Processing of Crawfish (*Procambarus clarkii*) for the Preparation of Carotenoproteins and Chitin

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Crawfish carotenoproteins and chitin are obtained by a combined process based on flotation−sedimentation and in situ lactic acid production. The carotenoprotein PF_1 obtained has a high content in essential amino acids, w-3-fatty acids, and carotene (mainly astaxanthin) and constitutes an excellent nutritional source for patients with malnutrition. The carotenoprotein PF_2 has a lower nutritional quality but with a substantial carotene content can be used as a feed for animals where coloration is required, such as salmon and trout bred under aquaculture. Chemical and spectrometric (FTIR and ^{13}C NMR) characterization shows the obtained chitin to be of high quality, similar to that available commercially, for medical and nutritional uses.

Keywords: *Carotenoproteins; chitin; essential amino acids; astaxanthin; crawfish; malnutrition*

INTRODUCTION

The breeding of crawfish (*Procambarus clarkii*) in the Marismas del Guadalquivir, Seville (Spain) originates a considerable amount, approximately 10^5 t/yr, of a byproduct that includes the complete animal (when not used for food), heads (thorax), and claws. This byproduct is a rich source of many valuable products such as protein, chitin, and pigments (e.g., astaxanthin) (*1, 2*). It is currently used as a supplement in animal feed, or—when not used—it is deposited on the land, constituting an important focus of environmental pollution. However, because of its high protein and chitin content (*1, 3*), this byproduct could be a good source of protein and chitin if appropriate processing is developed. Protein extracted from shellfish byproducts has been shown to be a good animal feed supplement (*4, 5*) and an excellent source for the preparation of protein hydrolyzates enriched in essential amino acids (EAAs) for human nutrition (Cremades, unpublished results). Chitin, a nitrogen-containing polysaccharide (poly-*N*-acetyl-D-glucosamine) that constitutes up to 55% of the byproduct, is one of the most promising biomaterials for the future (*6, 7*). The ketocarotenoid astaxanthin (3,3′-dihydroxy-β,β-carotene-4,4′-dione) is one of the most important and economically valuable carotenoids due to its biological functions as a vitamin A precursor (*8*). This ketocarotenoid also has a variety of applications in food technology and nutrition, such as for the pigmentation of salmon and trout bred under aquaculture (*8*) or as additive in poultry rations to improve the coloration of both flesh and eggs (*9*).

In this work, we describe a new, environment-friendly process based on the combination of flotation−sedimentation and semi-solid-state fermentation for the efficient preparation of crawfish protein concentrates (carotenoproteins) and raw chitin together with their chemical characterization.

MATERIALS AND METHODS

Samples. Crawfish (*Procambarus clarkii*) byproducts were obtained from Cangrimar S.L. (Lebrija, Seville, Spain) and included the complete animal (when not used for food), heads (thorax), and claws. The material was minced with an electronic mincer (Foss Tecator AB, Sweden) fitted with a plate perforated with holes of 4−5 mm and stored at −20 °C until required or air-dried at 50 °C in a fluid bed dryer and stored at room temperature. This product was named crawfish meal (CFM).

Sample Treatment. CFM was fractionated by sedimentation−flotation (*10*) into two fractions: a proteinaceous fraction (PF_1) and a chitinous fraction (CF_1). Fractionation was done by recirculating tap water in a reactor, designed by us (*10*), that facilitates the recovery of the flotating fraction (the PF_1) and the sedimented fraction (the CF_1). PF_1 and CF_1 were used as starting materials for carotenoprotein and chitin preparation, respectively.

Analyses. Moisture, ash, total nitrogen, and total fats were determined according to the standard methods of the AOAC (*11*). Nonproteinaceous nitrogen-containing substances (NPN-CS) (such as nucleotides, free *N*-acetylglucosamine, and *N*-acetylglucosamine oligosaccharides) were determined by HPLC methods as described elsewhere.

Protein Concentration. Protein concentration was determined by HPLC amino acid analysis after hydrolysis with 6 N HCl in the presence of phenol and under vacuum atmosphere at 105 °C for 18 h. The amino acid composition of the hydrolyzed fractions was determined by reversed-phase high-performance liquid chromatography (HPLC) analysis using the method of Bidlingmeyer et al. (*12*). Performic acid oxidation was used to determine cystine and methionine (*13*), and basic acid hydrolysis was used to determine tryptophan (*14*). The content of the different amino acids recovered is presented as milligram per gram of protein and compared with the FAO/WHO (*15*) reference pattern. The essential amino acid (EAA) score was calculated by the FAO/WHO

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method as shown below:

$$\text{essential amino acid score} = \frac{\text{mg of EAA in 1 g of test protein}}{\text{mg of EAA in 1 g of egg protein}} \times 100$$

Total Carotenoids. Total carotenoids were extracted with soybean oil (1:1, w/v), and a quantitative determination was made spectrophotometrically at 485 nm according to the procedure described by Chen and Meyers (*16*), using as extinction coefficient value of $E^{1\%}_{1cm} = 2155$.

Chitin Nitrogen. Nitrogen content of chitin was also estimated by the Kjeldahl method, after the sample (2−3 g, dry weight) had been purified of its calcium carbonate and protein by boiling with acid and alkali, respectively (*17*). Chitin was calculated by multiplying chitin nitrogen by 14.5, assuming that the pure chitin contained 6.9% nitrogen (*1*).

Mineral. Mineral content, mainly calcium, phosphorus, and potassium, was determined by an atomic absorption method, as described in ref *18*.

Solid-State ^{13}C NMR:CP/MAS ^{13}C NMR. Solid-state ^{13}C NMR:CP/MAS ^{13}C NMR spectra were recorded on a Chemagnetics CMX 360 NMR spectrometer (Chemagnetics Co. Ltd., Fort Collins, CO) at room temperature according to the procedure described by Struszczyk et al. (*19*).

FTIR Spectroscopy. FTIR spectra were recorded on a Jasco FT-IR 5300 spectrometer (Jasco Co. Ltd., Tokyo). Resolution was 4 cm^{-1}, and the scanning number was 15 (*20*).

Microorganism and Inoculum Preparation. Fermentation was carried out with the lactic acid bacterium, *Lactobacillus paracasei* strain A3, supplied by Dr. G. Hall (Loughborough University, Leicestershire, U.K.). *L. paracasei* was maintained on MRS agar slopes stored at 4 °C. A starter culture was prepared by transferring a loopful of cells from a slope of MRS agar into 5.0 mL of sterile MRS broth (Difco) and incubation at 30 °C for 24 h. The inoculum for fermentation was prepared by subsequent transfer of 2.0 mL of the starter culture into 100 mL of sterile MRS broth (2% inoculation), which was incubated statically for 24 h. Inoculum prepared by this procedure yielded a cell concentration of approximately 10^8 cfu/mL.

Semi-Solid-State Fermentation. Minced CF_1 (1.0 kg) was thoroughly mixed with 10% (w/w) dextrose and 10% (v/w) of the prepared inoculum. The semi-solid mixture was transferred to the reactor, and the entire reactor was placed in a temperature-controlled enclosure maintained at 30 °C. Rotation was controlled by an electronic timer set to provide 10 min of continuous operation every 6 h for 3 days. The gas vent was controlled by a Dreschel bottle approximately half-filled with water to provide a slight gas overpressure in the bioreactor with respect to the surroundings (*21*).

Size-Exclusion Chromatography. Proteins were separated using an Äkta purifier (Amersham-Pharmacia-Biotech, Sweden), by size-exclusion chromatography on a Sephacryl S-200 HR column (1.6 cm × 40 cm) equilibrated in 100 mM sodium phosphate buffer, pH 7.0, with 250 mM NaCl. The column was precalibrated with the following standard proteins: cytochrome *c* (12.2 kDa), α-chymotrypsinogen (25 kDa), carbonic anhydrase (31 kDa), ovalbumin (43 kDa), bovine serum albumin (67 kDa), and lipoxygenase (108 kDa). Samples were eluted at 0.2 mL/min, and proteins were detected by absorbance at two different λ: 280 and 215 nm.

Electrophoresis. SDS−PAGE was performed by the method of Laemmli (*22*). The gel system, containing 0.2% (w/v) SDS, consisted of a 15% polyacrylamide resolving gel (pH 8.8) and a 5% stacking gel (pH 6.8). The length of the resolving and stacking gels was 10 and 2.5 cm, respectively, with a gel thickness of 1.0 mm. Electrophoresis was performed at a constant current of 25 mA. Protein bands were stained by immersion of the gel in a 0.05% (w/v) Coomassie G-250 solution in a 50% methanol−10% acetic acid solution. Molecular masses were determined using the standard prestained protein broad-range molecular weight kit from New England BioLab Inc. (P7708S), comprising MBP-β-galactosidase (175 kDa), MBP-paramyosin (83 kDa), glutamic dehydrogenase (62 kDa), aldolase (47.5 kDa), triosephosphate isomerase 832.5 kDa), β-lactoglobulin A (25 kDa), lysozyme (16.5 kDa), and aprotinin (6.5 kDa).

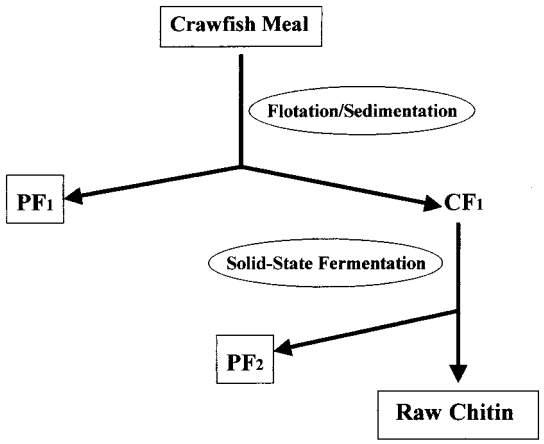

Figure 1. Schematic flowsheet for production of carotenoproteins and chitin from crawfish (*Procambarus clarkii*). PF_1: proteinaceous fraction obtained by flotation/sedimentation; CF1: chitinous fraction; PF_2: proteinaceous fraction obtained by solid-state fermentation.

RESULTS AND DISCUSSION

Crawfish Fractionation. Crawfish proteins were obtained by a two-step process, based on sedimentation−flotation and semi-solid-state fermentation, as summarized in the process flowsheet shown in Figure 1. In the first step, CFM was fractionated by a sedimentation−flotation process according to the procedure described by Bautista et al. (*10*) into two fractions: a proteinaceous fraction (PF_1), which is recovered by flotation, and a chitinous fraction (CF_1), which sediments at the bottom of the reactor. The process is carried out in a reactor that facilitates mixing, flotation, and sedimentation and allows easy recovery of both fractions.

The process yield, at this stage, was 83.5% with a recovery of 53.4% and 30.1% as PF_1 and CF_1, respectively, referred to as wet weight. However, when we expressed our results for PF_1 and CF_1 on a dry-weight basis, they were 11.0% and 20.6%, respectively, showing that the main component of crawfish is water (68.4%). The 16.5% (on wet-weight basis) of material lost during the flotation−sedimentation fractionation process comprises water-soluble substances such as sarcoplasmic proteins; peptides and oligonucleotides, originated by the proteolytic enzymes present in the digestive tract of the animals (Cremades, unpublished results); pigments; amines; vitamins; and enzymes.

Chemical Characterization of the Fractions. The chemical composition of PF_1 and CF_1 is shown in Table 1. PF_1—with a protein content, determined by amino acid analysis, of 57.22 ± 3.57% and a content of free amino acids and short peptides of 10.60 ± 0.72%—is an interesting protein source for both animal (*4, 5*) and human food (Cremades et al., unpublished results), but the main feature of this fraction is its amino acid composition, as will be discussed later. As the results of Table 1 also show, a substantial amount of protein (17.81 ± 0.87%) remains bound to the exoskeleton. If high-quality chitin is to be obtained, it is necessary to remove this protein (*1, 3, 23, 24*); thereby, we can also increase the global yield of protein recovered. This

Table 1. Chemical Composition of Crawfish Meal, PF_1, CF_1, PF_2, and Raw Chitin

	crawfish meal	PF_1	CF_1	PF_2	raw chitin
moisture (%)	7.02 ± 0.76	6.73 ± 0.41	6.90 ± 0.2	7.03 ± 0.62	6.43 ± 0.56
total nitrogen × 6.25 (%)	48.61 ± 3.72	70.1 ± 4.82	nd[a]	nd	nd
protein (%)[b]	34.51 ± 2.41	57.22 ± 3.57	17.81 ± 0.87	41.20 ± 3.12	2.07 ± 0.25
total fat (%)	12.84 ± 0.87	10.65 ± 0.72	0.92 ± 0.21	1.87 ± 0.31	nd
nucleotides (%)	nd	4.20 ± 0.35	nd	nd	nd
free AAs & short peptides (%)	5.56 ± 0.27	10.60 ± 0.72	2.76 ± 0.21	10.32 ± 0.98	nd
chitin (%)	16.11 ± 1.03	0.36 ± 0.05	28.90 ± 2.31	0.26 ± 0.04	89.12 ± 3.7
total carotenoids (μg/g)	162.27 ± 12.10	83.45 ± 6.34	147.78 ± 11.12	65.23 ± 12.42	<5
minerals (%)[c]	15.16 ± 1.03	nd	25.52 ± 2.43	nd	0.42 ± 0.06
others (%)[d]	15.80 ± 0.89	16.96 ± 0.89	24.07 ± 1.63	46.33 ± 3.23	8.39 ± 0.25
ash (%)	37.51 ± 2.49	4.12 ± 0.26	66.23 ± 3.82	20.22 ± 4.17	1.34 ± 0.25

[a] nd, not determined. [b] Determined by HPLC amino acid analysis after hydrolysis with 6 N HCl; >12 000 Da. [c] Ca + P + K. [d] Determined as difference: [100 − (protein + total fat + nucleotides + free AAs & short peptide + chitin + total carotenoids + minerals)]. Total carotenoids in 100 g.

bound protein is extracted mainly in alkaline medium with 40−50% NaOH solutions at 90−120 °C for several hours (*25*). Protease treatment has also been tried (*26*), but currently, its application is limited. The alkaline procedure is efficient, but with important drawbacks regarding amino acid modification (*27, 28*) and environmental problems. The modification of amino acids in highly concentrated alkaline media can reduce the nutritional properties of the proteins (*29*), and the disposal of the effluents causes considerable environmental deterioration (*30, 31*). Enzymatic procedures, based on the use of proteases, do not have these disadvantages: they do not modify amino acids and are environmentally friendly. However, enzymatic deproteinization is currently an expensive process, due to the high cost of enzymes, and the processes so far assayed are not as efficient as the alkaline process. After protease treatment, a significant amount of protein (2.3−4.8%) remains bound to the exoskeleton (*26*) and must be eliminated if the chitinous fraction is to be used as raw material for chitin preparation. Protease action is limited by steric hindrance of the access of protease to substrate bound to the exoskeleton support. Different approaches to overcome this problem are under investigation. One of these is solid- and semi-solid-state fermentation (*21*), which is the second step of our process.

Semi-Solid-State Fermentation. The treatment of CF_1, supplemented with dextrose, by semi-solid-state fermentation with *L. paracasei* has two effects that occur simultaneously during the fermentation process: demineralization due to the production of lactic acid and deproteinization due to the hydrolytic action of exoproteases excreted into the fermentation medium by the microorganism. The chitinous product (raw chitin) obtained shows a significant degree of demineralization, reflected as a mineral content of 0.42 ± 0.06%, and a low content of protein, 2.07 ± 0.25% (see Table 1). It is important to note that the degree of deproteinization achieved by this procedure is greater than that by the direct use of proteases, probably due to the demineralizing action of the acid, which helps the enzyme access to the exoskeleton-bound proteins. The protein obtained in this process (PF_2) does not show the drawbacks of protein obtained by alkaline deproteinization but does show the same advantages as that obtained by enzymatic treatments. The use of a cheap fermentation source, such as whey (*32*), cassava starch (*33*), or lignocellulose (*34*) instead of dextrose or glucose, might offer a commercial route for the deproteinization and demineralization of crawfish exoskeleton, or chitinous fraction, leading to the obtaining of raw chitin.

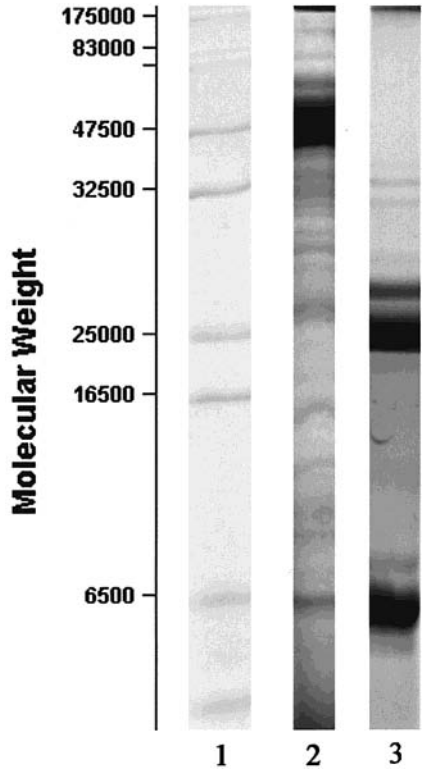

Figure 2. SDS−PAGE of crawfish PF_1 (lane 2) and PF_2 (lane 3). Lane 1, protein standards; for details, see text.

Crawfish Carotenoproteins. The analysis, by SDS−PAGE (gradient, T = 8−15%, C = 3%), of soluble proteins present in PF_1 and PF_2 is shown in Figure 2. The SDS−PAGE protein pattern of PF_1 (Figure 2, lane 2) shows an electrophoretic pattern constituted by numerous bands spread out over the whole gel in a range of molecular weight from less than 6.5 kDa to more than 175 kDa. The main bands are those corresponding to proteins between 200 and 160 kDa and those between 40 and 60 kDa, representing 12 and 73% of total proteins, respectively, which correspond to myosin, actin, tropomyosin, and troponins, indicating that PF_1 contained mainly sarcoplasmic and myofibrillar-soluble proteins.

The analysis, by HPLC size-exclusion chromatography, of soluble crawfish proteins present in PF_1 (results not shown) confirms this result. The HPLC profile of PF_1 is characterized by numerous peaks spread out over a large range of molecular weight from less than 10 kDa to more than 400 kDa, corresponding to sarcoplasmic and some myofibrillar-soluble proteins—for example,

Table 2. Amino Acid Composition of PF_1 and PF_2 (Expressed as Milligram of Amino Acid Residues/Gram of Protein) Comparison with FAO/WHO Requirement Pattern[a]

	PF_1	PF_2	FAO/WHO requirement pattern
Asp	101.2	95.6	
Glu	148.1	113.6	
Ser	45.5	55.7	
His	29.0	59.0	
Gly	52.1	149.4	
Thr	65.6 (1.93)	33.6 (0.98)	34
Arg	62.2	69.4	
Ala	70.6	59.7	
Pro	25.0	111.1	
Tyr	42.2	31.6	
Val	51.2 (1.46)	31.1 (0.88)	35
Met	31.4 (2.56)[b]	23.7(1.42)[b]	25[b]
Cys	32.6	11.7	
Ile	41.2 (1.47)	23.2 (0.83)	28
Leu	72.7 (1.10)	45.1 (0.68)	66
Phe	49.1 (1.45)[c]	35.2 (1.06)[c]	63[c]
Trp	7.3 (0.73)	5.4 (0.54)	11
Lys	73.0 (1.26)	45.9 (0.79)	58
Total EAA[d]	466.3 (1.46)	286.5 (0.89)	320

[a] Values are the average of three determinations. Numbers in parentheses are the essential amino acids score. [b] Met + Cys. [c] Phe + Tyr. [d] Total essential amino acids (mg of amino acid residues/g of protein).

actin (42 kDa) and myosin heavy chain (200 kDa) and its aggregates (400 kDa).

The electrophoretic pattern of PF_2 (Figure 2, lane 3) shows the presence of a main protein band of molecular weight around 18−20 kDa, which represents 27% of the total proteins, probably corresponding to domains of the main proteins bound to the exoskeleton and resistant to hydrolysis by the lactobacillus-excreted proteases (Cremades, unpublished result), and a group of low molecular weight proteins (<6.5 kDa) and peptides originated by the action of the endoproteases excreted by *L. paracasei* on the accessible parts of the main bound protein, which represents 35% of the total protein. The HPLC profile of PF_2 (results not shown) confirms this result. It shows two main peaks of 40 and 22 kDa and, at the end of the chromatogram, a fraction of several lower peaks constituted by low molecular weight peptides and free amino acids.

The amino acid profiles and the essential amino acid scores of both PF_1 and PF_2 are presented in Table 2. The amino acids analyzed represent both free and combined amino acids. PF_1 is an excellent protein source. It contains all the essential amino acids, which accounted for 46.63% of the total amino acid content, and is a good starting material for the preparation of high-quality products that require a high content in essential amino acids. Its composition (Table 2) meets all the FAO requirements for nutritional purposes. The high content in essential amino acids and in ω-fatty acids (Cremades, results not published) together with its content in carotenoids (mainly astaxanthin)—162.27 μg/g of product bound to protein, which stabilizes these antioxidants—make this product an important protein source for the nutrition of patients needing protein of high-quality rather than large amounts and/or an antioxidant source, as in the case of cancer, AIDS, hepatic, and renal patients and the elderly (*35*).

PF_2 is a protein source of lower amino acid profile quality than PF_1. However, due to its protein and carotene content of 41.20% and 65.23 μg/g, respectively, it can be used as a good protein source for animal nutrition where coloration improvement is required, such as for poultry or salmonoid fish (*8*, *9*). These proteins also have good functional properties due to their molecular weight profile, in the main being hydrolyzed proteins, soluble in a broad range of pH. Because of their high content in glycine and proline, 14.94 and 11.11%, respectively, these proteins can be considered collagen-like proteins.

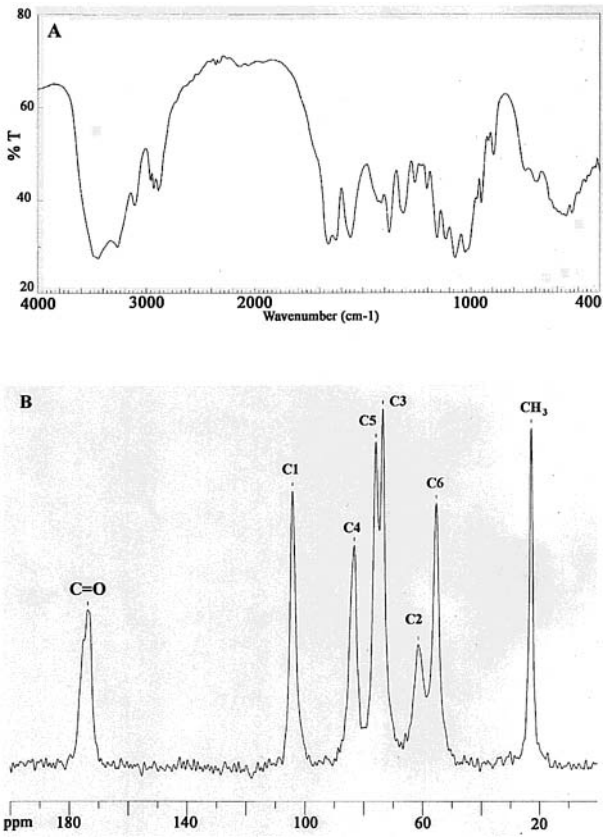

Figure 3. FTIR (A) and ^{13}C NMR (B) spectra of chitin demineralized by lactic acid fermentation and treated with 0.5 M HCl, 0.25 M NaOH, and 1:5 diluted hypochlorite (purified chitin).

Chitin Preparation. The raw chitin obtained, after semi-solid lactic acid fermentation followed by sequential treatment with 0.5 M HCl, 0.3 N NaOH, and a 1:5 dilution of hypochlorite at room temperature for 6 h each treatment, leads to a high-quality chitin (purified chitin) as demonstrated by its IR and ^{13}C NMR spectra (see Figure 3). The IR spectrum of crawfish chitin shows a low degree of deacetylation (30%) that is particularly evident from its well-resolved hydrogen-bonded NH bands of the amide group at 3120 cm^{-1} (symmetric) and 3340 cm^{-1} (asymmetric) bands. The NMR spectrum shows that both demineralization and deproteinization seem to be effective, as indicated by the presence of only one carbonyl signal at ca. 174 ppm (i.e., absence of carbonate) and the absence of any pronounced peak at ca. 30 ppm. Comparison of chemical shifts (results not shown) confirms that crawfish chitin is α-chitin. Another important characteristic of raw chitin is its property of acting as an excellent elicitor for the production of antifungal proteins, mainly chitinases, by *Trichoderma* strains (Bautista et al., unpublished data).

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