Milk clotting activity of proteinases produced by microorganisms

By

Samy Hassan Mohamed Ahmed

B. Sc. (Agric. - Biochem.) , 1967

Thesis

Submitted in Partial Fulfilment of the Requirements for the Degree of

Master of Science

in

Agricultural Microbiology

Agric. Microbiology Dept.
Faculty of Agriculture
Ain Shams University
Cairo, A. R. E.

1973

APPROVAL SHEET

MILK CLOTTING ACTIVITY OF PROTEINASE
PRODUCED BY MICROORGANISMS

Approved by:

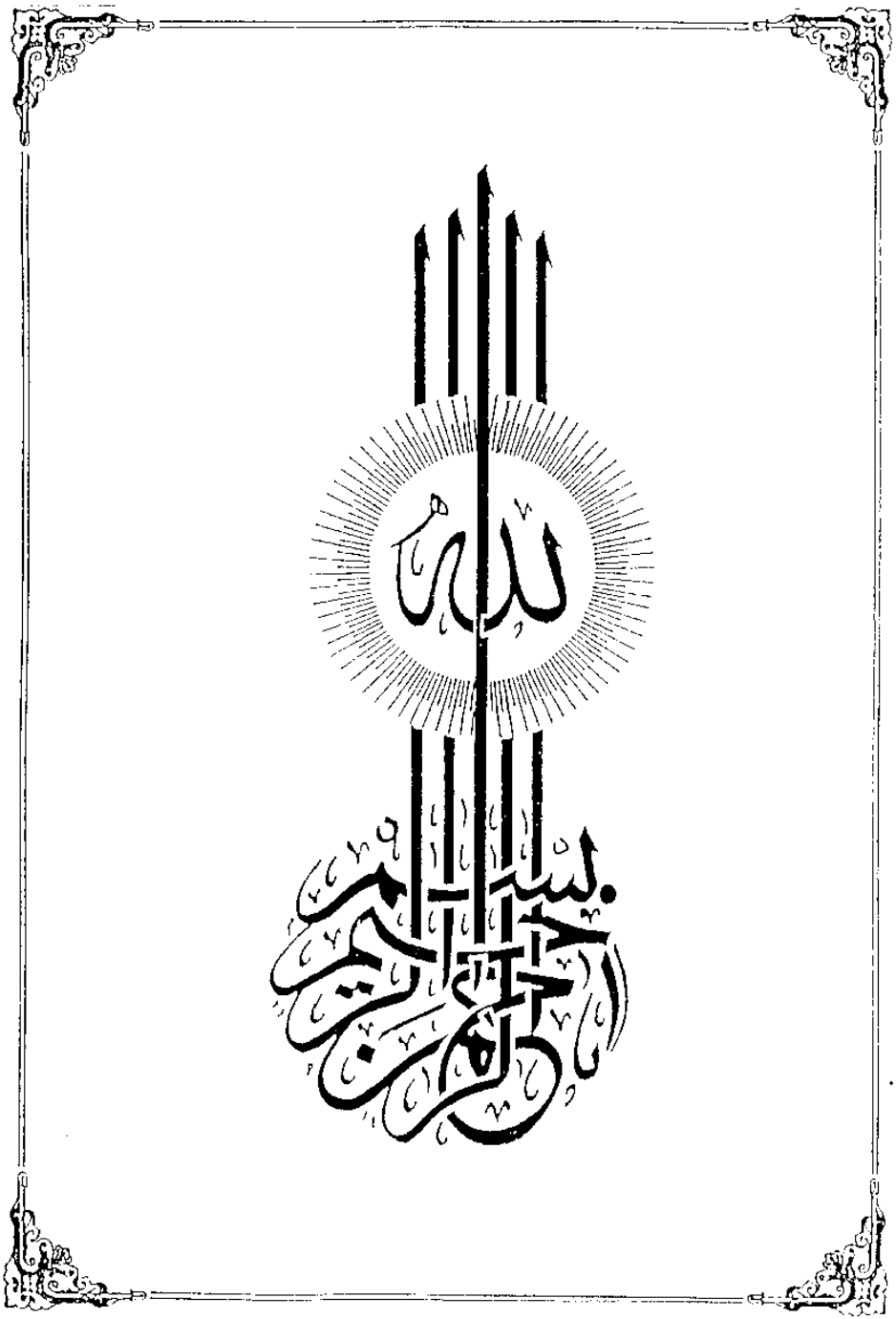
Salah Taha

A.M. Abdel Hafez

A.N. Ibrahim

17/12/73

1973



ACKNOWLEDGMENT

The author wishes to express his gratitude to [illegible] [illegible], former Head of Agric. [illegible] Dept., Faculty of Agriculture, Ain Shams University, Professor Dr. S[illegible] Mahmoud, Head of Agric. [illegible] Dept., Faculty of Agriculture, Ain Shams University, and Dr. [illegible]. Attia, Ass. Professor Microbiol. Research Unit, Agricultural Research Center for their deep interest in the problem valuable guidance, sincere helpfulness and supervision through the whole work.

The author is also indebted to Mr. Loutfy, the General Director of Agricultural Microbiology Research Div., and Dr. Amin El Nawawy, Director of Fermentation Research Section, Agric. Res. Center for facilities and moral encouragement. Thanks are also extended to Staff in the Fermentation Research Section, particularly Dr. Samir A. Ali, Professor of Industrial Microbiology and Mr. [illegible].M. Dokhan.

The author is also indebted to Staff in Grain and Bread Research Unit, Agric. Res. Center particularly Mr. A. Fahmy and Mr. El Housany Tawfik, for providing facilities throughout his work.

CONTENTS

INTRODUCTION

The use of microbial proteinases as milk clotting agents for rennet enzymes is of increasing import nce. As it has been stated by the USDA statistical reporting service (1966 a), the rapid increase in cheese production was forced by decrease in rennet enzymes produced from the fourth stomach of the calf. Thus the use of enzymes of microbial origin becomes of increasing importance.

The Food and Drug Administration (Federal Registration, 1966 a) has been petitioned for the clearance to use an enzyme produced by the fungus Endothia parasitica. The FAA (Federal Registration, 1966 b) has also been petitioned for a change in the cheese standards to include additional safe milk clotting enzymes.

In Egypt cheese manufacturers usually suffer from variations in the potency of rennet enzyme. Moreover, this enzyme which is marketed as liquid preparation is liable to microbial contamination and rapid inactivation especially in summer. This is because of the primitive procedure and non hygienic conditions under which the rennet is produced.

Therefore it was found of importance to produce milk clotting enzyme by fermentation for the first time in Egypt. Studies were focused on the nutritional and environmental factors that give high economical yield of the enzyme produced by the fungus.

REVIEW OF
LITERATURE

From this perplexity, which developed, [illegible] the commonly accepted nomenclature we use today, that the term 'rennet' would be used to designate the enzyme system extracted from the fourth stomach of [illegible] calf and the majority of this extraction would be the enzyme rennin. The milk-coagulating enzyme from other sources would be called rennet, prefixed by their origin as plant rennet, animal rennet and microbial rennet.

With the identification of the enzyme rennin, in the early 1920's, it appears that further interest in rennetic-enzyme substitutes fell off, except in India where, because of religious beliefs, work has and is being done.

The possibility of utilizing microbial rennet for the production of cheese, now made with rennet, should not be overlooked. The feasibility of such a substitute could readily be visualized when one realizes the fact that microorganisms in general play a major role in cheese production. Various microorganisms such as Streptococcus paracitiovorus contribute flavor (Brood, Murray and Smith, 1957); others are used in starter cultures. Why not, then, a microbial rennet for coagulation?

In the early 1920's, Hatano (1924), studied the coagulation of milk and caseinogen by "taka-rennin". Aspergillus oryzae, a fungus was the source of this

preparation. Hankiss (1927), a few years later, noted that this potential substitute had its optimum activity in the pH range of 5.2 to 5.7. The latest work reviewed on this subject was published by the team of Tsuchiya, Yamauchi (1959) who found the optimum temperature to be 60°C. After rather expensive study by electrophoresis, they concluded that the clotting action exhibited was not similar to rennet.

Another mold which will yield an enzymatic product for coagulating milk is Rhizopus oligosporus (Wang, Ruttle, and Hesseltine, 1969), Mucor pusillus (Mickelsen and Yancy, 1970) Mucor michei (Sternberg, 1971) and Penicillium citrinum (Abdel-Fattah, Souhair S. Mabrouk, and Nadia A. El-Hawwary, 1972).

In concluding this brief history a general outline follows of the desirable qualities investigators have looked for, either directly or indirectly, in searching for a substitute at least as good as rennet:

1 - The yield of curd produced should be the same as with rennet.

2 - The curd should possess adequate physical properties comparable to rennet curd.

3 - The loss of fat in the whey should be slight (as with rennet).

4 - There should be no detectable flavor defects.

Rennet Substitutes

The term "rennet" would be used to connotate the enzyme system extracted from the stomach of a suckling calf and the majority of this extraction would be the enzyme rennin. The milk coagulating enzymes from other sources would be called rennet prefixed by their origin as plant rennet, animal rennet and microbial rennet.

1) Plant rennet

Surveying the more promising plant rennet substitutes, the one which appears most prominently was the enzyme ficin from the latex of Ficus carica (Gerber, 1908). This enzyme because of its plentitute was "a natural" but it too had many drawbacks. Gerber (1908) noted that when using the juice of the Ficus carica in clotting raw milk, citric acid retarded coagulation, succinic acid first accelerated then retarded coagulation and butyric, phosphoric and hydrochloric acid all accelerated coagulation.

In 1948, ficin's potential as a rennet substitute was subjected to further study. This time not only the

enzyme extract of Ficus carica latex as well as those of Ficus religiosa, Ficus clemarta and Ficus krishna were investigated. The best source of a good rennet substitute was still the extract from ficus carica (Krishnaswamy, Jaya, Subrahmanyan, and Thomas, 1961, and Verinder, 1961).

2) Animal rennet

Gerber's (1908) early work in the field of rennet substitutes touched both the plant and animal kingdom. Although he pioneered in the investigation of plant rennet, he apparently favored the animal extracts. In 1908, he found an animal rennet decidedly different from other animal rennet studied in respect to its great resistance to heat and its peculiar behavior in the presence of acids.

Another proposed substitute was the enzyme trypsin, extracted from beef pancreas (Wahlgemuth , 1906). This enzyme ability to clot milk had been known for some time, but because of the fact that when it was used alone it did not give a firm clot its feasibility was disregarded. Clifford (1935), found that while trypsin alone did not give a firm clot with milk, trypsin used in conjunction with halogen salts did.

The enzyme chymotrypsin from best pancreas was the object of much research (Tsugo and Yamauchi, 1959). Without

doubt ... been given ... attention ... substitute for rennin ... (Sevi..., 1912; ...son, 1935 and ..., 1961).

3) Microbial rennet

According to Arima and Iwasaki (1965), ... production of milk clotting enzymes with low proteolytic activity was obtained from the extraction of ... culture of Mucor hemalis.

Srinivasan, Iyengar, Babbar, Chakravorty, Dudani, and Iya (1964); Schultz, Vose, Sell and Arowetz (1967); Melachouris and Tuckey (1966); Puraschel and Po...il (1968), and Tani, No..., Ashida, Matsumura and Ueda (1968), had studied a collection of bacteria isolated from a variety of different natural sources. They found that Bacillus brevis, B. cereus, B. mesentericus, and B. subtilis, were the most active strains for enzyme production.

Richardson, Nelson, Lubnow, and Schwarberg (1967), and Shovers and Basivotto (1967), used a partially purified fungal rennet elaborated by Mucor pusillus or Endothia parasitica for cheese manufacture.

Aunstrup (1968), concluded that milk clotting enzyme was prepared by cultivating Mucor miehei strain CBS 370 in a suitable culture media.

Sardinas (1968 and 1969), reported that, out of 921 organisms from his collection, one culture Endothia parasitica

was selected as a best strain for producing milk clotting enzyme.

Wang et al. (1965), stated that, forty-four strains of four species of Rhizopus including R. oligosporus, R. stolonifer, R. oryzae, and R. arrhizus, were tested for their ability to produce milk-clotting enzyme. All strains, except three strains of R. stolonifer, produce appreciable amounts of milk clotting enzyme. But the ability of Rhizopus to produce the enzyme varied greatly between different species. Among the culture tested, four strains of R. oligosporus excreted the greatest amount of milk-clotting enzyme. One strain of these four strains NRRL 3271 is used for enzyme production.

Arima, Yu, and Iwasaki (1970), reported that 60 strains out of 800 strains of microbes, which had a suitable milk clotting enzyme included Aspergillus sp., Bacillus sp., Mucor sp., Pseudomonas sp., Rhizopus achlamydospores, R. batatae, R. candidus, R. chinensis, R. chinniary, R. chungkuoensis, R. delemar, R. delemar var minimus, R. japanicus, R. nigricans, R. niveus, R. nodosus, R. oryzae, R. peka, R. pseudokiensis, R. salebrosus, and R. thermosus.

Kawai (1970), and Kawai and Mukai (1970), concluded that among 44 strains tested of Basidiomycetes, proteolytic observed, 2 strains, Fomitopsis pinicola (Fr.) karst, and