Properties

Stability. After 3 months of storage at −15°, during which time the enzyme was repeatedly frozen and thawed, the activity decreased approximately 10%; after 6 months, a decrease of 30% was noted.

pH Optimum. Alginate lyase activity exhibited maximal velocity in the pH range of 7.0–8.0.

Salt Activation. There is a striking dependence of enzymatic activity upon salt concentration.[3] Maximal activation was obtained at 0.05 *M* KCl; higher concentrations were inhibitory. Other salts giving activation were K_2SO_4, K_2HPO_4, NaCl, and Tris-HCl.

Specificity. Hyaluronic acid, chondroitin sulfate A, B, and C, and polygalacturonate were inactive. When the free uronic acids, D-mannuronate and L-guluronate, were incubated with the enzyme, no detectable reaction occurred.

Nature of the Products of the Enzyme Reaction

The crude extract can on exhaustive digestion convert 95% of the alginic acid to the monosaccharide, 4-deoxy-5-ketouronic acid. The DEAE-cellulose fraction however converts only 70% of the alginic acid to the monosaccharide. The rest of the alginic acid was degraded to oligosaccharides ranging in size from the trisaccharide to the nonasaccharide. These oligosaccharides contained an unsaturated uronic acid at the nonreducing end.

[111] Chitinases

By CHARLES JEUNIAUX

Definition and Nomenclature

The complete enzymatic hydrolysis of chitin to free *N*-acetyl-D-glucosamine is performed by a chitinolytic system, consisting of two hydrolases, the action of which is consecutive. The chitinase: poly-β-1,4-(2-acetamido-2-deoxy)-D-glucoside glycanohydrolase: EN[1] 3.2.1.14) hydrolyses the polymers of *N*-acetyl-D-glucosamine, including tetramers and, to a lesser extent, trimers.[2] Chitobiase (chitobiose acetamidodeoxyglucohydrolase: EN[1] 3.2.1.29) hydrolyses chitobiose (the dimer of *N*-acetyl-D-glucosamine) and chitotriose.

[1] Enzyme Nomenclature (International Union of Biochemistry), Elsevier, Amsterdam, 1965.

[2] L. R. Berger and D. M. Reynolds, *Biochim. Biophys. Acta* 29, 522 (1958).

Occurrence

1. Bacteria and Plants

Chitinase production is widely distributed among bacteria (*Chromobacterium, Klebsiella, Pseudomonas, Clostridium, Vibrio*),[3] and especially Streptomycetes.[4,5] Chitinases are also produced by molds and fungi (especially *Lycoperdon*[6]) and may be found in the "emulsin" of sweet almonds.[7] Chitinases may be synthesized in the absence of substrate ("constitutive enzyme")[3,8,9] or in its presence ("adaptive enzyme")[4] However, addition of chitin to culture media greatly enhances enzyme production.

2. Animals

Chitinases are synthesized by some protozoans, and by different glandular tissues of the digestive system of many coelenterates, nematodes, polychaetes and oligochaetes, molluscs, and arthropods. In vertebrates, chitinases are secreted by the pancreas and the gastric mucosa of insectivorous fishes, amphibians, and reptiles, as well as by the gastric mucosa of some insectivorous birds and mammals.[10] Chitinases are also secreted by the epidermis of nematodes during the hatching process[11] and by the epidermis of arthropods at the time of molting.[12] For an exhaustive review of chitinase distribution in animals, see Jeuniaux.[13]

Assay Methods

1. Methods Based on the Estimation of Substrate Degradation

a. Viscosimetric Methods

The action of chitinases on solutions of chitosan,[6] glycol-chitin,[14] or carboxymethylchitin[15] results in a decrease in viscosity, the rate of which is a function of enzyme concentration. However, the absolute rate of

[3] P. H. Clarke and M. V. Tracey, *J. Gen. Microbiol.* 14, 188 (1956).
[4] D. M. Reynolds, *J. Gen. Microbiol.* 11, 150 (1954).
[5] C. Jeuniaux, *Compt. Rend. Soc. Biol.* 149, 1307 (1955).
[6] M. V. Tracey, *Biochem. J.* 61, 579 (1955).
[7] W. Grassman, L. Zechmeister, R. Bender, and G. Toth, *Ber.* 67, 1 (1934).
[8] C. Jeuniaux, *Arch. Intern. Physiol. Biochim.* 66, 408 (1958).
[9] L. Claus, *Arkiv Mikrobiol.* 40, 17 (1961).
[10] C. Jeuniaux, *Nature* 192, 135 (1961).
[11] W. P. Rogers, *Nature* 181, 1410 (1958).
[12] C. Jeuniaux, *Bull. Soc. Zool. France* 86, 590 (1961).
[13] C. Jeuniaux, "Chitine et chitinolyse, un chapitre de la biologie moléculaire." Masson, Paris, 1963.
[14] A. Otakara, *Bull. Agr. Chem. Soc. Japan* 25, 50 (1961).
[15] E. Hultin, *Acta Chem. Scand.* 9, 192 (1955).

viscosity decrease may vary with the mode of preparation of the substrates and with other factors. Viscosimetric methods thus appear to be hardly useful for absolute standardization of chitinase measurement. Such methods have, however, been successfully used as a current tool for chitinase estimation during purification or kinetic studies.[6,14,16]

b. Nephelometric (= Turbidimetric) Method

This method[8] consists in the measurement of the turbidity variation of a colloidal chitin suspension during chitinolysis. This method is rapid and accurate, but is suitable only for the estimation of relatively high activities, in limpid and poorly colored solutions.

Preparation of Substrate. Shrimp shells are successively treated with cold 1 N HCl, 0.5 N NaOH at 100°, 0.5% $KMnO_4$ at 60° (20 minutes), cold half-saturated $Na_2S_2O_5$, hot water, and alcohol, then ground in water (Waring blendor). A 500 ml suspension containing 20 g (dry weight) of pure chitin is progressively mixed with 640 ml cold concentrated H_2SO_4, the temperature being kept below 10°. Viscous acid solution (100 ml) is filtered through glass wool into 900 ml distilled water or 50% aqueous ethanol, under continuous stirring. The chitin precipitate is washed with distilled water up to pH 5 and brought to a concentration of 1.8 mg/ml. This colloidal suspension is suitable for the nephelometric method if a 0.3 mg/ml chitin suspension has an absolute turbidity between 2.1 and 3.5, stable during at least 60 seconds.

Reagents and Procedure. Pyrex tubes (calibrated for nephelometer) receive 1 ml colloidal chitin suspension (1.8 mg/ml), 1 ml buffer (citric acid 0.6 M–Na_2HPO_4 1.2 M, pH 5.1), 0.1–4 ml enzymatic solution, and distilled water up to 6 ml. The tubes are stoppered with rubber corks. During the incubation at 37.5°, the chitin is brought back into suspension by simply turning the tubes upside down every 15 minutes. The relative turbidity (T) is measured immediately after mixing the reagents (T_0), then after 2 hours (T_2).

Units. Ten nephelometric units[8] correspond to a 50% turbidity decrease of the 6 ml colloidal chitin suspension (0.3 mg/ml) within 2 hours at 37.5° and pH 5.3. The amount (c) of chitinase, expressed in nephelometric units, is thus given by the formula

$$c = \frac{10}{0.3} \log \frac{T_0}{T_2}$$

2. Methods Based on the Measurement of End Products

Principle. The acetylglucosamine (AG) released from chitin may be measured by the colorimetric method of Morgan and Elson,[17] modified

[16] A. Otakara, *Bull. Agr. Chem. Soc. Japan* 25, 494 (1961).
[17] W. T. J. Morgan and L. A. Elson, *Biochem. J.* 28, 988 (1934).

by Reissig *et al.*[18] As chitobiose does not react, this method can be applied to chitinase estimation only if the chitinolysis proceeds in the presence of chitobiase in sufficient amount. In the case of enzymatic extracts poor in chitobiase (i.e., those of vertebrate glandular tissues) chitobiase has to be added. It has been calculated[13] that a chitobiase activity of 240 μg AG liberated per milliliter per hour is necessary in order to obtain by this method a correct estimation of a chitinase activity less than or equal to 30 μg hydrolyzed chitin per milliliter per hour using 1.25 mg native chitin per milliliter as substrate.

Reagents

"Native" chitin suspension prepared from cuttlefish bones (mechanical grinding of dried bones, decalcification of the powder by $0.5\,N$ HCl, treatment by $0.5\,N$ NaOH at 100°, and washing). The native chitin powder suspension is brought to 5 mg/ml.
Buffer: citric acid $0.6\,M$–Na_2HPO_4 $1.2\,M$, pH 5.1
Chitobiase solution: β-glucosidase (Nutritional Biochemicals Co.), 0.3 mg/100 ml, or lobster blood serum diluted 10 times with water
Potassium tetraborate ($K_2B_4O_7$), $0.8\,M$
p-Dimethylaminobenzaldehyde (DMAB): 1 g in 100 ml glacial acetic acid containing 1.25% (v/v) HCl $10\,N$ (prepared freshly)

Procedure. The test tubes contain 1 ml chitin suspension, 1 ml buffer, 1 ml chitobiase solution (if necessary), 0.1–2 ml enzymatic solution, and water up to 4 ml. After 0, 90, and 180 minutes of incubation at 37°, 1 ml is pipetted into a centrifuge tube containing 1 ml distilled water; it is covered with glass cap, boiled for 10 minutes, and centrifuged. The supernatant is used for AG measurement as follows: two samples of 0.5 ml supernatant, each receiving 0.1 ml tetraborate, are boiled for 3 minutes exactly (boiling water bath); after cooling, 3 ml DMAB solution is added, mixed, and allowed to stand for 20 minutes at 37°. The samples are cooled and read within 10 minutes at 585 mμ. A calibration curve is established with 3 solutions of AG (0.1, 0.2, and 0.4 μM).

Units. The activity is expressed as micrograms AG liberated per hour per milliliter or, better, as micrograms chitin hydrolyzed per hour per milliliter (correction factor: 0.92).

Purification Procedures

Chitinases have been purified from culture filtrates of *Streptomyces antibioticus*,[8,13,19] *Streptomyces griseus*,[1,4] and *Aspergillus niger*.[14,16] The former preparation has been shown to be homogeneous in the ultra-

[18] J. L. Reissig, J. L. Strominger, and L. F. Leloir, *J. Biol. Chem.* **217**, 959 (1955).
[19] C. Jeuniaux, *Arch. Intern. Physiol. Biochim.* **67**, 597 (1959).

centrifuge and in solubility tests in ethanol and ammonium sulfate, and is devoid of any trace of other enzymes (chitobiase, amylase, cellulase, proteolytic enzymes).

Preparation of Streptomyces Culture Filtrates. Culture medium composition: KCI 0.5 g; K_2HPO_4 1 g; $MgSO_4 \cdot 7\ H_2O$ 0.5 g; $FeSO_4$ 0.01 g; chitin (colloidal suspension) 10 g; pH adjusted to 7.5; diluted up to 1 l with distilled water. The medium is distributed in Erlenmeyer flasks (300 ml in 1-l flask), autoclaved, and inoculated with spores of *Streptomyces* (grown on agar plates). The flasks are incubated at 25° on a planetary agitator (120–180 rotations per minute). The production of chitinase is followed (nephelometric method) and generally reaches its maximum after 4–5 days. The preparation is centrifuged and filtered through glass wool.

Purification Procedure. The procedure is presented schematically in the table.[19]

PURIFICATION OF *Streptomyces antibioticus* CHITINASE

Step	Operation	Volume (ml)	Chitinase activity[a]	Yield (%)	Specific activity[b]
Culture filtrate ↓	Acidification by 0.02 *N* H_2SO_4 at 0°C to pH 5.2	1000	34	100	45
Filtrate pH 5.2 ↓	"Mass" adsorption on colloidal chitin (4.4 mg chitin for 200 units; blend 10 minutes at 0°; centrifuge 10 minutes at 0°)	1060	29	90	42
Chitin sediment ↓	Wash sediment with buffer (citric acid–Na_2HPO_4, 0.02 *M* pH 5.2) at 0° and centrifuge at 0°	—	—	75	—
Washed sediment ↓	Add thymol and buffer (same); allow to stand at 36° up to nearly complete hydrolysis of chitin; centrifuge	65	260	50	170
Supernatant "C D" ↓	Bring to 55% saturation ammonium sulfate, at 0° and pH 5.2; centrifuge and dissolve the precipitate in buffer	10	1400	40	380
Fraction SA_1-55 ↓	Collect the precipitate formed between 30 and 50% saturation ammonium sulfate at 0° and pH 5.2. Dissolve in water	4	2500	30	420
Fraction SA_2-50					

[a] In nephelometric units per milliliter.
[b] In nephelometric units per milligram protein.

Properties

Action. Chitinases are specific for linear polymers of *N*-acetyl-D-glucosamine, but do not split chitobiose. They hydrolyze chitin to chitobiose and, to a lesser extent, chitotriose; free acetylglucosamine may be produced, especially when the substrate is colloidal chitin.[1,13] Other substrates for chitinases include chitosan, carboxymethylchitin,[15] glycol-chitin,[14,20] and chitin sulfates.[21] The enzyme has no action on chitin nitrates,[21] cellulose, hyaluronic acid, alginic acid, or mucin.[13]

Sedimentation Constant. The value for *Streptomyces antibioticus* chitinases is 3.42 Svedberg units, and the molecular weight is approximately 30,000.[19]

Electrophoretic Mobility. The *S. antibioticus* chitinase SA_2-50 is made up of three distinct chitinolytic fractions, which migrate to the cathode with slightly different speeds, at pH 8.2 in Veronal–HCl buffer.[10] These chitinases have the same specific activity, but show a synergistic effect on chitin hydrolysis when recombined.[19] The *S. griseus* chitinase, at pH 7.0, separates into two different chitinolytic proteins, one migrating to the anode, the other to the cathode.[2]

Absorption Spectrum. The extinction coefficient of *Streptomyces antibioticus* chitinase is $E_{280}^{mg/ml} = 1.24$; the absorption spectrum shows a single band at 280 mμ.[13]

Michaëlis Constant. The value for *S. antibioticus* chitinase is 0.010–0.011 (g/100 ml).[13]

Effect of pH. Nearly all the chitinases so far studied have an optimum pH of about 5.0 when the substrate is chitin, *S. griseus* chitinase has an optimum pH of 6.3.[2] The activity is rapidly inhibited below pH 4.5, except for gastric chitinases of vertebrates, which are still very active at pH 3.0.[13,22]

Effect of Temperature. The optimum is 40°, except for *Aspergillus niger* chitinase acting on glycol-chitin which has an optimum of 50°.[18]

Activators. Cofactors are not known; dialysis hardly alters activity.[13] Cations, KCN, hydroquinone, pyrocatechin, reduced glutathione have no effect.[13] Serum albumin enhances the activity,[6] but this effect is apparent only after the first 2 or 3 hours of reaction.[13]

Inhibitors. Cu and Hg ions are inhibitors; *p*-chloromercuribenzoic acid and iodobenzoic acid have no apparent effect on purified *S. antibioticus* chitinase.[13]

Stability. Crude or purified chitinases are stable in frozen state for

[20] R. Senju and S. Okimasu, *J. Agr. Chem. Soc. Japan* **23**, 432 (1950).
[21] R. H. Hackman, *Australian J. Biol. Sci.* **7**, 168 (1954).
[22] C. Jeuniaux, *Ann. Soc. Roy. Zool. Belg.* **92**, 27 (1962).

at least two years. They are rapidly inactivated at 37° in the absence of substrate (half-life = 40 days at 37° and 230 days at 5°[6]). The stability is much greater in the presence of chitin. Chitinases are inactivated by oxygen[23]; inactivation constant at 20°: $k = 0.145$ hour^{-1}.

[23] G. Dandrifosse and E. Schoffeniels, *Arch. Intern. Physiol. Biochim.* **71**, 788 (1963).

[112] Bacterial Mucopolysaccharidases (Mucopolysaccharide Lyases)

By Alfred Linker

Mucopolysaccharidases isolated from bacteria differ from similar enzymes obtained from other sources by their mode of action. They degrade the uronic acid-containing substrates by an elimination reaction rather than by hydrolysis. The enzymes isolated from streptococci, clostridia, pneumococci, and some others appear to act on hyaluronic acid only and were therefore named hyaluronidases.[1] More recently enzymes degrading the chondroitin sulfates also have been isolated from a strain of flavobacteria[2] and *Proteus*.[3] When the flavobacteria are grown on heparin or heparitin sulfate, they will produce adaptive enzymes degrading these substrates.

Assay Method

Principle. Degradation by elimination, mentioned above, results in the formation of α,β-unsaturated uronides which show a strong absorption peak at 235 mμ.

Reagents

Substrate (hyaluronic acid, the chondroitin sulfates, heparin) at 10–20 mg/ml in the appropriate buffer (0.1 N, pH 5.0 acetate buffer for the staphylococcal enzyme; 0.1 N, pH 6.0 acetate buffer for the constitutive flavobacterial enzyme, and 0.1 N sodium acetate, pH 7.4 for the induced)
Enzyme at 2 mg/ml in the same buffer
Solution of 0.033 N HCl

Procedure. Equal amounts of enzyme and substrate solutions are mixed and incubated at the proper temperature (25° for the induced

[1] A. Linker, K. Meyer, and P. Hoffman, *J. Biol. Chem.* **219**, 13 (1956).
[2] A. Linker, P. Hoffman, K. Meyer, P. Sampson, and E. D. Korn, *J. Biol. Chem.* **235**, 3061 (1960).
[3] H. I. Nakada and J. B. Wolfe, *Arch. Biochem. Biophys.* **94**, 244 (1961).