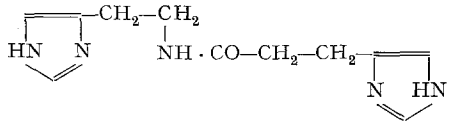
Materials and methods. 1500 specimens of *Drupa concatenata* were collected near Dumaguete City (Negros Oriental). The whole soft tissues (305 g) were removed from the living animals after cautious rupture of the shell and immediately extracted with 5 parts (w/v) of pure methanol. After 10 days the supernatant liquid was decanted and the tissue re-extracted for another 2 days



with 5 parts of 80% methanol. The extracts were mixed and filtered. Part of them was studied as such, but the greatest part, corresponding to 240 g tissue, was submitted to chromatography on alkaline alumina columns which were eluted with descending concentrations of ethanol. Both the crude extracts and the eluates from the alumina columns were submitted to paper chromatography, thin-layer chromatography, high-voltage electrophoresis and bioassay, using the isolated guinea-pig ileum.

Synthetic choline chloride, murexine chloride hydrochloride, senecioylcholine iodide, dihydromurexine dipicrate, urocanic acid, imidazolepropionic acid, the methyl and ethyl esters of these acids, histamine dihydrochloride, N-acetylhistamine and N-imidazolepropionylhistamine were available for comparison. The last histamine derivative was synthetized by one of us (V.).

Results and discussion. Following chromatography on alumina column, the histamine derivative supposed to be N-imidazolepropionylhistamine emerged in the 95–90% ethanol eluates. Its identification as N-imidazolepropionylhistamine was based on the following criteria:

a) both the unknown histamine derivative and synthetic N-imidazolepropionylhistamine showed the same colour shades with the Pauly reagent (pink red) and the Dragendorff reagent (lilac).

b) On high-voltage paper electrophoresis the unknown substance showed the same mobility towards the cathode as synthetic N-imidazolepropionylhistamine: $E_{1.2}$ = 1.1–1.2 histidine; $E_{5.8}$ = 1.7–2 histidine.

c) On paper chromatography the Rf values of the unknown derivative and those of synthetic N-imidazolepropionylhistamine were exactly the same in 5 solvent systems: Rf 0.26–0.28 in *n*·butanol:acetic acid:water (4:1:5), Rf 0.60–0.64 in *n*·butanol:methylamine 35% (8:3), Rf 0.51–0.54 in 1-pentanol:pyridine:water (40:40:10), Rf 0.43–0.46 in methylethylketone:pyridine:water:methylamine 35% (65:15:10:0.5) and finally Rf 0.65–0.72 in 20% KCl. Similar results were obtained in thin-layer chromatography: Rf 0.07–0.1 in *n*·butanol:acetic acid:water (4:1:5), Rf 0.64–0.70 in *n*·butanol:methylamine 35% (8:3), and Rf 0.28–0.32 in *n*·buthanol:ethanol:35% methylamine (22:7:1).

d) Upon hydrolysis with hydrochloric acid (6*N* HCl, 3–6 h at 100 °C) amounts of the unknown derivative and of synthetic N-imidazolepropionylhistamine showing, on paper chromatograms, a Pauly reaction and a Dragendorff reaction of the same intensity, yielded equal amounts of imidazolepropionic acid, as estimated by the Pauly reaction, and of histamine, as estimated by bioassay. For each mole of histamine, one mole of imidazolepropionic acid was liberated. The spasmogenic effect of the hydrolysate on the guinea-pig ileum was completely blocked by mepyramine (0.1–0.2 µg/ml).

The content in N-imidazolepropionylhistamine of the crude extract of total soft tissues was approximately 300 µg per g fresh tissue. In addition to N-imidazolepropionylhistamine, the extracts of *Drupa concatenata* contained more or less conspicuous amounts of choline, imidazolepropionic acid, methyl imidazolepropionate, free histamine and another histamine derivative, the isolation of which is in progress.

Research has been commenced to investigate whether imidazolepropionylhistamine is used by *Drupa concatenata*, a carnivorous gastropod, in the capture of the prey[6].

Riassunto. Gli estratti metanolici dei tessuti molli del mollusco gasteropode delle Fillppine *Drupa cancatenata* Lam. contengono cospicui quantitetivi (circa 300 µg per g di tessuto fresco) di un nuovo derivato dell'istamina, la N-imidazolpropionilistamina.

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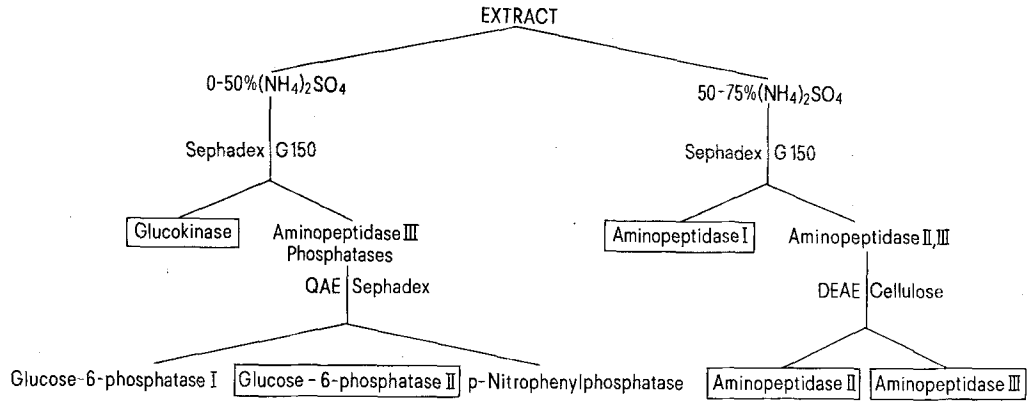
Isolation of Different Thermophilic Enzymes from *Bacillus stearothermophilus*

Several reports appeared recently from this laboratory about thermophilic proteolytic enzymes from *Bacillus stearothermophilus* [1–4]. We have now extended our studies and purified some additional enzymes from the same bacillus to a homogenious or nearly homogenious state, to get further information about these thermostable proteins by comparative studies. In this report we describe the method that allows us to purify 7 enzymes out of the same bacillus extract. These enzymes enclose the 3 aminopeptidases I, II and III that occur in *B. stearothermophilus*[5], two glucose-6-phosphatases, a *p*-nitrophenylphosphatase and a glucokinase.

Cells. B. stearothermophilus cells (strain NCIB 8924), grown as described[2], were a generous gift of the Ciba-Geigy AG, Basle.

Enzyme assays. p-Nitrophenylphosphatase: The hydrolyses of 0.01 *M p*-nitrophenylphosphate in 0.05 *M tris*-HCl, pH 9.0 at room temperature, containing 10^{-3} *M* Mg Cl_2 was followed at 405 nm.

Glucose-6-phosphatases: The assay mixture contained 2.5×10^{-2} *M* glucose-6-phosphate and 10^{-2} *M* Mg Cl_2 in 0.05 *M* sodium-N-morpholino-3-propansulfonate, pH 6.9. The released inorganic phosphate was determined by the method of Delsal and Manhouri[6].



Isolation Scheme of Glucokinase, Glucose-6-Phosphatase I and II, *p*-Nitrophenylphosphatase, Aminopeptidase I, II and III from *Bacillus stearothermophilus*.

Glucokinase: The method of STEIN et al.[7] was used with the following concentrations: 0.05 *M tris*-HCl, pH 9.0; 10^{-2} *M* glucose; 2×10^{-3} *M* ATP; 3×10^{-3} *M* $MgCl_2$; 10^{-3} *M* NADP and 0.028 units of glucose-6-phosphate dehydrogenase per ml.

Aminopeptidase I and II: Aminopeptidase I activity was determined as described elsewhere[4]. The same assay was used for the aminopeptidase II.

Aminopeptidase III: The hydrolysis of 10 *mM* gly-leu-tyr in *tris* buffer, pH 7.5, was used as a test for this enzyme. 10^{-3} *M* $CoCl_2$ was added. The glycine released was localized by thin layer chromatography in the system n-butanol: acetic acid: water = 4:1:1.

Results. 500 g of cells were suspended in 0.05 *M tris*-HCl buffer, pH 7.5, containing 10^{-3} *M* $MgCl_2$ and 10^{-2} *M* β-mercaptoethanol. The final volume was 1.6 l. The cells were disrupted in a Manton Gaulin press and centrifuged at 25,000 *g* for 30 min. The pellet was discarded. Solid $(NH_4)_2SO_4$ up to 50% saturation (313g/l) was added to the supernatant. The resulting precipitate contains the bulk of the 3 phosphatases, the kinase and part of the aminopeptidase III. Aminopeptidase I, II and partly aminopeptidase III are in the supernatant and are precipitated by the addition of $(NH_4)_2SO_4$ up to 75% saturation (176 g/l). The 0–50% $(NH_4)^2SO_4$ precipitate was dissolved after centrifugation in 0.05 *M tris*-HCl buffer containing again 10^{-3} *M* $MgCl_2$ and 10^{-2} *M* β-mercaptoethanol. This solution was applied to a 10×90 cm Sephadex G 150 column. This step separates the larger kinase from the 3 phosphatases. The glucokinase was purified to a homogenious state by ion exchange chromatography and preparative gel electrophoresis[8]. The 3 phosphatases were separated from each other by QAE-Sephadex A 50 chromatography in the same buffer. A column of 5×60 cm was eluted with a linear gradient from 0.15 to 0.3 *M* NaCl in a total volume of 2 l. One of the glucose-6-phosphatases was further purified by preparative gel electrophoresis[9].

The 50–75% $(NH_4)_2SO_4$ pellet was dissolved in 0.05 *M tris*-HCl, pH 7.2, and also applied to a 10×90 cm Sephadex G 150 column. This step separates aminopeptidase I from aminopeptidases II and III. Aminopeptidase I was further purified as described[10]. Aminopeptidases II and III were separated by DEAE-cellulose chromatography and further purified by ion exchange chromatography, gel filtration and preparative gel electrophoresis.

Discussion. The present communication describes the early steps of the purification of 7 enzymes from *B. stearothermophilus*. The Figure summarizes the whole procedure. The enzymes enclosed in the boxes have been purified to a homogenious state (Figure). The residual 2 enzymes were almost pure.

The losses due to the $(NH_4)_2SO_4$ fractionation are insignificant. 18% of the total activity of the glucokinase is in the 50–75% $(NH_4)_2SO_4$-fraction. The corresponding value for aminopeptidase II is 15% and less than 10% for aminopeptidase I and the glucose-6-phosphatases. Only AP III is distributed in both fractions. However, under our fermentation conditions at 50 °C, *B. stearothermophilus* synthesizes only small amounts of aminopeptidase III, quite in contrast to 37 °C cultures. It is therefore reasonable to work out a new purification procedure for this enzyme, starting with cells grown at 37 °C. But some preliminary information can surely be gained from the enzymes purified out of 50 °C cells as described.

The *p*-nitrophenylphosphatase was purified to an almost homogenous state. This enzyme hydrolyses only *p*-nitrophenylphosphate but none of the 20 physiological important phosphates we tested. The function of this enzyme is unknown. However it is also present in other microorganisms[11].

Zusammenfassung. Es wird eine Reinigungsmethode beschrieben, die es erlaubt, aus einem *B. stearothermophilus* Zellaufschluss fünf thermophile Enzyme in reiner Form zu isolieren und weitere zwei Enzyme stark anzureichern.

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