34. *Purified Proteases.*

I. Papain Protease :

*A Newly Devised Method of Purification and a New Method
for the Measurement of Proteolytic Power.*

*Physical and Chemical Properties of Purified Protease.
The Action of Proteolytic Power.*

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Contrary to the generally accepted idea that proteases are proteins in nature of large molecular weight which lie between 20,000~45,000, the present author has succeeded in obtaining a preparation of papain protease which is by no means a protein.

The procedure of the preparation was carried out as the following scheme :—

Crude papain protease (2 Kg)

Dissolved in water, filtered and acetic acid was added

Clear acidic solution

Insoluble residue, which was produced by adding a sufficient amount of lead acetate, removed

Filtrate

Precipitates obtained by saturating the solution with ammonium sulphate were collected on a filter paper

Precipitates

Dissolved in water and insoluble lead sulphate was removed

Solution

Precipitates were obtained by adding again ammonium sulphate to saturation and the precipitates were collected and washed with the saturated ammonium sulphate solution

Precipitates

Dissolved in water and alcohol was added up to 50% and after settling a while, the alcoholic layer was separated

Alcoholic solution

A sufficient amount of strong alcohol was added whereby precipitation taking place

Precipitates

Dissolved in water and the treatment of the solution with alcohol was repeated

Precipitates

Dissolved in water, dialysed through an animal membrane and the protease was precipitated by adding alcohol to the aqueous solution contained in the animal membrane, by collecting the precipitates and by washing the precipitates with alcohol and ether

Purified protease (10 g)

The preparation, thus purified is a colorless amorphous powder which is easily soluble in cold water and the aqueous solution has very strong proteolytic power, while no action of amylolytic and other enzymes is detected.

By analysis, it has been shown that the preparation contains N : 13.0%, C : 51.0%, H : 6.5%, S : 1.6~3.7%.

It has been the experience of laboratory-workers on protease that the well-known method of measuring the proteolytic power is rather troublesome and the results obtained have not always been precise.

In order to overcome these difficulties, the author invented a new method which is simple and fairly accurate.

This new method may be conveniently introduced here under the name of "Decomposition curve method."

The procedure of this method is as follows:—

Reagent.

(1) Standard suspension of coagulated egg white protein.

A fresh egg was broken and the white was separated from the yolk. After filtering the white through cloth, it was mixed with 20 times its volume of water, then heated upon the boiling water bath for 10 minutes and afterward a few drops of dilute acetic acid was added and shaken.

Two sets of several test tubes were used, one set containing 5 cc each of standard protein suspension and the other various increasing amounts of 10% aqueous solution of protease, i. e., 0.5 cc, 1 cc, etc.

The tubes were kept in a water bath at 40° C for 5 minutes. The warm solutions of protease in the second set of tubes were then added to the protein suspensions in the first set of tubes, which now containing the mixture, were returned to the water bath.

At stated intervals the tubes of the mixture were taken out of the bath, and the degrees of the transparency of the tubes were compared with the standard suspensions, i. e., the blank solution, $\frac{1}{2}$ diluted, $\frac{1}{3}$ diluted, $\frac{1}{4}$ diluted solutions, etc.

The observed changes of the transparency plotted against the time constituted the "Decomposition curve" of the author and the curves thus obtained were compared with each other as shown in the following charts :

Thus, the proteolytic powers upon protein were easily measured even at the little intervals of the operations of purifying protease.

Physical and chemical properties of purified protease.

The purified papain protease was in the form of colorless amorphous powder which was dissolved quickly in the cold water.

This aqueous solution exhibited a strong proteolytic power, but never the power of diastase and other enzymes.

On adding inorganic or organic acids beyond the destruction point, protease was destroyed and precipitated out of the solution. By heating beyond the destruction temperature, protease was also destroyed and was precipitated out.

Furthermore, protease was destroyed in alkaline solution such as sodium hydroxide, potassium hydroxide, barium hydroxide, etc.

It is important to note that protease is easily destroyed in a dilute ammoniacal solution, in which diastase is only inactivated without any

Chart 1. Purified papain protease (10% solution) [at 40° C].

Chart 2. Purified papain protease (10% solution) [at 40° C].

destruction. There exists a quite different property against ammonia between protease and diastase.

The destroyed precipitated out of the alkaline solution was protease never dissolved in cold water again.

Protease was neither inactivated in nor precipitated out of the water solution acidified with acetic acid, with lead acetate, copper sulphate, etc., though a great of impurities was precipitated.

The purified protease contained only a little inorganic matters and gave a trace of ash when burned in crucibles. Nevertheless, no reaction of phosphorous was given.

It is most important to note that the purified protease gives strong organic sulphur reactions, i.e., when the protease was boiled with alkali and afterward added with a few drops of lead acetate solution, a brownish black precipitate was formed, and when the protease was boiled with sulphuric acid over 2%, hydrogen sulphide gas was evolved out of the solution and changed the colour of lead acetate paper to brownish black, and furthermore nitropruside reaction.

Chart 3. Proteases (10% solution) [40° C].

These organic sulphur reactions of protease are important reactions which detects the existence of protease. Diastase never gives such reactions.

The pure preparation did not reduce Fehling's solution, the reduction which was always given intensively by crude samples. From this, it was obvious that the purified protease had no free sugar group and the substances which gave the reduction were all impurities.

Purified protease gave also no pentose reactions, i.e., Bial reaction, etc., and never gave any reduction of Fehling's solution, though it was decomposed with 1-2% sulphuric acid, showing no saccharide nature.

Furthermore, the purified protease gave no α -Naphthol reaction, and this negative reaction was also important in distinguishing protease from diastase, which gave a strong α -Naphthol reaction.

The pure preparation gave Liebermann's pirrole reaction and gave red colouration with concentrated hydrochloric acid when heated strongly in test tubes.

It is noteworthy that these pirrole reactions are important ones

which detect the existence of protease, as diastase never gives such reactions.

Protease gave biuret reaction and ninhydrine reaction and gave red precipitate with Millon's reagent.

The important reactions of purified protease and diastase are conveniently summarized in the following table:—

Reaction	Protease	Diastase
Biuret reaction	+	+
Ninhydrine reaction	+	+
Millon's reaction.....	red precipitate	red precipitate
α -Naphthol reaction.....	—	+
Sulphur reaction.....	+	—
Liebermann's pirrole reaction...	+	—

Properties of proteolytic action.

The preparation of purified papain protease showed many newer properties, as already shown in the case of purified diastase by the author.

The purified protease, prepared by the method previously mentioned, indicated a much higher destruction temperature than previously reported. The destruction temperature of papain protease was about 85° C or slightly over.

The destruction temperature of papain protease, however, falls down more and more with the increased quantities of acid added, the curve going down as shown in the following chart:—

① Destruction temperature.

② Destruction curve of purified papain protease.

Chart 4. Destruction curve of papain protease.

The purified protease has a strong decomposing power upon protein in the solution acidified with acetic acid, producing amino acids and peptides at the same time.

Protease acted most intensively when acid was added up to near the destruction point at every temperature. Without acid, protease acted more and more intensively as the temperature was brought up to near destruction temperature.

The manner of action thus observed, could be affirmed to be quite similar to that of purified diastase.

All the alkaline solutions destroyed protease instantaneously and even in dilute ammoniacal solution, protease was easily destroyed. We see thus, that there is a remarkable difference of property against ammonia between protease and diastase.

It is noteworthy in this connection that the buffer solution, previously assumed to be most suitable for the action of impure protease was not always good for the protease in pure state.

It has been generally accepted that by dialysis through the animal membrane, certain amounts of protease may be lost or the power of protease somewhat decreased. Against this conclusion, the results of experiments with the purified protease led the author to believe that protease, like diastase, did not dialyse through the animal membrane, the power of protease remaining entirely undiminished even after a complete dialysis.

On the contrary, dialysis may be regarded as an useful method for removing inorganic impurities from protease solution.

Adsorption was practiced as one of the most important methods for purifying protease just like in the case of diastase.

In spite of this fact, critical examinations showed that protease was only partially adsorbed by the usual adsorbents adhering with impurities and the extracting alkaline solutions, which were thought as good buffer solutions were harmful to the purified protease.

These experimental results showed the alleged adsorbability of protease, although so generally accepted hitherto, should be further critically examined.

Finally, the author has to propose the suppression of the idea of certain confusing enzymes, the existence of which in crude materials have been supposed under certain conditions, but never actually demonstrated.

The purified papain protease has the powers to produce polypeptides and amino acids at the same time. In reality, however, the two powers of the purified protease are differently influenced by the impurities adhering to.

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