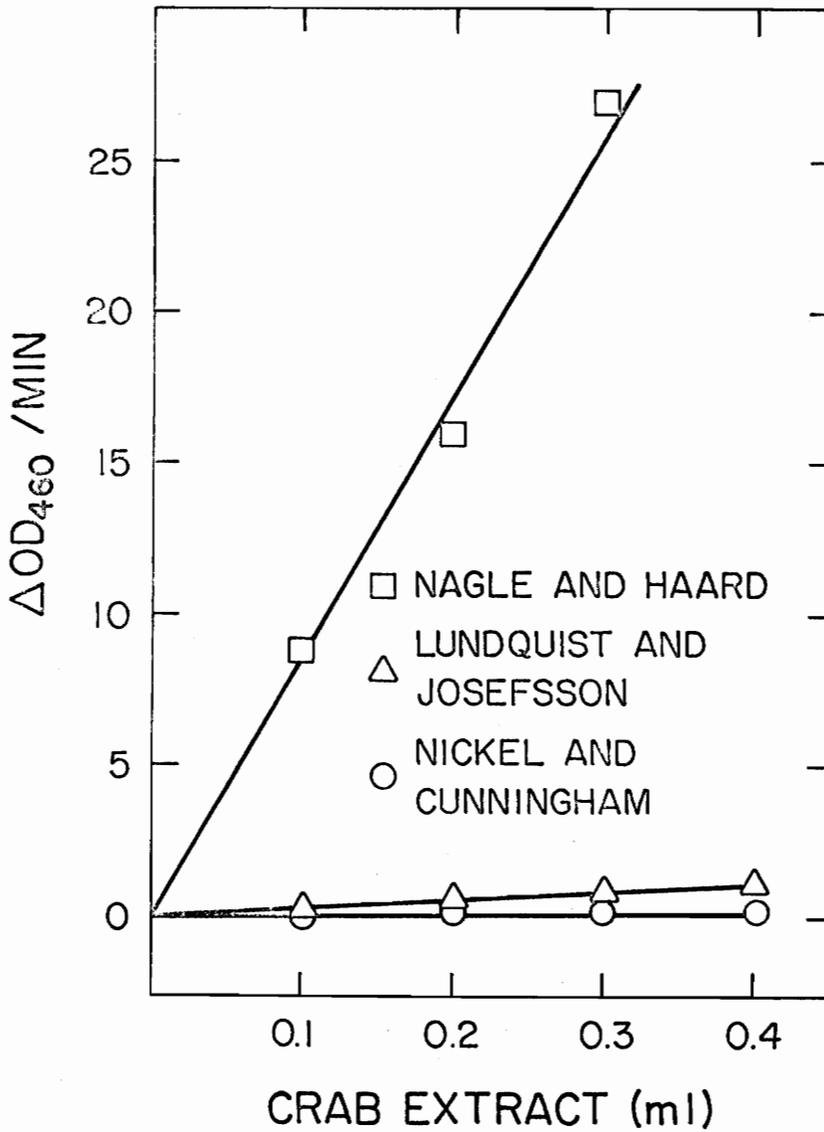


Figure 2: Comparative sensitivity of three assays for the determination of peroxidase activity in raw blue crab extracts.

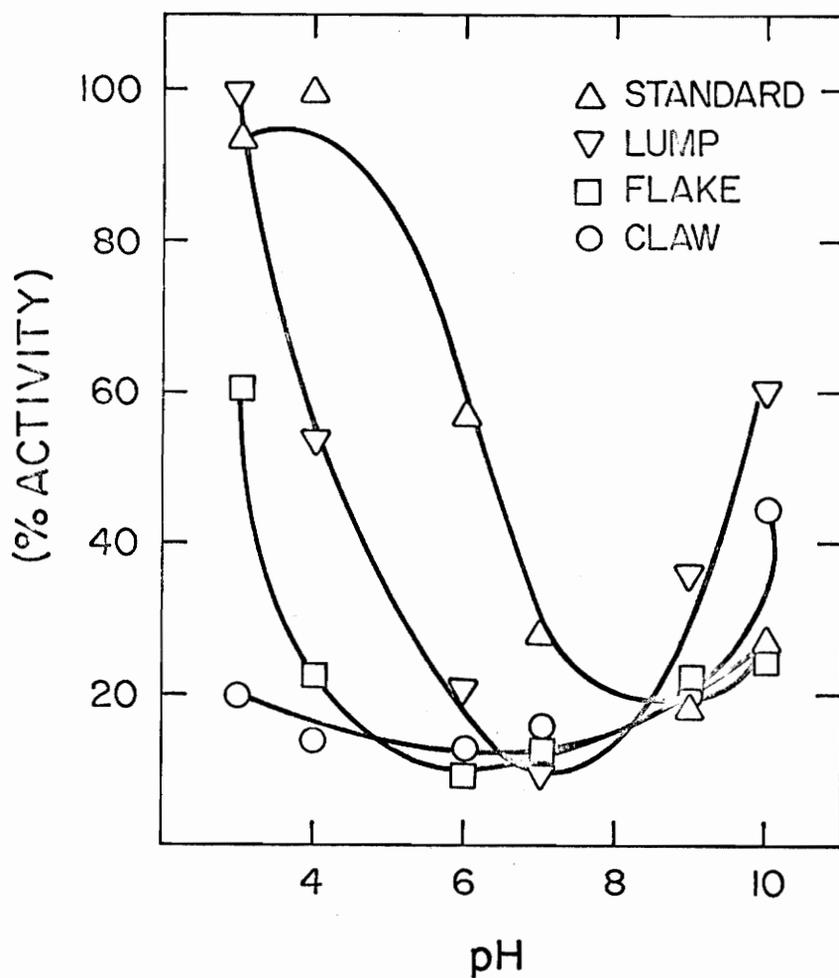


Figure 3: Percent peroxidase activity in a purified horseradish peroxidase standard and in extracts of lump, flake and claw meat from the blue crab at different pH intervals. The data is based on the percent activity of the purified standard at pH 4.0 equaling 100%.

data for initial activity.

Aqueous extracts of claw, flake and backfin meat extracted from raw blue crabs exhibited similar activity profiles. All fractions produced activity over a wide range of pH with the greatest activity at a pH less than 4.0 or greater than 7.0. The actual pH of raw crab meat determined immediately following extraction fell in a range from pH 6.0 to pH 7.0, therefore, the enzyme assays were performed at the pH of the extract itself.

C. Isozymes of Peroxidase:

Polyacrylamide disc gel electrophoresis is a widely used technique for protein studies (Zak and Keeney, 1974). Polyacrylamide starch gel electrophoresis, containing an iodide stain, was used to distinguish the different isozymes in the male and female blue crab (Figure 4).

Disc gels of the male crab revealed 9 bands and the female 8. The male crab used to prepare the gel shown in Figure 4 weighed 115 g, the female 116 g. The extra band shown in the gel containing the male crab extract was separated in the gel containing the female crab extract by extending the time of electrophoresis by 1 hr. The mobility of the bands from the male and female were similar. Staining near the gel origin, which is not indicated in Figure 4, was possibly due to enzyme bound to the precipitate which forms on the gel upon standing (Nagle and Haard, 1975). The starch iodide stain used was also tested with catalase to determine if catalase could oxidize iodide to iodine which in the presence of starch would form blue-black

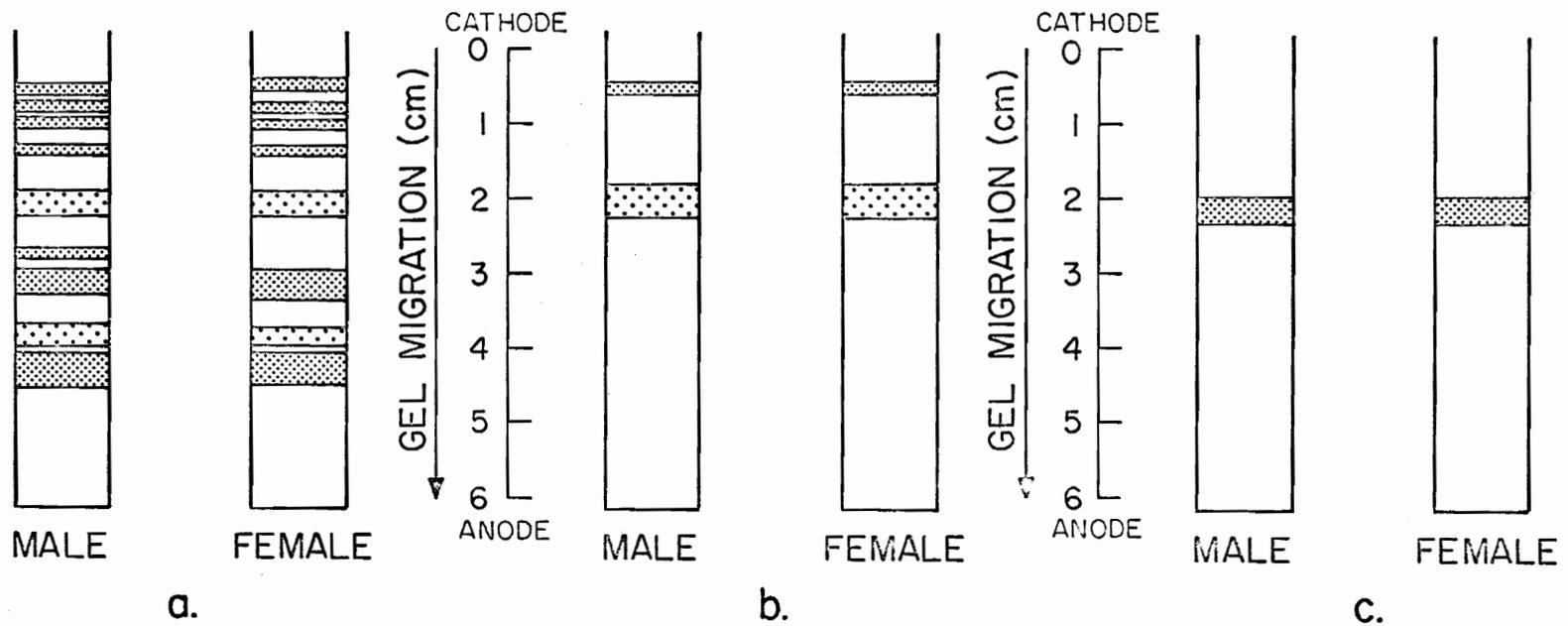


Figure 4: Diagrams of gel patterns for peroxidase in extracts of male and female blue crabs:
 (a): Raw extracts
 (b): Extracts cooked at 200 F for 4 min
 (c): Extracts cooked at 200 F for 30 min

bands. These results were negative.

Polyacrylamide starch gels were also used to determine if the weight of the crab was relative to the number of bands produced and to the size and intensity of these bands (Figure 5). Female crabs which weighed 155, 121 and 96 g were tested. Eight bands appeared on the gels containing an extract from the 155 g and the 121 g females, however, only seven bands appeared on the gel containing an extract from the 96 g female. The bands produced in the gel containing an extract from the 155 g female were more intense than those produced in the gels containing extracts from the 121g and the 96 g females, but not necessarily any larger.

D. Peroxidase Activity in Male and Female Blue Crabs:

A comparison of the initial peroxidase activity in male and female blue crabs is given in Table 4. Male crabs usually exhibited a greater initial activity ($\Delta OD_{460}/\text{min}$) than did females of equal size. The larger the crab for a given sex, the greater the initial activity. Size, however, is not directly proportional to weight. A 115 g male was 4 3/4 inches in length from point to point of the carapace, while a 116 g female was 5 1/4 inches.

E. Peroxidase Inactivation by Heat:

A comparison of the six gels shown in Figure 4 reveals that as the time of heating is increased at a specific temperature (200 F) there is a loss of isozyme bands and the remaining bands become less intense.

Data on the effects of time and temperature on the initial

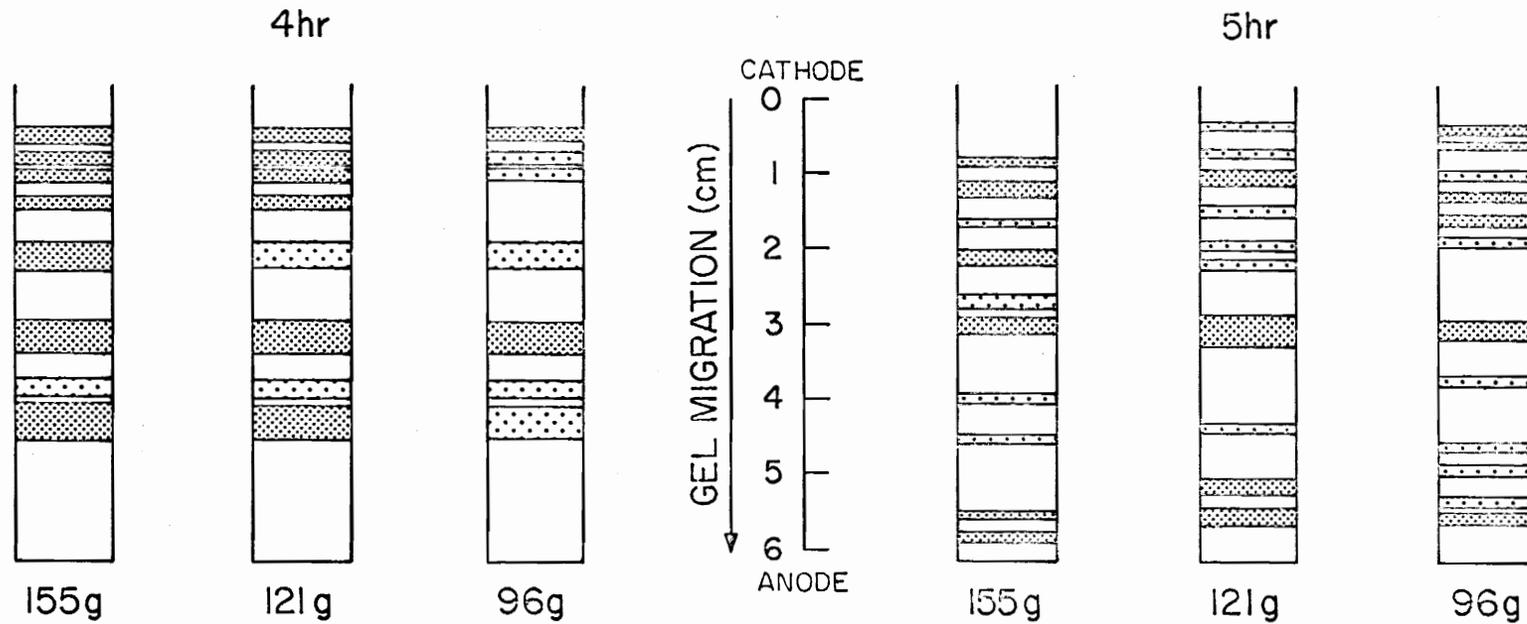


Figure 5: Diagrams of gel patterns for peroxidase in fresh extracts of female blue crabs of various weights (155, 121 and 96 g) following 4 and 5 hr of electrophoresis.

Table 4

Peroxidase Activity in Male and Female Blue Crabs

Sex	Crab Size ^a (inches)	Crab Weight (g)	$\Delta OD_{460}/\text{min}$
Male	5 1/2	145	11.2
Female	6	144	7.6
Male	5	125	6.8
Female	5 3/4	136	5.2

^a Crab measured from point to point of the carapace.

concentration of peroxidase where $\log C$ equals percent activity in blue crabs is given in Figure 6. There was an increase in percent activity after the initial drop in activity during the first heating stage for all temperatures studied. The percent activity continued to increase for 8 to 10 min, then it decreased. From these data, the following D values (time required for a 90% reduction in enzyme activity) were determined using the second decreasing line segment: $D_{80}=47$ min, $D_{110}=18.2$ min and $D_{150}=11.2$ min.

Figure 7 contains the relationship between temperature and $\log D$ values. A "z" value (number of degrees F on the temperature scale over which the thermal inactivation curve passes in transversing one log cycle on the time scale) of 92 F was obtained for the blue crab enzyme.

A heat penetration curve for canned blue crab meat is given in Figure 8. There was a 15 min time lag period during the initial heating of the product, then a relatively linear log increase in temperature occurred until the product reached an internal temperature at the can cold point of 185 F. The temperature of the water bath dropped during the first 15 minutes of the process to 187 F due to the dissipation of cooler temperatures in the water bath from the cans of crab meat which were at a temperature of 64 F. It then increased back to 195 F and remained at that temperature for the duration of the heating process (67 min). The decrease in water bath temperature was partially responsible for the time lag in heating the product.

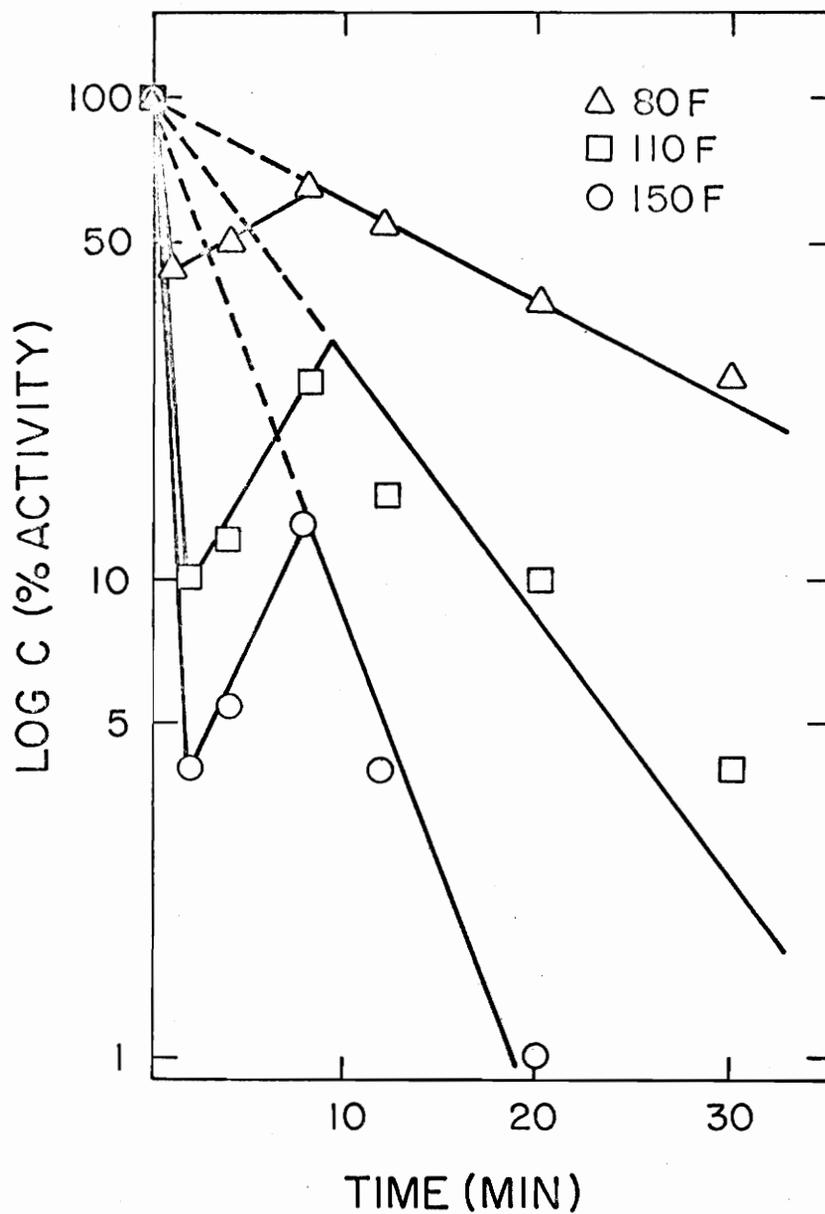


Figure 6: Percent peroxidase activity in raw backfin extracts following heat treatments at 80, 110 and 150 F for 2, 4, 8, 12, 20 and 30 min.

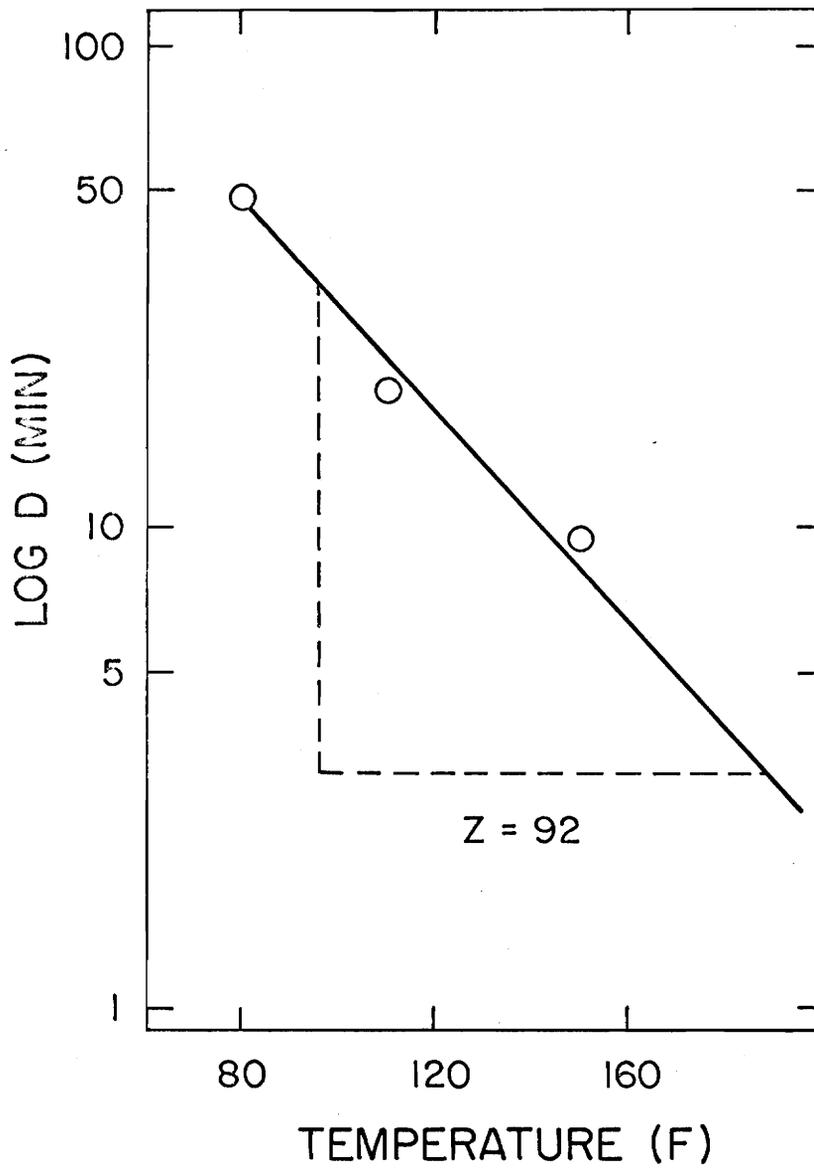


Figure 7: Relationship between log D values and temperature for raw blue crab peroxidase.

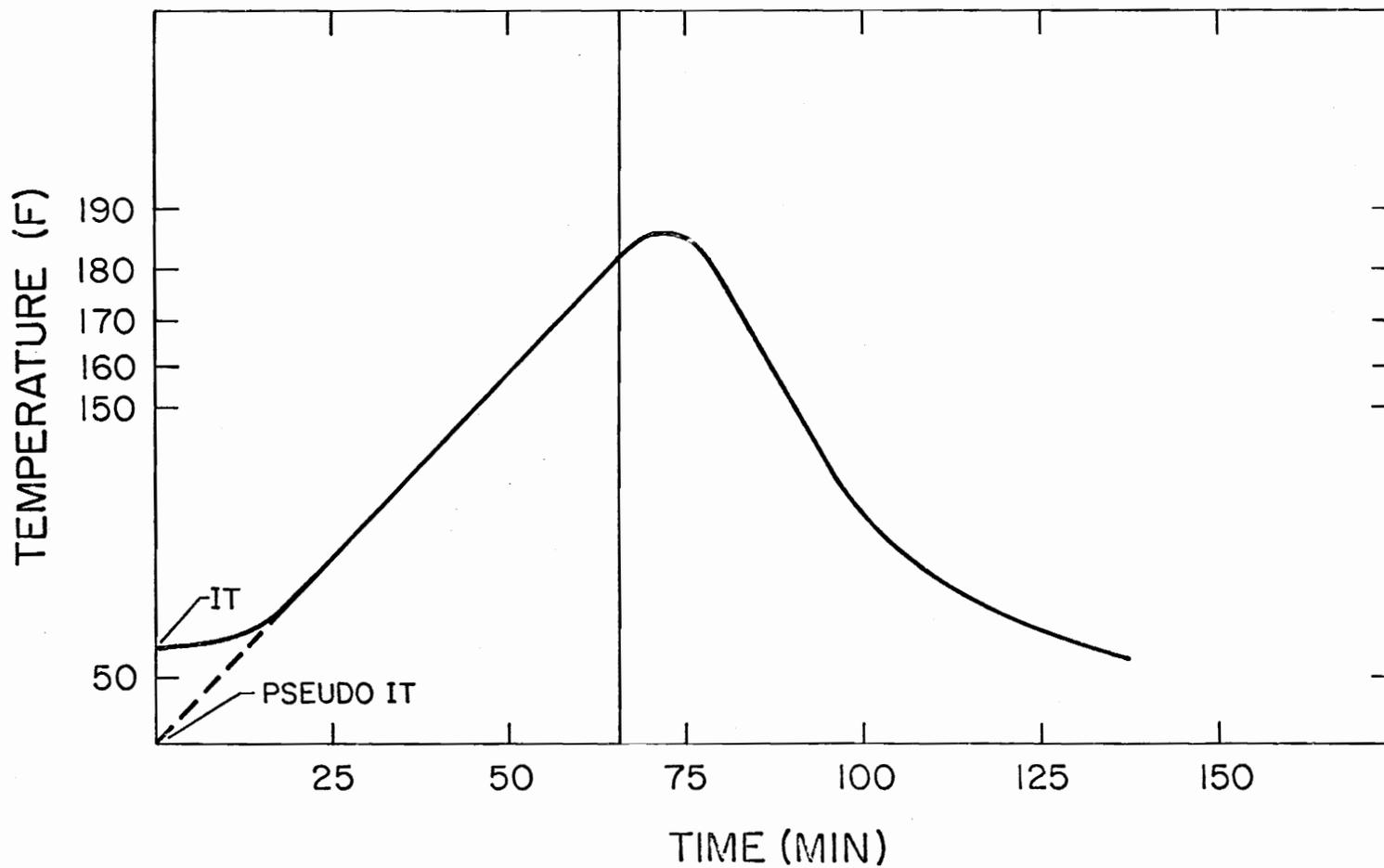


Figure 8: A heating and cooling penetration curve for canned blue crab meat (backfin) obtained from the geometric center of a 401 x 301 can during water bath heating at 195 F and cooling at 32 F. (IT represents initial temperature).

F. Regeneration:

The histogram in Figure 9 shows that after 2 weeks of storage the pasteurized backfin, flake and claw meat showed very similar peroxidase activity when compared to the raw backfin, flake and claw meat. Fresh pasteurized crab meat assayed immediately after heating had considerably less activity than either the raw crab meat or the pasteurized crab meat stored for 2 weeks. In all cases, the flake extract exhibited the most activity and the claw extract the least. Since the activity illustrated in the histogram is based per mg of nitrogen, the percent nitrogen in all samples is given (Table 5).

By basing percent activity of peroxidase on $\Delta OD_{460}/\text{min}/\text{mg N}$, the more nitrogen present, the less the $\Delta OD_{460}/\text{min}$. The percent extractable nitrogen was less for pasteurized flake and lump than for the raw samples due to coagulation of the soluble protein. Percent nitrogen in the pasteurized claw extract and the raw claw extract was the same. This could not be explained. The percent extractable nitrogen should be less for the pasteurized extracts than for the raw due to coagulation of soluble proteins during heating.

The $\Delta OD_{460}/\text{min}$ for pasteurized and raw samples of the blue crab are given in Table 6. The flake extract showed the greatest peroxidase activity and the claw extract the least.

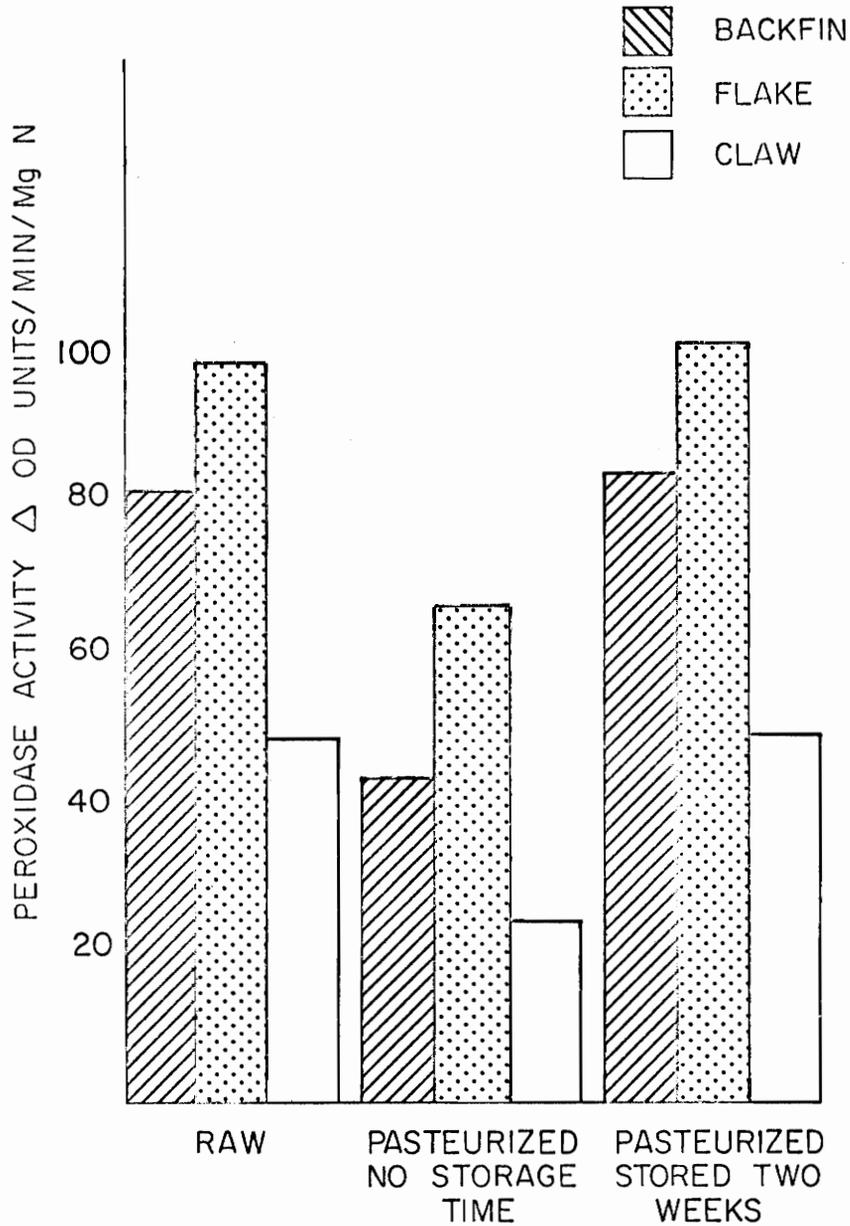


Figure 9: Comparison of peroxidase activity in various crab meat extract samples to show regeneration of the enzyme.

Table 5

Percent Nitrogen in Crab Meat Extracts			
	Pasteurized Stored 2 Weeks ^a	Pasteurized No Storage Time	Raw
Flake	0.30	0.29	0.34
Lump	0.26	0.26	0.38
Claw	0.39	0.39	0.39

^aProduct was stored under refrigerated conditions (1 C).

Table 6

$\Delta OD_{460}/\text{min}$ for Peroxidase Activity in
Crab Meat Extracts

	Pasteurized Stored 2 Weeks ^a	Pasteurized No Storage Time	Raw
Flake	3.27 ± 0.75	2.05 ± 0.34	3.70 ± 0.57
Lump	2.40 ± 0.52	1.30 ± 0.23	3.37 ± 0.55
Claw	2.10 ± 0.10	1.00 ± 0.18	2.07 ± 0.06

^a Product was stored under refrigerated conditions (1 C).

DISCUSSION

This research appears to be the first preliminary study of peroxidase in lump, flake and claw meat of the blue crab (Callinectes sapidus). The enzyme exists as several isozymes in the crab, therefore, the activity detected was a total of all the isozyme activity.

A modified Nagle and Haard (1975) method gave the best results when used to determine the initial activity present in each component of the crab (backfin, flake and claw) when compared to two other assays tested. The other assays studied were the Nickel and Cunningham (1969) assay and the Lundquist and Josefsson (1971) assay. These assays appeared to function best in detecting a pure enzyme. Nickel and Cunningham (1969) stated that the reactivity of leuco-TIP may be disadvantageous in assaying crude systems, however, it is useful in comparing relatively pure peroxidase systems. Results obtained in this study agree with the findings of Nickel and Cunningham (1969). According to Lundquist and Josefsson (1971), although their assay is very sensitive and good for detection of peroxidase in animal tissue, the enzyme must be highly purified for optimum results. Since the objective of this study was to examine the enzyme in crude extracts rather than to purify the enzyme, the Nagle and Haard (1975) procedure was used with modification.

The oxidation of o-dianisidine increased linearly with enzyme concentration (Kay et al., 1967). Oxidation was negligible when the enzyme or H_2O_2 were omitted. The initial rate in the linear portion of the reaction was used to determine the enzyme activity so the

effects of products formed from the enzyme substrate reactions would be miniscule.

Peroxidase activity in three types of market styles of crab meat was found to be active at the high and low ranges of the pH scale. The high relative percent activity at both ends of the pH range could be due to the denaturation of some proteins exposing heme groups (Eriksson and Vallentin, 1973; Lu and Whitaker, 1974) and to the release of NH_3 which will increase the rate of peroxidation of o-dianisidine (Fridovich, 1963). Weinryb (1966) indicated that the active site of peroxidase (using horseradish peroxidase) involved an apoprotein as well as a heme group.

Polyacrylamide starch gel electrophoresis performed with male and female blue crab extracts indicated 9 isozyme bands for the male and 8 for the female crab. The visualization of isoperoxidases in polyacrylamide gels is strongly influenced by substrate concentration used in the "staining procedure" (Novacky and Hampton, 1968). Using a relatively high substrate concentration may actually mask some of the sites of enzyme activity because of substrate or product inhibition. Since varying enzyme concentrations will also yield greater or fewer bands, there is a need to obtain an optimum crab extract concentration for peroxidase activity determination. More research is needed in purifying the enzyme concentration in crabs to determine the actual number of isozymes.

Polyacrylamide starch gel electrophoresis performed with female crabs which varied by weight indicated 8 bands on the gels containing

extracts from the 155 g and the 121 g females. The gel containing an extract from the 96 g female revealed only 7 bands. The mobility of the bands produced from the three female crab extracts varied, as did the intensity of the bands. The larger the crab, the more isozymes detected. Also, the greater the concentration of enzyme in the extract, the darker the bands produced in the gel. The spectrum of peroxidase isozymes revealed on starch gel and acrylamide gel electrophoresis has been shown to be relatively constant for a given species under specific conditions and is apparently related to age, species, variety, growth regulating substances and disease (Novacky and Hampton, 1968). Previous studies showed that in several cases isoperoxidases that were not detected in young healthy plant tissues were later detected in tissues upon aging or after infection. Such changes have been attributed to biosynthesis of new proteins (Novacky and Hampton, 1968). The "new" isoperoxidases may simply represent an increase in activity of isoperoxidases normally present at low activity or low concentrations in younger tissues.

Additional isozyme bands appeared on the gels prepared from extracts of the 155, 121 and 96 g female blue crabs by extending the time of electrophoresis 1 hr. Isozymes are separated on the basis of size, charge and molecular weight, therefore, any two or more isozymes which are quite similar in physical and chemical properties may appear as one band in the usual 4 hr electrophoresis period. When the current (2 mA/gel) is extended for an additional hour, the isozymes can be separated.

Male blue crabs generally showed greater peroxidase enzyme activity than females. Also, the larger the crab within a given sex, the more activity was detected. Size can be related to age, therefore, one can postulate that the older the crab, the more peroxidase activity will be present. These data support the work of Novacky and Hampton (1968).

Information as to relative activity in different sexes of the blue crab is not advantageous to the seafood processor at this time since no discrimination is made as to sex prior to picking or pasteurizing the crabs. These are basically informative data that could be of possible future significance.

During the heating of crab meat, certain isoenzyme bands are lost and those remaining are reduced in intensity. Maier et al. (1955) have shown that various isozymes of peroxidase have different heat constants. Heat will cause the denaturation of proteins and the longer the heat treatment at a particular temperature, the greater the loss in activity. This agrees with research data published by Zoueil and Esselen (1959).

There was a decrease in peroxidase activity for the first min of heating, then an increase in activity for the next 7-9 min for all temperatures included in this study, followed by another decrease in activity. Yamamoto et al. (1962) suggested that during heat inactivation of peroxidase in sweet corn, two independent first-order inactivation reactions took place. The initial inactivation line represented inactivation of the heat-labile enzyme and the second represented inactivation of the heat-stable enzyme. Another reason

suggested by Yamamoto et al. (1962) for the two reactions was that a protective enzyme-substrate complex was formed which affected the heat stability of a portion of the enzyme. The initial rapid inactivation represented the disappearance of the unprotected peroxidase.

The heat inactivation reactions for peroxidase in the blue crab appear to be time dependent rather than temperature dependent. The increase in activity after approximately 1 min could be due to an increase in NH_3 in the crabs which will increase the rate of oxidation of o-dianisidine (Fridovich, 1963). A high level of ammonia in the blue crab is not surprising since the end product of purine metabolism in fish and many invertebrates is allantoin which is further broken down to ammonia (Lehninger, 1975). Free pyrimidines are finally degraded to urea and ammonia in most species of aquatic animals.

A "z" value of 92 F has been determined for peroxidase in crab meat by using the D values calculated for the second decreasing straight line segment of the enzyme at different heating temperatures. By using this "z" value plus data from the heat penetration curves for different types of crab meat in cans of specified size, a time and temperature can be determined for pasteurizing crab meat whereby all peroxidase can be inactivated. More research needs to be done to determine times and temperatures needed during processing to prevent peroxidase regeneration.

Peroxidase will regenerate in pasteurized crab meat. By basing the activity on $\Delta\text{OD}_{460}/\text{min}/\text{mg N}$, any loss of nitrogen will increase

the $\Delta\text{OD}_{460}/\text{min}$. The loss of nitrogen in the pasteurized flake and backfin samples as compared to the raw samples could be due to a loss of NH_3 during the heating process prior to pasteurization and due to a loss of nitrogen during denaturation of proteins. A greater percent nitrogen in the pasteurized claw as compared to the raw claw extract could not be explained. Since $\Delta\text{OD}_{460}/\text{min}$ for the pasteurized samples increased after 2 weeks of storage, one can assume that regeneration has occurred.

More research is needed to ascertain if peroxidase is a major cause of off-flavor and off-odor development in blue crabs. The enzyme is present in blue crabs and past research has shown it to be one of the most heat stable enzymes present in food products which can cause off-flavors and off-odors in food products. An almost 100% regeneration of the enzyme in crab meat that was processed following commercial times and temperatures proves that present processing methods are inadequate to completely inactivate the enzyme. It is possible, therefore, that peroxidase may cause off-flavor development in blue crab meat during refrigerated (32-36 F) or frozen (<32 F) storage. By extending the times and temperatures of processing, the enzyme could be inactivated, however, the temperatures required to inactivate peroxidase may cause denaturation of the proteins in crab meat and destroy the texture of the product. Due to this problem, it may not be possible to satisfactorily inactivate peroxidase in blue crab meat and still retain good quality.

LIST OF REFERENCES

- Acker, L. 1962. Enzymatic reactions in foods of low moisture content. *Adv. Food Res.* 11: 263.
- Agner, K. 1943. Verdoperoxidase. *Adv. Enzymol.* 3: 137.
- AOAC. 1975. "Official Methods of Analysis," 12th ed. Association of Official Analytical Chemists, Washington, D. C.
- Balls, A.K. and Hale, W.S. 1935. Peroxidase in the darkening of apples. *Ind. Eng. Chem.* 27: 335.
- Bedford, C.L. and Joslyn, M.A. 1939. Enzyme activity in frozen vegetable string beans. *Ind. Eng. Chem.* 31(1): 751.
- Bruemmer, J.H., Roe, B. and Bowen, E.R. 1976. Peroxidase reactions and orange juice quality. *J. Food Sci.* 41: 186.
- Campbell, H. 1940. *West. Canner Packer.* August. Quoted in Tauber, H. 1949. Enzymes in the meat, egg, vegetable, and fruit industries. In "The Chemistry and Technology of Enzymes." p.440. John Wiley & Sons, Inc., New York.
- Cherry, J.P. and Ory, R.L. 1973. Characterization of isoperoxidase activity in Arachis Hypoganea cultivars. *Phytochemistry.* 12: 1581.
- Cherry, J.P., Ory, R.L. and Mayne, R.Y. 1972. Proteins from peanut cultivars (Arachis Hypoganea) grown in different area. VI. Changes induced in gel electrophoretic patterns by Aspergillus contamination. *J. Am. Peanut Res. Edu. Assoc.* 4(1): 32.
- Cruess, W.C., Friar, H.G. and Lew, M.S. 1944 *West. Canner Packer.* June and July. Quoted in Reed, G. 1975. Oxidoreductases. In "Enzymes in Food Processing," p. 219. Academic Press, New York.
- Danner, D.J., Brignac, P.J., Arceneau, D. and Patel, V. 1973. The oxidation of phenol and its reaction product by horseradish peroxidase and hydrogen peroxide. *Arch. Biochem. Biophys.* 156: 759.
- Davis, W.B. 1942. The distribution and preparation of citrus peroxidase. *Am. J. Bot.* 29: 252.
- Dutcher, R.A., Jensen, C.O. and Althouse, P.M. 1951. Enzymes. In "Introduction to Agricultural Biochemistry," p. 111. John Wiley & Sons, Inc., New York.
- Eriksson, C.E. and Vallentin, K. 1973. Thermal activation of peroxidase as a lipid oxidation catalyst. *J. Am. Oil Chem. Soc.* 50(7): 264.

- Esselen, W.B. and Anderson, E.E. 1956. Thermal destruction of peroxidase in vegetables at high temperatures. *Food Res.* 21: 322.
- Farkas, D.F., Goldblith, S.A. and Proctor, B.E. 1956. Stopping storage off-flavors by curbing peroxidase. *Food Eng.* 28(1): 52.
- Fridovich, I. 1963. The stimulation of horseradish peroxidase by nitrogenous ligands. *J. Biol. Chem.* 238(12): 3921.
- Furia, T.E. (editor). 1968. "CRC Handbook of Food Additives," The Chemical Rubber Company, Cleveland, Ohio.
- Gallagher, P.H. 1924. VII. Mechanism of oxidation in the plant. Part III. Peroxydase observations on the thermostability of the peroxydase of the mangold. *Biochem. J.* 18: 39.
- Gardner, H.W., Inglett, G.E. and Anderson, R.A. 1969. Inactivation of peroxidase as a function of corn processing. *Cereal Chem.* 46: 626.
- Ghiretti, F. 1956. The decomposition of hydrogen peroxide by hemocyanin and by its dissociation products. *Arch. Biochem. Biophys.* 63: 165.
- Godbillon, G. and Frentz, R. 1972. Cellulose acetate electrophoresis of the peroxidase-active protein fraction for the hemolymph of Carcinus maenas, a decapod crustacean. *C. R. Acad. Sci., Ser. D.* 275(14): 1531.
- Guyer, R.B. and Holmquist, J.W. 1954. Enzyme regeneration in high temperature short-time sterilized canned foods. *Food Technol.* 8: 547.
- Haard, N.F. 1973. Upsurge of particulate peroxidase in ripening banana fruit. *Phytochemistry.* 12: 555.
- Hayaishi, O. 1974. Peroxidase. In "Molecular Mechanisms of Oxygen Activation," p. 535. Academic Press, New York.
- Jacobs, M.B. 1951. "The Chemistry and Technology of Food and Food Products," Vol 3, 2nd ed., p. 2363. Interscience Publishers, Inc., New York.
- Joslyn, M.A. 1949. Enzyme activity in frozen vegetable tissue. *Adv. Enzymol.* 9: 613.
- Joslyn, M.A. 1955. Report on peroxidase in frozen vegetables. *J. Assoc. Offic. Agric. Chemists.* 38: 583.

- Joslyn, M.A. 1966. Cryobiology. In "Freezing of Fruits and Vegetables," p. 583., Academic Press, New York.
- Joslyn, M.A. and Bedford, C.L. 1940. Enzyme activity in frozen vegetables, asparagus. *Ind. Eng. Chem.* 32(1): 702.
- Joslyn, M.A. and Ponting, J.D. 1951. Enzyme-catalyzed oxidative browning of fruit products. *Adv. Food Res.* 3:1.
- Karlson, P. 1965. IX. Porphyrins and hemins. In "Introduction to Modern Biochemistry," p. 173. Academic Press, New York.
- Kay, E., Leland, M., Shannon, M. and Lew J.Y. 1967. Peroxidase isozymes from horseradish roots. *J. Biol. Chem.* 242(10): 2470.
- Kiermeyer, F. 1951. *Proc. Int. Congr. Reigrig.* 7th, London, p. 344. Quoted in Reed, G. 1975. Oxidoreductases. In "Enzymes in Food Processing," p. 219. Academic Press, New York.
- Kobrehel, K. and Gautier, M.F. 1974. Variability in peroxidase isozymes in wheat and related species. *Can. J. Bot.* 52(4-6): 755.
- Kon, S. and Whitaker, J.R. 1965. Separation and partial characterization of the peroxidase of Ficus glabrata Lates. *J. Food Sci.* 30: 977.
- LaBerge, D.E., Kruger, J.E. and Meredith, W.O.S. 1973. Peroxidase isozymes in mature barley kernels. *Can. Pl. Sci.* 53(4): 705.
- Lee, Y.B., Rickanrud, D.A., Hagberg, E.C. and Forsythe, R.H. 1976. Detection of various non-meat extenders in meat products. *J. Food Sci.* 41: 589.
- Lehninger, A.L. 1975. Chapter 8 and Chapter 20. In "Biochemistry," p. 477 and 543. Worth Publishers, Inc., New York.
- Livshits, O.D. 1968. Control over the quality of thermal processing of meat products by determining the peroxidase activity. *Vop. Pitan.* 27(2): 80.
- Lopez, A., Bocklet, M.F. and Wood, C.B. 1959. Catalase and peroxidase activity in raw and blanched wouthern peas, Vigna sinensis. *Food Res.* 24(5): 548.
- Lu, A.T. and Whitaker, J.R. 1974. Some factors affecting rates of heat inactivation and reactivation of horseradish peroxidase. *J. Food Sci.* 39(6): 1173.

- Lundquist, I. and Josefsson, J. 1971. Sensitive method for determination of peroxidase activity in tissues by means of coupled oxidation reaction. *Annal. Biochem.* 41(2): 567.
- Maier, V.P., Tappel, A.L. and Volman, D.H. 1955. Reversible in-activation of enzymes at low temperatures. Studies of temperature dependence of phosphatase- and peroxidase-catalyzed reactions. *J. Amer. Chem. Soc.* 77: 1278.
- Manwell, C. and Baker, C.M. 1963. Starch gel electrophoresis of sera from some marine arthropods: Studies on the heterogeneity of hemocyanin and on a "ceruloplasmin-like protein". *Comp. Biochem. Physiol.* 8: 193.
- Masure, M.P. and Campbell, H. 1944. Rapid estimation of peroxidase in vegetable extracts - An index of blanching adequacy for frozen vegetable. *Fruit Prod. J. Am. Food. Manuf.* 23(12): 369.
- McCune, D.C. 1961. Multiple peroxidases in corn. *Ann. N. Y. Acad. Sci.* 94: 723.
- Murphy, M.J. 1970. Starch-iodide as a stain for peroxidases. *J. Chromatogr.* 50: 539.
- Nagle, N.E. and Haard, N.F. 1975. Fractionation and characterization of peroxidase from ripe banana fruit. *J. Food Sci.* 40: 576.
- Nebesky, E.A., Esselen, W.B., Jr., Kaplan, A.M. and Fellers, C.R. 1950. Thermal destruction and stability of peroxidase in acid foods. *Food Res.* 15: 114.
- Nickel, K.S. and Cunningham, B.A. 1969. Improved peroxidase assay method using leuco 2,3',6-Trichloroindophenol and application to comparative measurements of peroxidatic catalysis. *Anal. Bio.* 27: 292.
- Novacky, A. and Hampton, R.E. 1968. The effect of substrate concentration on the visualization of isoperoxidases in disc electrophoresis. *Phytochem.* 6: 1143.
- Peterson, W.H. and Strong, F.M. 1953. Enzymes. In "General Biochemistry," p. 260. Prentice-Hall, Inc., New York.
- Pinsent, B.R.W. 1962. Peroxidase regeneration and its effects on quality in frozen peas and thawed peas. *J. Food Sci.* 27: 120.
- Ponting, J.D. and Joslyn, M.A. 1948. Ascorbic acid oxidation and browning in apple tissue extract. *Arch. Biochem.* 19: 47.

- Price, J.F. and Schweigert, B.S. 1971. "The Science of Meat and Meat Products," p. 455. W. H. Freeman & Co., San Francisco.
- Reed, G. 1975. Oxidoreductases. In "Enzymes in Food Processing," p. 216. Academic Press, New York.
- Rucker, W. and Radola, B.J. 1971. Isoelectric patterns of peroxidase isoenzymes from tobacco tissue cultures. *Planta*. 49: 192.
- Scandalios, J.G. 1969. Genetic control of multiple molecular forms of enzymes in plants: A Review. *Bio Genetics*. 3: 37.
- Schwimmer, S. 1944. Regeneration of heat-inactivated peroxidase. *J. Biol. Chem.* 154: 487.
- Sullivan, B. and Anderson, J.A. 1946. Chap. VII. In "Interscience," New York. (In *Can. Pl. Sci.* 1973. 53(4): 705.)
- Sumner, J.B. and Gjessing, E.C. 1943. A method for the determination of peroxidase activity. *Arch. Biochem.* 2(3): 291.
- Sumner, J.B. and Howell, S.F. 1936. Hematin and the peroxidase of fig sap. *Enzymologia*. 1: 133.
- Sumner, J.B. and Somers, G.F. 1947. The iron enzymes. In "Chemistry and Methods of Enzymes," 2nd Ed. p. 207. Academic Press, New York.
- Sweet, C.W. 1973. Activity of Antioxidants in Fresh Fish. *J. Food Sci.* 38: 1260.
- Tauber, H. 1949. VIII. The iron-porphyrin enzymes. XXVI. Enzymes in the meat, egg, vegetable and fruit industries. In "The Chemistry and Technology of Enzymes," pp. 189 and 440. John Wiley & Son, inc., New York.
- Theorell, H. 1940. *Arkiv Kemi, Mineral. Geol.* 14B(20): 1. Quoted in Sumner, J.B. and Somers, G.F. 1947. The iron enzymes. In "Chemistry and Methods of Enzymes", 2nd ed. p. 207. Academic Press, New York.
- Thurlow, S. 1925. XXV. Studies on xanthine oxidase. IV. Relation of xanthine oxidase and similar oxidizing systems to Bach's oxygenase. *Biochem. J.* 19: 1975.
- Tressler, D.K. 1947. Enzymes in the Food Industry. Presented at the Chem. Research Conf., Colby Jr. Coll., Aug 7. (In *Food Res.* 1950. 15: 114).
- Uglow, R.F. 1969. Haemolymph protein concentrations in protunid crabs - I. Studies on adult Carcinus maenas. *Comp. Biochem. Physiol.* 39(6):

- Wagenknecht, A.C. and Lee, F.A. 1958. Enzyme action and off-flavor in frozen peas. *Food Res.* 23: 25.
- Weaver, M.L. and Hautala, E. 1970. Study of hydrogen peroxide, potato enzymes and blackspot. *Am. Potato J.* 47: 457.
- Weinryb, I. 1966. Behavior of horseradish peroxidase at high hydrogen peroxide concentrations. *Biochem.* 5: 2003.
- Willstatter, R. and Stoll, A. 1918. *Ann. Chem.* 416: 21. Quoted in Sumner, J.B. and Somers, G.F. 1947. The iron enzymes. In "Chemistry and Methods of Enzymes," 2nd ed. p. 207. Academic Press, New York.
- Willstatter, R. and Weber, H. 1926. *Ann. Chem.* 449: 175. Quoted in Tauber, H. 1949. The iron-porphyrin enzymes. In "The Chemistry and Technology of Enzymes," p. 189. John Wiley & Sons, Inc., New York.
- Wood, C.B. and Lopex, A. 1963. Evaluation of quantitative methods of determining peroxidase in vegetables. *Food Tech.* 17(4): 497.
- Wood, K.R. 1971. Peroxidase isoenzymes in leaves of cucumber (*Cucumis sativus* L.) cultivars systemically infected with the W strain of cucumber mosaic virus. *Physiol. Pl. Pathol.* 1: 133.
- Woods, A.E. 1901. *Bull.* 18, U.S. Dep. Agric. (In *J. Biol. Chem.* 1944.) 154: 487.
- Yamamoto, H.Y., Steinberg, M.P. and Nelson, A.I. 1962. Kinetic studies on the heat inactivation of peroxidase in sweet corn. *J. Food Sci.* 27: 113.
- Zak, D.L. and Keeney, P.G. 1974. Disc gel electrophoresis. A technique involving fluorescent staining prior to separation. *J. Agric. Food Chem.* 22(5): 800.
- Zoueil, M.E. and Esselen, W.B. 1959. Thermal destruction rates and regeneration of peroxidase in green beans and turnips. *Food Res.* 24: 119.

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DETECTION, ACTIVITY AND RESISTANCE TO THERMAL INACTIVATION OF
PEROXIDASE IN THE BLUE CRAB (CALLINECTES SAPIDUS)

by

Florence Scheulen Burnette

(ABSTRACT)

Peroxidase is one of the most heat resistant enzymes and may cause undesirable quality changes in thermally processed foods. Peroxidase activity and its resistance to thermal inactivation in fresh and pasteurized lump, claw and flake meat of both male and female blue crabs was determined spectrophotometrically. Activity was greatest in the flake and least in the claw. Male crabs usually exhibited a greater initial activity ($\Delta OD_{460}/\text{min}$) than did females of equal size. The larger the crab for a given sex, the greater the initial activity.

Eight isozymes of peroxidase were detected in raw extracts of a 115 g female blue crab following starch gel electrophoresis and nine in a 116 g male. A smaller female crab (96 g) revealed seven bands which were less intense than those of larger females. By extending the time of electrophoresis, twelve bands were detected in the gel containing an extract from the 96 g female crab.

The optimum thermal processing times needed to denature peroxidase and to prevent regeneration were studied. Heat inactivation curves indicated two straight line decreasing segments which varied by rate of descent. The first segment which decreased at a faster rate was considered due to heat-labile isozymes and the second segment

which decreased at a slower rate due to heat stable isozymes. D values obtained for the enzyme based on the second straight line segment were $D_{80}=47$ min, $D_{110}=18.2$ min and $D_{150}=11.2$ min. A "z" value of 92 F was also obtained for the enzyme.