

The Purification and Characterization of the  
Chitinase and Chitobiase Enzymes Produced by

EF4a

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Chitin, predominantly a polymer of B(1-4) linked N-Acetyl-D-glucosamine molecules, is found within the cell walls of fungi, bacteria, the exoskeletons of insects, and the coverings of crustacea. Chitin is degraded by two classes of enzymes, the chitinases and the chitobiases. Chitinases primarily cleave chitin into the dimer known as chitobiose and other soluble chitodextrins. Studies involving the chitinase of *Serratia marcescens* have indicated that its activity relies on the acetyl group of the N-acetylglucosamine molecule. The enzyme has no activity against modified chitin whose acetyl groups have been replaced by H or OH groups (12). Whether or not the chitobiase enzyme has the same specificity has not been determined. This enzyme degrades the chitobiose and other soluble chitodextrins produced by the action of chitinase into the monomer N-acetyl-D-glucosamine molecules.

Chitinases have been isolated from a variety of organisms. Their molecular weights have been determined by electrophoretic techniques, column chromatography, and density centrifugation. The molecular weights of *Serratia*, *Aeromonas*, *Vibrio*, and several *Streptomyces* species have been determined as 52,000 and 58,000; 110,000; 63,000; 30,000 and 56,000 daltons respectively (16)(20) (14)(6)(3)(2). Chitinases have also been isolated from sources such as Yam, Wheat germ, Spider, and Stable fly with the following respective molecular weights 33,500; 30,000; 48,000±4,000 and 48,500 daltons (19)(10)(11)(4).

Not a lot of regard has been given to the chitobiase enzyme which is normally a part of the chitin degradation system. Isolations from *Streptomyces* and *Aeromonas* species indicate molecular weights of 50,000 and 105,000, respectively (6)(3)(20). The Spider produces a chitobiase having a molecular weight of 108,000±6,500 daltons which is very similar to the chitobiase of the *Aeromonas* species(20). It should also be noted that the molecular weights of the chitobiases are very close to those found for the chitinases in those systems.

Purification procedures attempt to separate these two enzymes which is difficult due to molecular weight similarities and the substrate of the chitobiase enzyme is the product of the chitinase enzyme's action. Some chitinase purification procedures have led to over a five fold reduction in the level of chitobiase activity found in the purified extract. Total elimination of chitobiase activity has not been accomplished; some researchers theorize that this activity is intrinsic to the chitinase enzyme (16).

Attempts have been made to isolate the chitinase and chitobiase enzyme produced from yet another source, a bacterial pond isolate identified by CDC as an EF4a. This paper describes the purification procedures used in the separation of these enzymes and the methods employed to determine their molecular weights.

#### Methods

Enzyme purification. Cultures of Ef4a were maintained on chitin agar plates and grown in mineral salt chitin broths as described by Smucker (18). Broth cultures were monitored throughout their growth and harvested by centrifugation at 10,000rpm once the culture entered the stationary phase. The supernate was then subjected to ~~the~~ ultrafiltration using a size exclusion filter which allowed passage of proteins with molecular weights less than 10,000 daltons. The retentate was then purified by the Affinity Chromatography method described by Jeulinaux(8). This method relies on the binding of the chitinase enzyme to the insoluble chitin particles followed by decantation of the unbound proteins. The resuspended pellet is then allowed to digest the chitin resulting in unbound chitinase.

Assays. Protein concentraion was determined using the Biorad protein dye and by absorption at 280nm. Both methods employed Bovine Serum Albumin as a standard.

Chitinase activity was determined by the method of Molano(9). In this reaction, .5ml of enzyme is reacted with 20 $\mu$ l of tritiated chitin for one hour at 37°C. The reaction is stopped by the addition of .2ml of a 10% solution of TCA. Each sample is then filtered to remove any undigested particulate chitin. The filtrate is suspended in liquid scintillation fluid and counted. The level of chitinolytic activity is determined by the radioactivity of the soluble chitodextrins present within the filtrate.

Chitobiase activity was determined colorimetrically by warming .1ml of enzyme and .1ml of Na<sub>2</sub>PO<sub>4</sub> buffer to 37°C followed by the addition of 1ml of a solution of 20mg of p-Nitrophenyl-N-acetyl-B-D-glucosaminide dissolved in 100ml of water also warmed to 37°C. After incubation at 37°C for 10 minutes, the reaction is halted by the addition of 2ml of .1M Na<sub>2</sub>CO<sub>3</sub>. Absorbance was measured at 420nm.

Electrophoresis. Polyacrylamide gel electrophoresis was carried out under denaturing conditions using SDS. The gels were 12% polyacrylamide and 1%SDS. Protein samples were denatured by suspending .3ml of sample in 1ml of a buffer solution consisting of 4ml distilled water, 1ml .5M Tris-Cl pH 6.8, .8ml glycerol, 1.6ml of a 10% SDS solution, .4ml 2-Mercaptoethanol and .2ml of a .05% solution of Bromophenol blue. The 1.5mm slabs were then run with a current of 20mA per gel through the stacking gel, increasing the current to 30mA per gel once the separating gel was reached. An electrode buffer consisting of 3g Tris base, 14.4g of Glycine, and 1g of SDS, was adjusted to pH 8.3 in one liter of water was used for conduction of the current. Gels were stained with Commassie Brilliant Blue R and destained by a 7% acetic acid solution. R<sub>F</sub> values were determined by dividing the distance traveled by the protein by the distance traveled by the tracking dye. The following standards were used for determination of molecular weights of the sample proteins: Bovine serum albumin 66,000d, Egg albumin 45,000d, Glyceraldehyde-3-P 36,000d, Carbonic anhydrase 29,000d, Trypsinogen 24,000d, Trypsin inhibitor 20,100d, and  $\alpha$ Lactalbumin 14,200d.

All enzyme samples were reacted with the sample buffer for a period of 3-4 hours at room temperature or for 5 minutes in a 98°C water bath.

Column chromatography was carried out using a column packed by gravity and also by a peristaltic pump. TSK HW 55 fractogel was used as the packing material based on its size exclusion separating abilities. .1M and .01M  $\text{Na}_2\text{PO}_4$  buffers were used as eluants.

## Results

Ultrafiltration of Crude supernate followed by affinity chromatography resulted in a 45 fold purification of the chitinase. See Table 1. The majority of chitobiase activity was decanted after the first centrifugation in the affinity procedure. A lot of chitinase activity was also lost in this supernate. It is interesting to note, crudes which were frozen for a brief period, about 5 days, prior to ultrafiltration, had very high chitobiase activity in the first supernate. Crudes which were only refrigerated for an equivalent length of time, showed less chitobiase activity overall. Cultures grown for longer periods of time also exhibited lower levels of chitobiase activity.

Although in a crude form, the chitobiase activity found within the affinity chromatography supernate 1 has been maintained for over 5 months by refrigeration with little loss in activity.

The retentate sample used for the source of crude enzyme for the affinity procedure also exhibited a very high chitobiase activity as well as a high chitinase activity. Freezing of this sample for several months resulted in a loss of almost all chitobiase activity but a retention of chitinase activity.

SDS electrophoretic studies of fresh retentate samples exhibit numerous protein bands with a very prominent band at 42,000d.

Table 1: Enzyme Purification

Sample	Volume ml	Protein mg/ml	Activity*			Purity
			Total	Total	Specific	
Crude	460	.27	124mg	68098	549	1
Retentate	18	5.22	94	6189	66	.12
Affinity Sup. 1	13.8	3.26	45	4433	98	.18
Affinity Product	18	.009	.162	3988	24620	44.8

\*Activities are in thousands.

This band was also observed with electrophoretic studies done after several months of frozen storage. The majority of other bands observed when the sample was fresh were either greatly diminished in their intensity or absent totally. A band at this location of 42,000d is also present in the first affinity chromatography supernate. Application of the affinity supernate to a TSK HW 55 column elutes a number of chitinolytic fractions which trail off in their activity. SDS electrophoresis of these chitinolytic fractions also produced a band at the 42,000d location. Fig. 1, 2.

An affinity chromatography product when fresh also exhibited a band at the 42,000d position, as well as, one at 35,000d. The 35,000d band was solely observed in the affinity chromatography products obtained from other crudes. This band was also seen in electrophoresis of the retentate and affinity supernate 1. However, in these two samples the 42,000d band was by far larger.

To ensure that the 35,000d band was not solely an impurity some how concentrated by the affinity chromatography procedure, a product was placed on the column. Absorption at 280nm of the fractions revealed a single peak eluted. Assays for chitinase activity in the fractions comprising the peak were positive. Fig. 3.

Column chromatography was also utilized in attempts to isolate the chitobiase enzyme. The first supernate of the affinity chromatography procedure was employed as a source for this enzyme. Like the chitinase enzyme, the chitobiase enzyme was

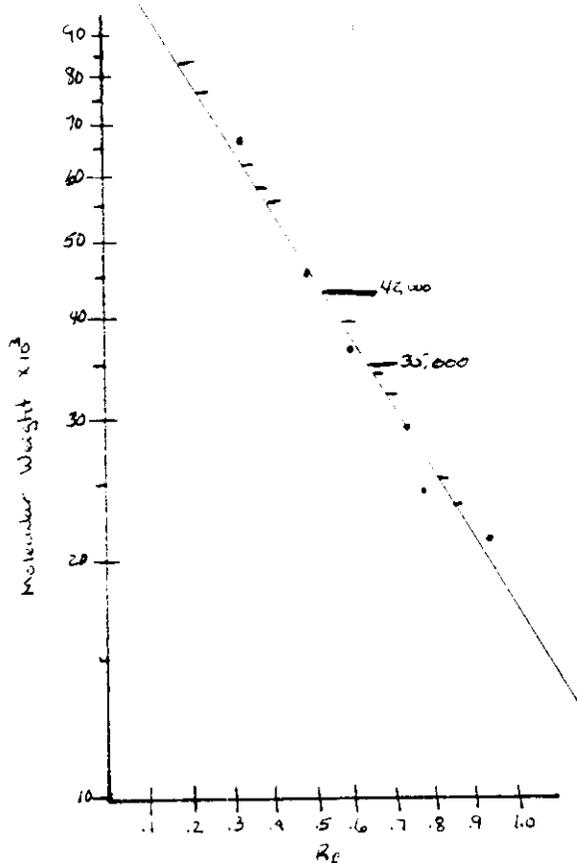


Fig 1. SDS electrophoresis of a retentate when fresh. The 42,000 band was large and retained after frozen storage. The 35,000 band was also exhibited at both times.

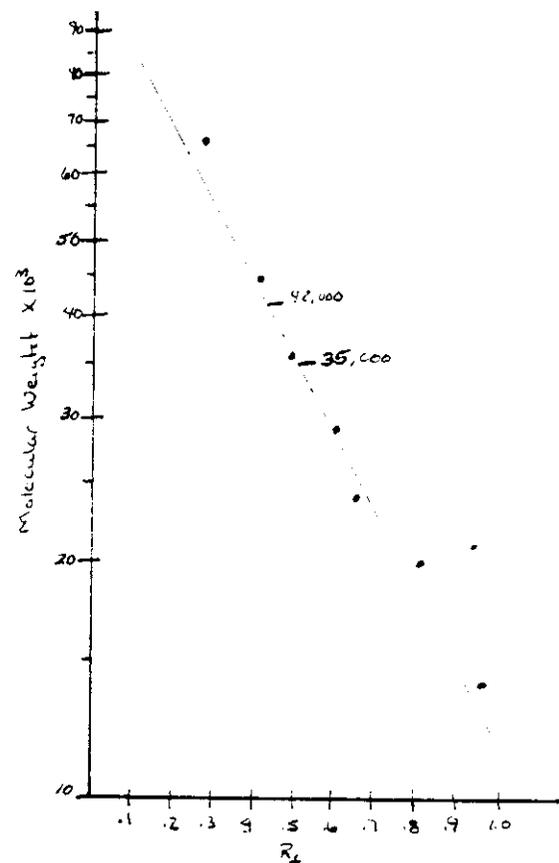


Fig. 2. SDS electrophoresis of the products of affinity chromatography. Product 4 exhibited both bands all others only showed the 35,000 band.

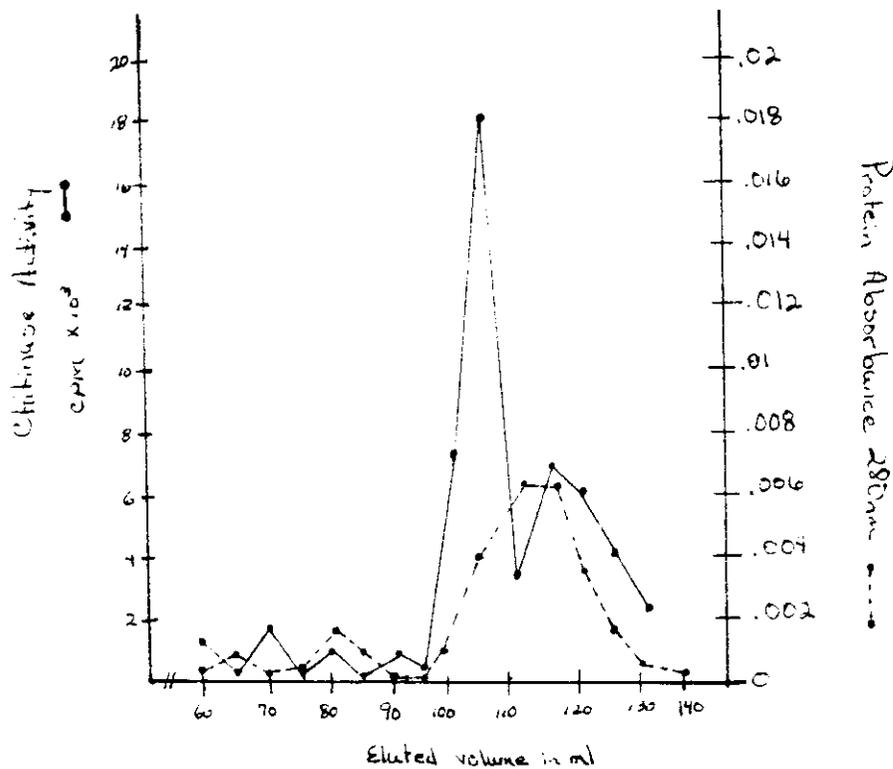


Fig 3. Column chromatography using .01M  $\text{Na}_2\text{PO}_4$  buffer to elute affinity chromatography product two from the TSK HW 55 column.

eluted over several fractions. The elution of chitobiase was always prior to chitinase, but a region of overlap existed where fractions exhibited some activity of both enzymes. Fig 4, 5. Attempts to determine the molecular weight of the chitobiase enzyme by subjecting fractions high in chitobiase activity to SDS electrophoresis were unsuccessful.

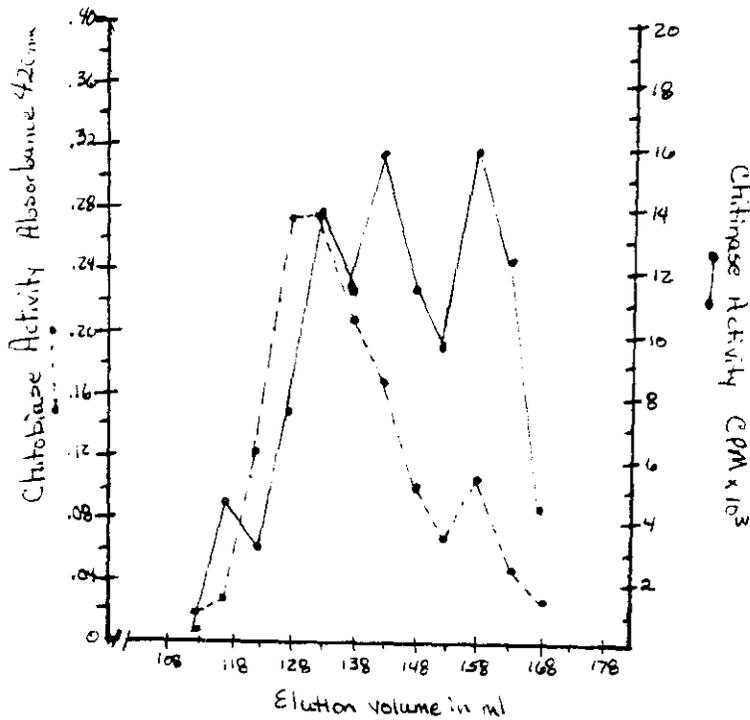
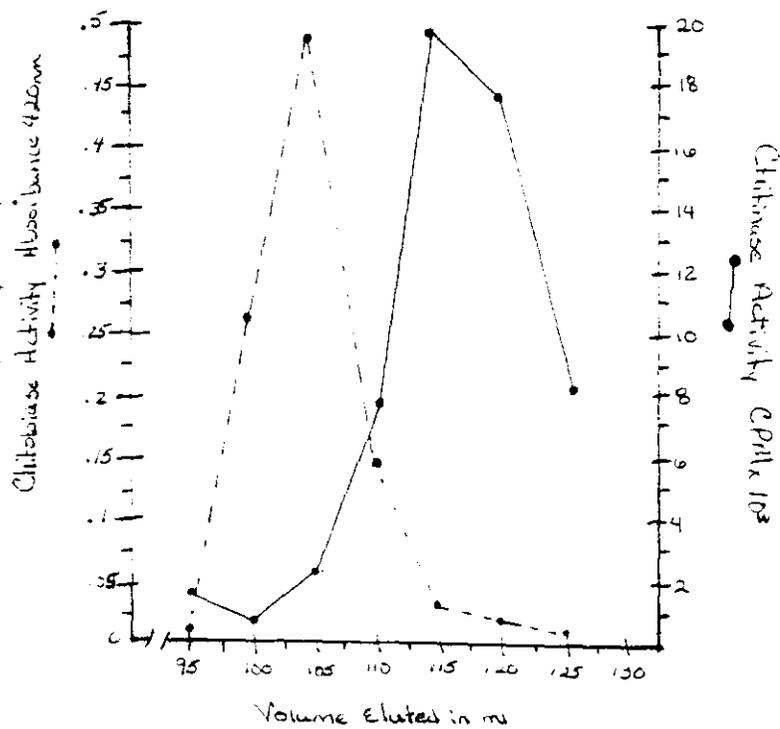


Fig. 4. Column chromatograph using a gravity packed TSK HW 55 column and .1M Na<sub>2</sub>PO<sub>4</sub> buffer. The chitinase and chitobiase enzymes both trail over several fractions.

Fig. 5. Column chromatography of affinity supernate 1. using .01M Na<sub>2</sub>PO<sub>4</sub> buffer was used, the column was still under gravity packing, but had been repacked since the data presented in Fig. 4 was obtained. This obtained better separation of the two enzymes.



## Discussion

Molecular weight studies of the various fractions obtained during the chitinase purification procedure indicate the presence of two chitinases having molecular weights of 42,000 and 35,000d. The absence of the 42,000d band in affinity chromatography products which were frozen prior to electrophoresis possibly indicates this enzyme is more temperature sensitive or the 35,000d band represents a chitinase with a greater specificity for the substrate. The later may be indicated since the 35,000d band was present in all affinity chromatography products obtained, as well as, in the retentate and affinity supernate 1. The 42,000d band also persisted in retentate samples which had been frozen for prolonged periods of time. However, the size of the band was somewhat diminished. Further evidence for the 42,000 band being a chitinase comes from the chitinolytic fractions obtained by column chromatography of the affinity chromatography supernate 1. Using a  $\text{Na}_2\text{PO}_4$  buffer at pH 7 at either a .1M or a .01M concentration, fractions exhibiting chitinolytic activity produced bands at 42,000d when subjected to SDS electrophoresis. An early column fractionation attempt done when the column had been packed by gravity, produced a fraction having chitinolytic activity but which produced bands at 83,000d, 55,000d, 42,500d and 26,750d when subjected to electrophoresis. This same fraction after several weeks of storage in the refrigerator retained its chitinolytic activity but when electrophoresied exhibited only the band at 42,000d. Within other systems, evidence for the existence of several chitinases having similar molecular weights exists. Research involving *Serratia* and *Streptomyces* have indicated the presence of two or more chitinases with close molecular weights (2)(3).

Although unable to isolate the chitobiase enzyme by SDS electrophoresis, the fact that it eluted in close proximity to the chitinase enzyme on a size exclusion column indicates the closeness of their molecular weights. Using gel filtration techniques on a Bio-Gel P 150 column, the chitinase, chitobiase,

and chitosanase were eluted almost together with molecular weights ranging from 40-50,000d (2). Chitobiase and chitinase enzymes with close molecular weights have also been reported for *Aeromonas* (20).

The observed stability of the EF4a chitinase under frozen conditions is similar to yeasts. Correa found that purified yeast chitinases remained stable for several months when frozen at 0-5°C in a sodium citrate buffer with .02% sodium azide (5).

Chitobiase activity is also retained for several months if samples exhibiting this activity are refrigerated. Freezing leads to a great reduction in the activity of this enzyme.

For the conclusions drawn in this paper to be acceptable without question, electrophoretic studies under nondenaturing conditions need to be carried out. Attempts at this so far have been unsuccessful. Presumably because the proteins were denatured due to overheating of the glass surrounding the slab gels. Disc electrophoretic studies showed no bands at all, possibly due to errors in gel content or migration of the protein to the anode or cathode causing elution from the gel. A *Streptomyces griseus* chitinase has been observed to separate into two different chitinolytic proteins one migrating to the anode, the other to the cathode at pH 7. (12) Possibly, a similar mechanism is happening within our system resulting in the absence of any bands under nondenaturing conditions.

It would also be interesting to measure the amount of activity lost over time by freezing and refrigeration of highly chitinolytic samples, highly chitobiotic samples, and samples containing activities of both enzymes. I would anticipate retention of chitinase activity with loss of chitobiase activity in a more purified sample than the retentate when the sample is subjected to freezing. Some data was acquired which indicated the loss of chitinolytic activity over a period of refrigeration, it would be interesting to compare the loss of activity under frozen conditions with this and with retention of chitobiase activity. Chitobiase may be the more stable enzyme under the milder temperature.

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