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Isolation and Characterization of Chitin from Crawfish Shell Waste

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Crawfish shell waste is an excellent source of chitin (23.5% on a dry basis). Procedures for isolation of chitin have been developed, along with investigation of its distinctive physicochemical properties. Optimal conditions for deproteinization of crawfish waste were 3.5% NaOH at 65 °C for 2 h with a solids to solvent ratio of 1:10 (w/v). Optimal demineralization involved treatment with 1 N HCl at ambient temperature for 30 min with a solids to solvent ratio of 1:15 (w/v). Removal of the carotenoid astaxanthin from the shell matrix required extraction with acetone before bleaching with 0.315% sodium hypochlorite solution for 5 min with a solids to solvent ratio of 1:10 (w/v). Proximate and amino acid analyses indicate the potential of the recovered protein fraction in various feed applications. Fractionation of whole crawfish meal has permitted comparison of chemical composition changes with differential particle size, resulting in significant percent increase in protein and reduction in calcium.

Investigations from our laboratory (Chen and Meyers, 1982a,b; Meyers and Chen, 1985) have demonstrated the commercial feasibility of recovery and utilization of astaxanthin from Louisiana crawfish processing waste. A significant amount of shell is separated in the initial processing stage for extraction of the pigment (Meyers and Chen, 1985). Compared with whole crawfish waste, this shell portion is an excellent source of chitin in view of the higher chitin and lower protein content. A preliminary study has demonstrated that crawfish shell waste contains 23.5% chitin, on a dry basis.

To date, the majority of attempts to isolate chitin from crustacean processing operations have been with shrimp and crab waste shell (Johnson and Peniston, 1982; Muzzarelli, 1977). Ashford and co-workers (1977) have demonstrated that chitin represents 14–27% and 13–15% of the dry weight of shrimp and crab processing waste, respectively.

Several salent features of crawfish waste processing indicate its appliability as a feasible source of chitin. These include, among others, the multimillion ton volume available and the potential cost-effective integration of the treatment process with existing commercial pigment recovery (Meyers and Chen, 1985). Furthermore, preliminary data suggest that the crawfish exoskeleton, or shell, may possess distinctive physicochemical properties, possibly providing polymers different from, or superior to, those obtained from other crustacean wastes.

In view of the increasing need for reliable and renewable sources of raw material for chitin production and its promising commercial applications (Hirano and Tokura, 1982; Knorr, 1984; Muzzarelli, 1985; Muzzarelli and Pariser, 1978; Rha et al., 1984; Zikakis, 1984), effective exploitation of crawfish shell waste for production of chitin appears realistic. The present research notes development of a composite process for effective isolation of chitin from crawfish shell and characterization of the physicochemical properties of the chitin product. Differential sieving of the whole meal also is reported for product fractionation to separate proteinaceous and shell portions.

EXPERIMENTAL SECTION

Sample Collection and Preparation. Dried ground crawfish waste (>2-mm particle size), designated as crawfish whole meal, and wet crawfish shell (>2-mm particle size), separated through a commercial vertical hammer mill, were obtained from Acadiana Processors, Inc. (Henderson, LA). The crawfish meal and shell were placed separately into double black polyethylene bags and stored at −20 °C until use.

Prior to use, the frozen shell was thawed at ambient temperature, treated under running hot water to remove soluble organics and adherent protein, and dried in a forced-air oven at 60 °C for 24 h. This was done to minimize batch dissimilarities due to the adherent protein of the shell. To obtain a uniform size product, the dried shell was ground through a Wiley mill (Standard Model No. 3) with a 2-mm mesh screen and shifted with 10- (2.00-mm),

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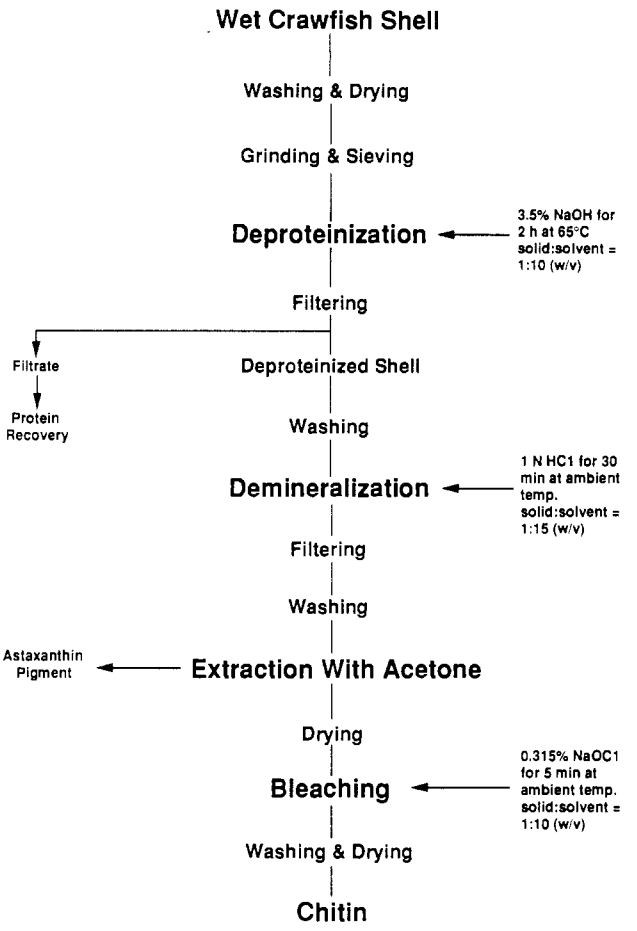

Figure 1. Overall process for preparation of chitin from crawfish shell.

20-(0.841-mm), and 35-mesh (0.500-mm) sieves with a portable Tyler sieve shaker (Model RX-8). This allowed evaluation of the effect of three different particle sizes on the quality of the final chitin products. Dried ground shell was placed in opaque plastic bottles and stored at ambient temperature. Crawfish meal was reground in a Wiley mill to pass through a 2-mm mesh screen, packed in plastic bags, and stored at −20 °C. In the meal-fractionation study, two additional mesh screens, i.e., 42- (0.354-mm) and 60-mesh (0.250-mm) sieves, as well as the aforementioned three screens were used.

Isolation of Chitin. Production of chitin involved basic steps of demineralization, deproteinization, and decoloration, comparable to those applied to other chitinous sources such as crab wastes. However, the aforementioned process, based on the distinctive features of the crawfish shell, required appropriate modification to achieve maximal recovery efficiency with crawfish waste. Dried ground shell of 0.841–0.500-mm size was used throughout the chitin research.

The shell was demineralized with 1 N HCl for 0.5–6 h at ambient temperature with constant stirring and a solids to solvent ratio of 1:10 or 1:15 (w/v). Following demineralization, the decalcified shell was collected on a 80-mesh (0.177-mm) sieve, washed to neutrality in running tap water, rinsed with deionized water, and filtered to remove excess moisture.

Deproteinization involved stirring of the demineralized shell with dilute NaOH (2.0%, 3.0%, 3.5%) with a solids to solvent ratio of 1:10 (w/v) for 1–6 h at ambient temperature, 65 °C, and 100 °C. The residue was then washed and filtered as above.

The chitin residue was bleached with reagents to obtain a white product: These included sodium hypochlorite solution (Blumberg et al., 1951), absolute acetone (Kamasastri and Prabhu, 1961), 3% hydrogen peroxide (Brine and Austin, 1981), and ethyl acetate (Brzeski, 1982). The decolorized product was collected, washed as above, and dried at 60 °C in a forced-air oven for 4 h.

Optimal conditions were designated as those giving a nitrogen content closest to the theoretical value of 6.9% for pure chitin, with minimum ash content under minimum reagent usage and reaction time/temperature. The overall process for preparation of crawfish chitin is given in Figure 1.

Table I. Chemical Composition of Crawfish Whole Meal and Shell[a]

composition	whole meal	shell
crude protein, %	35.8	16.9
fat, %	9.9	0.6
fiber, %	16.5	23.6
chitin, %	15.9	23.5
ash, %	38.1	63.6
minerals		
Ca, %	12.3	24.8
P, %	0.8	1.0
K, %	1.0	0.1
Mg, %	0.2	0.3
Mn, ppm	545	200
Fe, ppm	1611	180
astaxanthin, ppm	78	108

[a] Average of three determinations.

Protein Recovery. After deproteinization, the reaction mixture was filtered and the filtrate centrifuged (Sorvall RC-5C automatic superspeed refrigerated centrifuge) at 16300*g* for 15 min to remove extraneous solids. The clarified extract was adjusted with 1 N HCl to pH 4.5 with stirring and allowed to settle for 3 h at ambient temperature. The protein precipitates were separated by centrifugation at 16300*g* for 15 min, washed with deionized water by repeated centrifugation, and dried in a vacuum oven (Model 10, Precision Scientific Group) at 50 °C for 8 h. The dried protein was analyzed for protein, ash, and amino acids.

Chemical Analysis. Crude protein was calculated by multiplying nitrogen content of the sample by 6.25. Nitrogen was determined by a semiautomated method (AOAC, 1980); fat, fiber, and ash were determined by standard methods (AOAC, 1980); chitin was determined by the method of Black and Schwartz (1950). In addition to proximate analysis, levels of calcium, potassium, magnesium, manganese, and iron were analyzed with a Perkin-Elmer atomic absorption spectrometer, and phosphorus content was analyzed according to the AOAC method (1980).

Astaxanthin Analysis. The extractable astaxanthin was determined following the solvent extraction procedure described by Lee (1985), using the formula of Kelly and Harmon (1972).

Amino Acid Analysis. Each 0.1 g of sample was hydrolyzed with 6 N HCl for 24 h at 110 °C under vacuum. Hydrolysates were filtered and evaporated to near dryness and made up to 25 mL with sodium citrate buffer to pH 2.2. A 0.2-mL aliquot was used for analysis on a Beckman Model 116 amino acid analyzer.

Acetyl Value. Acetyl value was estimated by the method of Lemieux and Purves (1947).

Solubility. Percentage of soluble material was determined in *N,N*-dimethylacetamide (DMAc) containing 5% lithium chloride (LiCl) following procedures described by Rutherford and Austin (1978).

Statistical Analysis. Data were subjected to analysis of variance. Mean separation was accomplished by use of the Duncan's multiple-range test (Steel and Torrie, 1980).

RESULTS AND DISCUSSION

Chemical Composition of Crawfish Whole Meal and Shell. Chemical analyses of crawfish whole meal and mechanically separated shell are shown in Table I. Both products are excellent sources of chitin at 15.9% and 23.5% by dry weight, respectively. Compared with whole meal, the shell contains a higher content of chitin and less protein. An important aspect is the level of carotenoid pigments present. Crawfish shell contained a relatively high concentration, 108 ppm, of astaxanthin compared with 78 ppm in the meal portion. Thus, an appropriate pigment extraction procedure was developed to obtain a white chitin product comparable to that of other available chitins.

The effects of differential particle sizes on chemical composition of crawfish meal are summarized and com-

Table II. Effect of Particle Size on the Composition of Crawfish Meal[a]

composition	whole	particle size, mm 2.00	0.841	0.500	0.354	0.250
protein, %	36.0 ± 0.96	27.8 ± 2.57	33.5 ± 3.76	40.4 ± 1.01	44.7 ± 2.26	45.4 ± 2.32
fat, %	9.1 ± 1.05	5.6 ± 1.60	7.4 ± 1.11	9.4 ± 1.01	10.8 ± 0.93	11.7 ± 1.23
fiber, %	13.8 ± 0.51	18.8 ± 1.47	17.6 ± 0.93	14.4 ± 0.50	10.2 ± 0.24	7.2 ± 0.36
ash, %	38.9 ± 2.01	51.0 ± 2.93	44.6 ± 2.84	35.2 ± 2.07	29.6 ± 2.36	28.1 ± 3.15
Ca, %	13.6 ± 1.13	20.2 ± 1.91	17.5 ± 1.69	12.4 ± 0.89	9.6 ± 0.83	8.6 ± 1.19
P, %	0.8 ± 0.02	0.8 ± 0.04	0.8 ± 0.06	0.9 ± 0.03	0.9 ± 0.03	0.9 ± 0.06
K, %	1.1 ± 0.19	0.9 ± 0.08	1.0 ± 0.07	1.2 ± 0.03	1.3 ± 0.03	1.3 ± 0.06
Mg, %	0.2 ± 0.01	0.3 ± 0.03	0.2 ± 0.02	0.2 ± 0.02	0.2 ± 0.01	0.2 ± 0.01
Mn, ppm	589 ± 138	364 ± 39	469 ± 48	576 ± 125	707 ± 167	876 ± 197
Fe, ppm	1745 ± 742	580 ± 90	1025 ± 247	1637 ± 588	2320 ± 760	3293 ± 1323
astaxanthin, ppm	76 ± 3.8	119 ± 5.9	93 ± 4.6	72 ± 3.6	68 ± 3.4	47 ± 2.3

[a] Mean ± standard deviation of six determinations.

Table III. Protein and Chitin Nitrogen of Crawfish Shell

composition	percent
total crude nitrogen (A)	2.7
crude protein	16.9
chitin nitrogen (B)	1.6
chitin[a]	23.2
corrected nitrogen[b]	1.1
corrected protein[c]	6.9

[a] Based on 6.9% N present in chitin. [b] Corrected nitrogen = A − B. [c] Corrected protein = corrected N × 6.25.

pared with crawfish whole meal in Table II. Changes in mineral composition probably are due to separation of larger amounts of shell portion from the meal with decreased particle size. Compared with the original whole meal, the overall increase of protein with particles smaller than 0.500 mm was 23%, with a 29% reduction of calcium. Fractions larger than 0.500 mm contained increased fiber (primarily chitin), i.e., 13.8% vs 18.1%, and this portion of the whole meal (58%) may lend itself as a source of chitin. An approximate 42% recovery was obtained by sieving the meal through a 0.500-mm mesh screen. This higher protein fraction may have more feasible application as a meal for animal ration purposes.

Crude and corrected protein values for crawfish shell are given in Table III. The average uncorrected crude protein content of the shell was 16.9%. However, the extracted chitin contained 59.3% of the total nitrogen of the shell, leaving 40.7% of the total nitrogen presumably in the form of protein. The nonchitinous nitrogen, multiplied by 6.25, gives 6.9% corrected crude protein.

Isolation of Chitin. In establishment of optimal conditions for chitin isolation, a demineralization step was followed by deproteinization. However, for actual protein recovery, the overall process followed a sequence of deproteinization and demineralization steps to maximize protein yield and quality.

Demineralization. Optimum demineralization was performed by constant stirring of the dried ground crawfish shell with 1 N HCl for 30 min at ambient temperature and a solids to solvent ratio of 1:15 (w/v). The demineralization of the shell with 1 N HCl, at a solids to solvent ratio of 1:15, was effective in decreasing the ash content, compared with a 1:10 (w/v) solids to solvent ratio (Figure 2). Decalcification for 30 min with the former ratio resulted in a 99.5% decrease in the original ash content, i.e., from 63.6% to 0.3%.

Deproteinization. Optimal conditions for deproteinization involved treatment of the decalcified shell with 3.5% (w/w) NaOH solution for 2 h at 65 °C with constant stirring and a solids to solvent ratio of 1:10 (w/v). Effects of alkali concentrations and extraction times at 65 °C on nitrogen content showed that nitrogen levels of the decalcified shell gradually decreased with an increase in alkali concentration and extraction time (Figure 3). With 3.5% NaOH and 2-h extraction time, nitrogen levels decreased to 6.86%, which approximates the theoretical value of

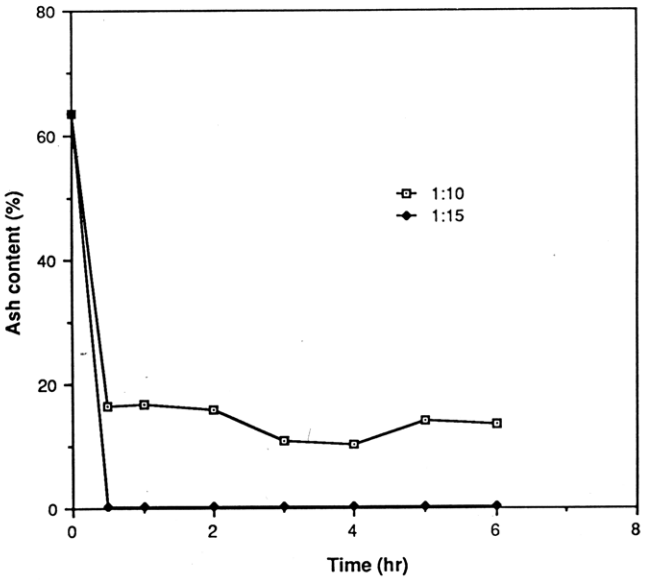

Figure 2. Effect of solid to solvent (1 N HCl) ratios and extraction times on reduction of ash content in crawfish shell at ambient temperature.

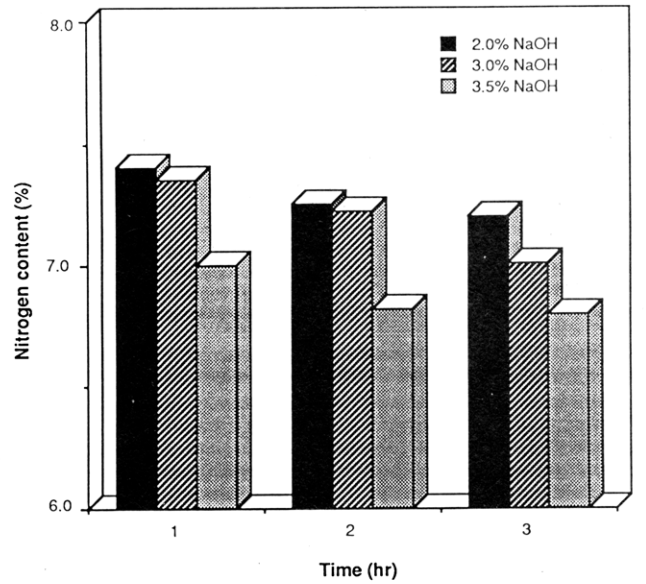

Figure 3. Effect of alkaline concentrations and extraction times on reduction of nitrogen content in the decalcified shell at 65 °C with a solid to solvent ratio of 1:10 (w/v).

Table IV. Comparison of Analytical Data for Chitins Prepared from Three Different Particle Size Ranges of Crawfish Shell and from Crawfish Whole Meal

source	particle size range, mm	percent N[a]	N[b]	fat	ash	yield
shell	2.00–0.841	7.00[a]	7.01[a]	ND[c]	0.1[a]	28.6
	0.841–0.500	7.00[a]	7.01[a]	ND	0.1[a]	25.4
	<0.500	6.98[a]	6.99[a]	ND	0.2[a]	*d*
whole meal		6.94[a]	6.99[a]	ND	0.7[b]	17.6

[a] Calculated on a moisture-free basis. [a,b] Means with the same letter in the columns are not significantly different at the 5% level. [b] Calculated on a moisture-free and ash-free basis. [c] ND = not detectable. [d] Not determined.

6.9% for pure chitin. Further treatment did not result in significant changes in nitrogen content.

Decoloration. The protocol that produced the most acceptable bleached product involved the following steps. Chitin residue, firmly complexed with the carotenoid pigment (107 ppm), was extracted with acetone and dried for 2 h at ambient temperature, followed by bleaching with 0.315% (v/v) sodium hypochlorite solution (containing 5.25% available chlorine) for 5 min with a solids to solvent ratio of 1:10 (w/v), based on dry shell. Without prior acetone extraction, bleaching for more than 1 h was needed to obtain a commercially acceptable white product.

Several workers have used reagents (as discussed in the Experimental Section) to eliminate pigments from crustacean exoskeleton, usually crab. However, with crawfish shell the reagents alone were not as effective as the procedure developed currently. This suggests that carotenoids, in all likelihood, are more strongly bound to the crawfish shell matrix than are those reported from other crustacea. Earlier, Fox (1973) found one 4-keto- and three 4,4′-diketo-β-carotene derivatives firmly bonded to the exoskeletal chitin of red kelp crab.

Effect of Particle Size and Different Raw Materials. The effects of particle sizes of the crawfish shell on composition of subsequent chitin preparations were investigated to compare extraction efficiency. Chitin from whole meal was compared with that from the shell. The compositions of nitrogen, fat, and ash in the resulting chitin products are given in Table IV.

Chitins, from three different mesh sizes of crawfish shell, did not show significant differences ($P > 0.05$) in nitrogen and ash compositions. In comparison of chitin from crawfish shell with that from whole meal, no significant differences ($P > 0.05$) were observed in nitrogen levels, but highly significant differences ($P < 0.01$) were seen in ash contents. Yields of chitin from the shell portion were higher than those from whole meal since the former contained higher contents of chitin than did the latter. Yields from particles smaller than 0.500 mm were not determined because loss of fine particles occurred during washing on a 80-mesh screen.

Characterization of Crawfish Chitin. To characterize the quality of the chitin product prepared from 0.841–0.500-mm-mesh sizes of crawfish shell, physicochemical properties were determined with results shown in Table V.

Nitrogen Content. Crawfish chitin contained 7.01% nitrogen on a moisture-free and ash-free basis, which is slightly higher than the theoretical value of 6.9% for pure chitin. This probably is due to the presence of protein residues in the crawfish chitin, as mentioned by Rutherford and Austin (1978). Numerous workers (Attwood and Zola, 1967; Austin et al., 1981; Brine and Austin, 1981; Hackman, 1960) have reported that protein is bound by covalent

Table V. Characterization of Crawfish Chitin

specification	description
nitrogen,[a] %	7.01
fat, %	ND[b]
ash, %	0.1
acetyl, %	19.6[c]
deacetylation, %	7.5
solubility,[d] %	26.4
color	white
residual amino acids, mg/g	6.5

[a] Calculated on moisture-free and ash-free basis. [b] ND = not detectable. [c] Theoretical value = 21.2%. [d] *N,N*-Dimethylacetamide containing 5% LiCl (DMAc–5% LiCl).

Table VI. Amino Acid Composition of Crawfish Chitin, Shell, and Protein Removed from Shell after Deproteinization[a]

amino acid[b]	content, mg/g chitin	shell	protein from shell
aspartic acid	0.2	4.3	24.6
threonine	0.1	2.1	6.8
serine	+[c]	2.4	1.3
proline	0.1	3.8	3.1
glutamic acid	0.1	5.2	16.9
glycine	0.3	4.1	7.1
alanine	0.1	2.6	7.4
valine	0.1	2.1	6.4
cystine	–[d]	0.2	–
methionine	–	0.3	1.5
isoleucine	0.1	1.4	6.0
leucine	0.1	2.5	11.8
tyrosine	4.7	28.4	246.4
phenylalanine	2.3	2.3	7.3
lysine	0.3	2.2	3.6
histidine	+	0.7	2.0
arginine	+	2.6	2.4
total	6.5	67.2	354.6

[a] Average of three determinations. [b] Tryptophan was destroyed in the acid hydrolysis. [c] Trace. [d] Not detectable.

bonds to chitin, forming stable complexes. Thus, it is highly improbable that pure chitin samples can be prepared without some residual protein remaining.

Ash and Fat Content. The ash content of chitin, indicative of the effectiveness of the method used for removal of calcium carbonate, was 0.1%; fat was not detectable.

Acetyl Value. The acetyl value (19.6%) of the crawfish chitin indicates that it has been partially deacetylated to 7.5%, naturally or during preparation, based on the theoretical acetyl value for chitin of 21.2%. Relatively high acetyl value of the chitin is indicative of the mild alkali treatment used.

Solubility. Crawfish chitin showed lower solubility (26.4%) in DMAc–5% LiCl compared with other chitins. Those from horseshoe crab, blue crab, red crab, pink shrimp, and brown shrimp had high solubilities ranging from 58% to 92% (Rutherford and Austin, 1978). On the other hand, the solubility (30%) of Dungeness crab chitin was comparable to that of the crawfish chitin. Rutherford and Austin (1978) noted that the low solubility of Dungeness crab chitin was due to formation of swollen gel particles, which were removed by filtration. This also was observed in the present study. Brine and Austin (1981) mentioned that relatively lower solubility values would suggest incomplete removal of protein.

Residual Amino Acids. Table VI shows the amino acid composition of crawfish chitin compared with that of the shell. Chitin isolates contain 6.5 mg/g of residual amino acids, with the most predominant being tyrosine (4.7 mg/g). Significantly higher content of tyrosine, compared with the other amino acids, also was observed in the shell.

A comparison of total amino acid content of the shell with that of chitin revealed that approximately 98% of the total amino acids in the former had been removed by mild alkaline treatment.

There are isolated reports of amino acid or protein residues in chitin preparations. Hackman (1960) reported aspartyl and histidyl amino acid residues in chitin from insects, crustacea (crab and crawfish), cuttlefish, and squid after hot alkali treatment. Karlson et al. (1969) found that the horseshoe crab (*Limulus polyphemus*) shell complex was relatively rich in glycine and alanine even after mild alkali treatment. More recent findings by Brine and Austin (1981) indicated that chitin isolates from several marine invertebrates all contained significant amounts of covalently bound amino acids (0.7–34.8 mol %), indicating substantial species-related variability.

Protein Recovery. Proximate analyses of protein from crawfish shell show concentrations of 34.1% protein and 12.8% ash. Amino acid analysis (Table VI) reveals an unusual content of tyrosine (246.4 mg/g), representing 69% of the total amino acids. Aspartic acid and glutamic acid are the other major amino acids found. Further analyses of the crawfish shell complex are in progress.

It has been shown that crawfish waste shell, as well as the meal itself, is an excellent source of the biopolymer chitin. Combination of chitin production with an existing pigment recovery process, and introduction of product fractionation approaches, represents a total byproduct utilization concept with realistic implication in other crustacean waste recovery industries. Further significance can be seen in utilization of the initial decalcified shell as a potentially valuable source of protein, chitin, and pigment for a variety of feed applications. Research from our laboratory (Lee, 1985) has demonstrated the value of the astaxanthin-rich shell as a natural red intensifier in pigmentation of egg yolk. Additional investigations are in progress to document the nutritional value of the composite substrate in various feeding trials.

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