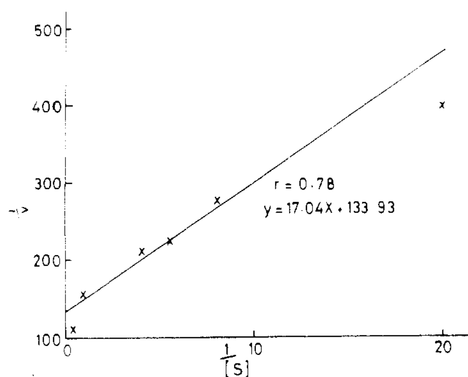

Figure 7. Lineweaver-Burk plot of the activity of sorghum β-amylase.

7). This relatively less affinity of our purified enzyme for the substrate may be an inherent difference in the β-amylases isolated from the two strains of sorghum, *Sorghum bicolor* (Linn) (*S. caffrorum* and *S. bicolor*). These values also compare favorably with those reported for wheat and barley malts (Meyer et al., 1953; Tkachuk and Tipples, 1966).

Registry No. β-Amylase, 9000-91-3; starch, 9005-25-8.

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Received for review April 25, 1983. Accepted August 19, 1983.

Enzymatic Determination of Urea and Ammonia in Refrigerated Seafood Products

Wai L. Cheuk and Gunnar Finne*

An enzymatic method for the determination of both urea and ammonia in fresh seafoods was investigated. The method involves the transamination of α-ketoglutarate in the presence of glutamate dehydrogenase and NADH. The consumption of NADH as measured at 340 nm is stoichiometrically equivalent to the amount of ammonia in the test sample. Urea is determined by the same reaction scheme after the addition of urease. When this enzymatic method was used to determine the quality of refrigerated shrimp and crab meat, both ammonia and urea were shown to increase during storage and there was also good correlation between the concentration of both these compounds and traditional spoilage indicators.

Quality changes that occur in fresh seafood products during refrigerated or iced storage are the combined results of both microbiological and tissue enzyme activities. Both give rise to a number of different volatile compounds of which ammonia can be most detrimental. Due to the accumulation during storage, the ammonia concentration has been suggested as an objective index of fresh seafood quality. Ward et al. (1979), using an ammonia-specific electrode, demonstrated the relationship between storage time, total microbial numbers, and ammonia concentration during refrigerated storage of fresh shrimp. Ammonia has also been suggested as an index of quality for crab meat (Burnett, 1965; Steinbrecher, 1973), dogfish, and thornback ray (Vyncke, 1978). Post-mortem ammonia production is especially a problem in elasmobranch species (sharks, dogfish, rays, and skates). These fishes contain high levels of urea both in blood and in tissue, and during iced or refrigerated storage urease-positive microorganisms will rapidly convert urea to ammonia. Both France and Belgium, where dogfish is an important food fish, have stringent quality standards based on the ammonia content of this species.

A number of quantitative methods for the determination of ammonia in seafood products have been developed. Most of these methods rely on microdiffusion, distillation, and/or ion exchange. After being liberated from the tissue by strong alkali, ammonia is trapped in weak acids and measured titrimetrically (Conway and Cooke, 1939) or photometrically by either Nessler's reagent (Seligson and Hirahara, 1957) or the Berthelot reaction (Chaney and Marbach, 1962).

When researching the kinetics of conversion of urea to ammonia in sharks held on ice, we adapted an enzymatic method for the simultaneous determination of urea and ammonia. The method is based on the enzymatic deter-

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mination of ammonia in body fluids and tissue as reported by Fawaz and Dahl (1964) and later by Mondzac et al. (1965). Knight and Toom (1980) showed that the enzymatic method was suitable for the determination of ammonia in seafoods. They also demonstrated that, unlike other methods, the enzymatic technique was specific for ammonia and other decomposition products and certain food additives did not interfere with the assay. Talke and Schubert (1965) coupled the enzymatic ammonia determination in blood with urease, thus providing a convenient way to measure both ammonia and urea in the same sample aliquot in a single step.

The objectives of this research were (1) to test the applicability of the enzymatic determination of urea and ammonia in seafood products and, if applicable, (2) to adopt the enzymatic method and correlate it with traditional spoilage indicators commonly used for seafood products.

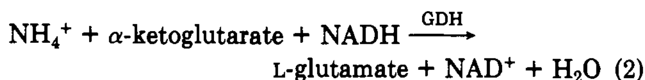
EXPERIMENTAL PROCEDURES

Preparation of Seafood Samples. Tails of brown shrimp (*Penaeus aztecus*) and freshly picked crab meat (*Callinectes sapidus*) obtained from a seafood retail store in Aransas Pass, TX, were packed on ice and shipped to the laboratory in College Station, TX. The shrimp tails were stored refrigerated at 3.5 °C, and the crab meat was stored in plastic bags on ice. At regular intervals during the storage period, samples were analyzed for ammonia and urea by the enzymatic assay, total volatile nitrogen (TVN), and trimethylamine (TMA) as described by Cobb et al. (1973). Aerobic plate counts were determined according to the American Public Health Association (APHA, 1970).

The extraction procedure for the enzymatic assay and assay conditions used in this study were similar to those described in "Methods of Enzymatic Food Analysis" (Boehringer Mannheim Biochemicals, 1980). Composite samples, weighing approximately 50 g, were prepared by mixing tissue from various parts of the seafood products in a beaker with a glass rod. Ten-gram samples of the composite homogenate were extracted with 20 mL of 1 N perchloric acid for 5 min in a blender. Each homogenate was centrifuged at 5000 rpm for 15 min and 10 mL of the supernatant was transferred to a beaker and neutralized with a 2 N potassium hydroxide solution. The volume of KOH used was recorded. The mixture was stored in a refrigerator for at least 10 min and then filtered through a Whatman No. 1 filter paper. After appropriate dilutions, 0.1 mL of the filtrate was used for the enzymatic assay.

Analytical Procedures. *Principles.* The reaction schemes for the enzymatic determination of urea and ammonia are outlined by eq 1 and 2. Abbreviations used

$$\text{urea} + H_2O \xrightarrow{\text{urease}} 2NH_3 + CO_2 \quad (1)$$



$$NH_4^+ + \alpha\text{-ketoglutarate} + \text{NADH} \xrightarrow{\text{GDH}} \text{L-glutamate} + \text{NAD}^+ + H_2O \quad (2)$$

were as follows: GDH = L-glutamate dehydrogenase; NAD^+ = nicotinamide adenine dinucleotide; NADH = nicotinamide adenine dinucleotide, reduced.

Reagents Needed. (1) Buffer. The buffer was as follows: triethanolamine, 0.5 mol/L; α-ketoglutarate, 35 mmol/L; pH 8.6. This was made by dissolving 9.3 g of triethanolamine hydrochloride (Boehringer Mannheim Biochemicals, Indianapolis, IN) and 670 mg of α-ketoglutarate disodium salt (BMB) in 70 mL of redistilled water, adjusting to pH 8.6 with sodium hydroxide, 5 mol/L, and filling up to 100 mL with distilled water. The buffer is stable for at least 4 weeks at 4 °C. (2) Reduced Nicotinamide Adenine Dinucleotide Solution (NADH), 6 mmol/L. This was prepared by dissolving 30 mg Na_2 of NADH (BMB) and 60 mg of sodium bicarbonate in 6 mL of redistilled water. It was stable for 4 weeks at 4 °C. (3) Glutamate Dehydrogenase (GDH), 10 g/L. Commercial GDH in glycerol solutions (BMB) can be used undiluted. It is stable for at least 1 year at 4 °C. (4) Urease, 2.5 g/L. Urease was prepared by dissolving 10 mg of lyophilisate urease [available as 2.5 mg of enzyme protein (BMB) in 1 mL of glycerol (50% v/v)]. It is stable for 4 weeks at 4 °C.

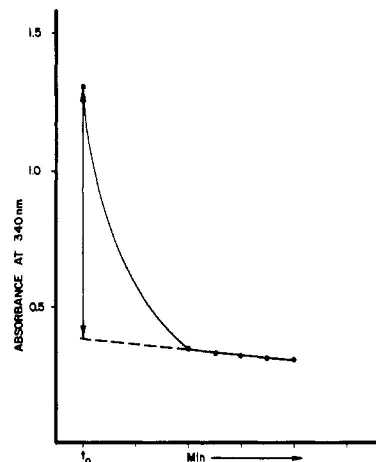


Figure 1. Graphical extrapolation of absorbance during enzymatic assay.

Enzymatic Assay. One milliliter of buffer, 0.10 mL of NADH solution, 0.10 mL of filtrate (sample), and 1.90 mL of redistilled water were pipetted into a quartz cuvette and mixed thoroughly. After approximately 3 min, the absorbance of the mixture was read against water at 340 nm (A_1 ammonia). The reaction (eq 2) was started by the addition of 0.02 mL of GDH solution, the contents were mixed well, and the reaction was allowed to be completed (15–25 min). The absorbance was recorded (A_2 ammonia). If the reaction is still running after 25 min, the absorbance should continue to be recorded at 2 min intervals until a constant rate. The ammonia concentration was calculated from the absorbance difference between A_1 and A_2 or from a constant rate graph as shown in Figure 1. For the determination of urea in the same mixture, the reaction (eq 1) was started by the addition of 0.02 mL of urease solution and allowed to proceed as described for ammonia. The final absorbance (A_3 urea) was recorded and the urea concentration calculated from the absorbance difference ($A_2 - A_3$).

Calculations. The concentration (c) of ammonia or urea in the assay solution can be calculated according to the general equation

$$c = \frac{V \times \text{EW} \times \Delta A}{E \times d \times v \times 1000} \text{ (g/L)} \quad (3)$$

where V = final cuvette volume (mL), v = sample cuvette volume (mL), EW = equivalent weight of the substance to be assayed, d = light path (cm), E = extinction coefficient of NADH at 340 nm (6.3 L mmol^{-1} cm^{-1}), c = concentration, and ΔA = absorbance difference.

When the final concentration of ammonia and urea in seafood products is calculated, the quantity of moisture in the samples must be taken into consideration. For instance, in the following calculation example it is assumed that the moisture content of fresh shrimp is 78%. The weight of the sample is 10 g and the moisture added by the sample is thus 7.8 mL.

An example of the calculation is as follows: weight of sample, W_s, = 10.0 g; KOH used to neutralize = 6.9 mL;

Table I. Recovery of Ammonia from Shrimp Crab Meat of Various Degrees of Decomposition

sample	initial NH_3, mg/100 g	added NH_3, mg/100 g	assayed NH_3, mg/100 g	recovered NH_3, mg/100 g	% recovered
shrimp 1	31.21	37.59	69.61	38.40	102
shrimp 2	28.81	37.59	65.34	36.53	97
shrimp 3	85.34	37.59	121.35	36.01	95
shrimp 4	25.07	37.59	62.94	37.87	100
crab meat 1	30.81	37.59	72.81	42.00	111
crab meat 2	37.07	37.59	79.21	42.14	112
crab meat 3	25.07	37.59	68.01	42.94	114
crab meat 4	40.20	37.59	80.81	40.61	108

Table II. Recovery of Urea from Shrimp and Crab Meat of Various Degrees of Decomposition

sample	initial urea, mg/100 g	added urea, mg/100 g	assayed urea, mg/100 g	recovered urea, mg/100 g	% recovered
shrimp A	36.18	70.47	110.88	74.70	106
shrimp B	20.20	70.47	93.02	72.82	103
shrimp C	37.12	70.47	108.06	70.94	101
shrimp D	20.20	70.47	90.21	70.01	99
crab meat A	1.87	70.47	67.65	65.78	93
crab meat B	1.41	70.47	74.23	72.82	103
crab meat C	29.13	70.47	97.72	68.59	97
crab meat D	15.03	70.47	82.69	67.66	96

total volume (20 mL of $HClO_4$ + 7.8 mL of H_2O), V_1, = 27.8 mL; volume of supernatant, V_2, = 10.0 mL; volume of sample mixture (V_2 + KOH), V_3 = 16.9 mL, volume taken for sample = 0.1 mL absorbance difference ($A_1 - A_2$) = 0.405 OD. Thus

$$\% \text{ ammonia in shrimp} = C\ (\text{g/L}) \times \frac{V_1 \times V_3 \times 100}{V_2 \times W_s \times 1000}$$

$$= \frac{3.22 \times 17.03 \times 0.405}{6.3 \times 1 \times 0.1 \times 1000} \times \frac{27.8 \times 16.9 \times 100}{10 \times 10 \times 1000}$$

$$= 0.01653\% \text{ or } 16.53 \text{ mg/100 g} \quad (4)$$

Standard Curves, Recovery, and Confirmatory Tests. Instead of using the extinction coefficient of NADH to calculate ammonia and urea, standard curves showing the absorbance difference as a function of concentration can also be used. When anhydrous analytical-grade ammonium sulfate is used, a standard curve for ammonia can be constructed by accurately weighing 32.97 mg of $(NH_4)_2SO_4$ into a 100-mL volumetric flask and bring it to volume with redistilled water. This stock solution is diluted into a series of appropriate concentrations. The effect of substrate concentration on the absorbance difference is determined by performing the enzymatic assay on each dilution. A standard curve for urea can also be constructed by using analytical-grade urea.

To determine recovery efficiency of the enzymatic assay, shrimp and crab meat of different degrees of decomposition (Tables I and II) were spiked with either ammonium sulfate or urea. The spiked samples were subsequently analyzed by the enzymatic assay and the recovery was determined.

Confirmatory experiments were performed by using both aqueous standard solutions and freshly obtained shrimp and crab meat. Samples were prepared by supplementing with ammonium sulfate to increase the ammonia concentration by 20, 40, 60, and 80 mg of NH_3/L (assay solution) and with urea to increase the urea concentration by 30, 60, 90, 120, and 150 mg of urea/L (assay solution). The aqueous solutions and the supplemented samples were analyzed for ammonia and urea by the enzymatic assay.

RESULTS AND DISCUSSION

In order to test the relationship between substrate concentration and decrease in absorbance at 340 nm, a large number of aqueous standard solutions of ammonia and urea were analyzed by using the enzymatic assay method. The decrease in absorbance was shown to be linearly proportional to both ammonia and urea up to the highest concentrations of 85 and 150 mg/L (assay solution) for ammonia and urea, respectively. Instead of using the extinction coefficient for NADH, a standard curve constructed this way can be used to calculate the concentrations of both ammonia and urea.

Recovery tests were performed by spiking shrimp and crab meat with known amounts of ammonium sulfate and urea. Initially shrimp and crab meat of different degrees of decomposition were analyzed for ammonia and urea by using the enzymatic method. To the same samples were added anhydrous analytical-grade ammonium sulfate equivalent to 37.59 mg of NH_3/100 g of sample and analytical-grade urea equivalent to 70.47 mg of urea/100 g of sample. Tables I and II show the recovery percentage of ammonia and urea for the spiked samples. The average recovery of ammonia added to shrimp and crab meat of various degrees of decomposition was 98.5 and 111.2%, respectively. Although there is no immediate explanation for the excessive recovery of ammonia from crab meat, this could possibly be due to bacterial ammonia production during preparation and mixing of the spiked sample. The average recovery of urea from shrimp and crab meat of different degrees of spoilage was 102 and 97%, respectively.

Confirmatory tests were performed by adding different concentrations of ammonia and urea to shrimp and crab meat of the same quality. Samples were prepared by supplementing shrimp and crab meat with analytical-grade ammonium sulfate and urea at different concentrations. Aqueous samples of the same concentrations were prepared simultaneously. After being mixed, both the spiked and the aqueous samples were analyzed by the enzymatic methods. The confirmatory recoveries were within 1–5% of theoretical values for both ammonia and urea. Furthermore, the relationship between added ammonia or urea and the decrease in absorbance (ΔA) was linear (Figures 2 and 3).

In order to further evaluate the applicability of the enzymatic method for determining seafood quality, duplicate lots of fresh shrimp were placed in a refrigerator at 3.5 °C and duplicate lots of crab meat in plastic bags were placed on ice. At regular time intervals, subsamples were removed and analyzed for urea, ammonia, total volatile nitrogen (TVN), trimethylamine (TMA), and total aerobic plate

Table III. Development of Urea, Ammonia, Total Volatile Nitrogen, Trimethylamine, and Aerobic Plate Count in Shrimp Tails Held at 3.5 °C[a]

storage period, days	urea, mg/100 g	ammonia,[b] mg/100 g	TVN,[b] mg of TVN N/100 g	TMA, mg of TMA N/100 g	aerobic plate count
1	0.74	4.92	7.51	traces	1.49×10^5
3	1.05	10.35	8.34	traces	1.16×10^6
5	1.38	18.09	15.42	2.45	3.40×10^6
7	2.43	23.98	28.79	4.52	1.69×10^7
9	2.70	40.05	43.40	7.82	2.29×10^7

[a] All data are the average of duplicate lots analyzed in triplicate. [b] Correlation coefficient 0.98.

Table IV. Development of Urea, Ammonia, Total Volatile Nitrogen, Trimethylamine, and Aerobic Plate Count in Crab Meat Held in Plastic Bags on Ice[a]

storage period, days	urea, mg/100 g	ammonia,[b] mg/100 g	TVN,[b] mg of TVN N/100 g	TMA, mg of TMA N/100 g	aerobic plate count
1	70.30	41.73	43.85	4.79	
3	92.60	49.66	51.18	3.62	2.8×10^7
5	96.70	72.39	74.68	6.80	
7	113.20	123.47	98.94	12.98	1.9×10^8
9	119.20	132.72	115.94	13.60	2.7×10^8
11	147.67	185.72	119.73	15.21	3.9×10^8

[a] All data are the average of duplicate lots analyzed in triplicate. [b] Correlation coefficient 0.98.

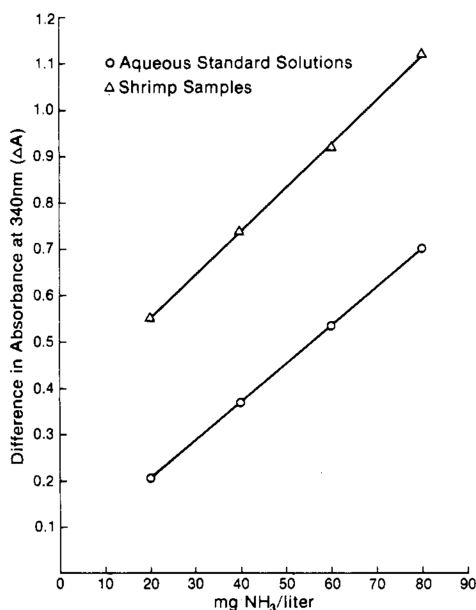


Figure 2. Difference in absorbance (ΔA) resulting from the addition of various amounts of ammonia to fresh shrimp.

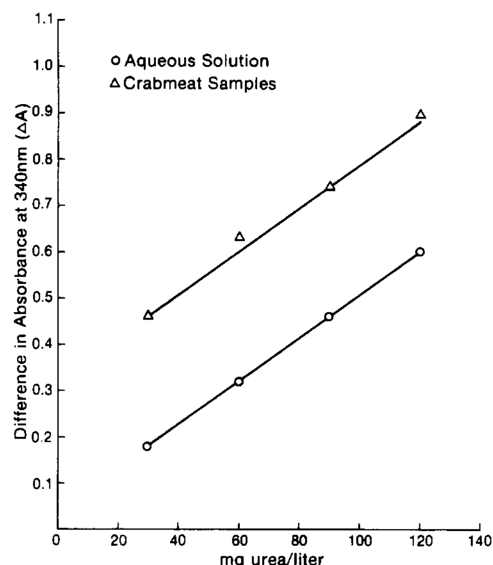


Figure 3. Difference in absorbance (ΔA) resulting from the addition of various amounts of urea to fresh crab meat.

count (APC). Table III shows the development of the different quality parameters for shrimp tails held at 3.5 °C. Using TVN as an index of quality, Stansby (1976) suggested the following values for different degrees of freshness of fish: 12 or less, fresh fish; 12–20, edible with only slight decomposition; 20–25, borderline; above 25, inedible and decomposed. Even though such a classification may not be directly applicable to shrimp, a value of 7.51 mg of TVN N/100 g indicated good initial quality. During storage at 3.5 °C, ammonia, urea, TVN, and TMA increased as the microbial population increased. As expected, there was a close relationship between ammonia and total volatile nitrogen. These two quality indexes had a correlation coefficient of 0.98 during storage over a 9-day period. When a value of 25 mg of TVN N/100 g was used as an indicator of acceptability, the shrimp used for this study were spoiled after 7 days at 3.5 °C. At this time the aerobic plate count had reached 1.69×10^7 organisms/g, the TMA concentration was 4.52 mg of TMA N/100 g and the ammonia content was 23.98 mg of NH_3/100 g. The urea content had also increased slightly, which is in agreement with Cobb et al. (1973).

On the basis of organoleptic evaluations, Hillig et al. (1959) defined three stages of decomposition for fresh crab meat. In a later collaboratory study, Steinbrecher (1973) showed statistically significant differences in ammonia between the three stages of decomposition and recommended that ammonia be adopted as an index of quality for fresh crab meat. Table IV shows the development of ammonia and other quality indexes during storage of crab meat on ice. The most noticeable difference between crab meat and shrimp held under refrigerated storage was the dramatic buildup of urea in the crab meat. Since crab meat is a cooked product, endogeneous enzymes associated with the urea cycle cannot explain the buildup of urea during storage. The high ammonia content and rapid spoilage of fresh crab meat is most likely due to the invasion of urea-positive psychrotrophic microorganisms that will convert urea to ammonia. The initial ammonia content of the crab meat used for this study was 41.73 mg of NH_3/100 g. Using a value of 45.00 mg of NH_3/100 g as a cutoff point as suggested by Steinbrecher (1973), the crab meat would be placed within class 1. However, spoilage proceeded rapidly and after only approximately 6 days on ice the crab meat had reached class 3 quality (94.30 mg of NH_3/100 g) at which point the microbiological popu-

lation was more than 10^8 organisms/g. As was true for shrimp, there was also good correlation between ammonia production and other quality indexes for fresh crab meat held on ice.

The direct enzymatic determination of ammonia and urea has a number of advantages over other common methods used to assess seafood quality. The practical advantage is that both compounds can be determined in the same sample by using the same technique. The technical advantage lies in the specificity of the enzymes for the substrates (Mondzac et al., 1965; Kaplan, 1969). While total volatile nitrogen analysis includes a number of amines in addition to ammonia, the enzymatic assay is specific for either ammonia or urea. No special apparatus is required with the exception of a narrow bandwidth UV spectrophotometer.

Registry No. Urea, 57-13-6; GDH, 9001-46-1; NADH, 58-68-4; urease, 9002-13-5; NH_3, 7664-41-7; N_2, 7727-37-9; TMA, 75-50-3.

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Received for review June 20, 1983. Accepted September 19, 1983. Technical article 17685 from the Texas Agricultural Experiment Station. The research was supported by a grant from the Gulf and South Atlantic Fisheries Development Foundation.

Characterization of the Cauliflower Peroxidase Isoenzyme

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Cauliflower peroxidases were separated into three isoenzymes by using hydrophobic chromatography. Some characters of the major isoenzyme, which consists of over 94% of the total peroxidase activity, were studied. Optimal pH and temperature with guaiacol as a substrate were found to be pH 6.5 and 40 °C. Kinetic studies indicated an apparent K_m of 3.1 mM for guaiacol at an optimal hydrogen peroxide concentration and a K_m of 8.7 mM for hydrogen peroxide at an optimal guaiacol concentration. The heat inactivation rate followed first-order kinetics with the activation energy, E_a = 46.4 kcal/mol. As this indicated, the isoenzyme preparation was very heat labile; at 50 °C, it took only 15 min to reduce its activity by 98%.

Peroxidase (EC 1.11.1.7, donor:hydrogen-peroxide oxidoreductase) is known to occur in most higher plants and is considered to have a direct relationship to the adverse changes in flavor and color of raw underblanched vegetables. Peroxidase is the most heat-stable enzyme in plants, and under certain conditions of limited heat treatment of vegetables it may regain activity during storage (Schwimmer, 1944; Lu and Whitaker, 1974). Any reactivation of peroxidase that does occur is probably due to the enzyme not being wholly inactivated by heat (Burnette, 1977). Recently an extensive review on peroxidase in fruits and vegetables was presented by Vamos-Vigyazo (1981).

Rosoff and Cruess (1949) were the first workers to report the presence of peroxidase in cauliflower. Since then a very limited number of papers on this vegetable and enzyme have been published. Bottcher (1975) reported that when cauliflower was heated at 95 °C for 2 min, less than 13% of residual activity was observed. When residual peroxidase activity ranged from 2.9 to 8.2% after hot water blanching, they observed that frozen cauliflower maintained its best quality. This and other reports were all based on peroxidase activity within cauliflower. In the present study, we isolated peroxidase in a homogeneous form from cauliflower and examined some of its chemical characters.

Institute of Food Science (C.Y.L. and A.P.P.) and Department of Seed and Vegetable Sciences (M.H.D.), New York State Agricultural Experiment Station, Cornell University, Geneva, New York 14456.

MATERIALS AND METHODS

Persistent white cauliflower selections (Dickson and Lee, 1980) were grown during the 1981 and 1982 seasons at this experiment station. The peroxidases were extracted from cauliflower, harvested at maturity, by homogenizing with acetone. The isoenzymes were separated on a hydrophobic chromatographic column, phenyl-Sepharose CL-4B (Pharmacia), according to the methods of Flurkey and Jen

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