PRODUCTION, PURIFICATION, AND CHEESE-MAKING PROPERTIES OF A MILK CLOTTING PROTEASE FROM THERMOACTINOMYCES VULGARIS

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"PRODUCTION, PURIFICATION, AND CHEESE-MAKING PROPERTIES OF A MILK CLOTTING PROTEASE FROM THERMOACTINOMYCES VULGARIS"

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

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Production, Purification, and Cheese-making Properties of a Milk Clotting Protease from Thermoactinomyces vulgaris.

Major Professor; Ronald Roy Pereira.

A milk clotting protease(rennet) was produced by the fermentation of a synthetic medium using Thermoactinomyces vulgaris. The crude enzyme was partially purified, and its biochemical properties as well as its suitability in the manufacture of Cheddar cheese, were investigated. These properties of the T. vulgaris rennet were compared with those of commercial calf rennet.

The optimum conditions of pH, temperature, aeration rate, and nutrient concentration for rennet production, were different than those observed for bacterial cell production. A pH of 5.5 was optimum for efficient rennet production. Of the two systems(each of 12 liter working capacity) employed in batch fermentation process, "System B" was efficient in rennet production, while "System A" produced higher cell density and poor rennet yields.

Partial purification of T. vulgaris rennet, present in the extracellular fraction of the fermented broth, resulted in 49% recovery and an 8.6 fold purification. The product (in form of freeze dried flakes)

was water soluble and free of antibiotic activity.

The T. vulgaris rennet and calf rennet showed maximum stability at pH 5.5-8.0, and pH 5.0 respectively. Of the two rennets, T. vulgaris rennet exhibited relatively higher degree of thermostability. The optimum pH for proteolysis of casein substrate was 6.6 for T. vulgaris rennet and 5.0 for calf rennet.

The suitability of T. vulgaris rennet for Cheddar cheese manufacture was investigated on a laboratory scale. The experimental cheeses were evaluated for organoleptic qualities, cheese yields and chemical composition of cheese and whey. Following classification into mild, medium, and old age categories, the test batches of cheese manfactured with 100%, 75%, 50%, and 25% replacement of commercial calf rennet with T. vulgaris rennet, were compared with the samples manufactured with 100% use of calf rennet(i.e. experimental controls) and locally available commercial cheeses(i.e. commercial controls). All of the above cheeses examined were free of bitterness. For medium cheeses, while the differences in the flavor scores were insignificant, the total scores of cheeses from 100% T. vulgaris rennet were significantly lower than those of the experimental and commercial control samples. Also the yields of cheese from 100% T. vulgaris rennet were significantly lower than those of experimental controls from calf rennet. However, the differences

between the yields, of the cheese batches from 75%, 50%, and 25% replacement of calf rennet by T. vulgaris rennet, and those of experimental controls, were insignificant. The total solids and fat contents of cheese in above test and control batches were comparable. The data on whey analysis revealed that although the total solids and fat contents did not differ significantly, the protein contents of whey, from cheese batches manufactured with 100% T. vulgaris rennet, were significantly higher than those of experimental and commercial controls. The average tyrosine content of medium cheeses from 100% T. vulgaris rennet was 29% higher than that of experimental controls from calf rennet, thus indicating the possibility of inducing the ripening of cheese by T. vulgaris rennet.

In general, the use of T. vulgaris rennet resulted in slightly higher losses of nutrients into whey and consequently slightly lower cheese yields. Evidently however, considering the statistically significant and insignificant differences, substitution of calf rennet by T. vulgaris rennet up to an extent of 75% appeared highly promising. The conclusions regarding the commercial feasibility of process(es), employing T. vulgaris rennet to replace calf rennet in cheese manufacture, can possibly be drawn from future studies involving relatively large scale cheese manufacturing trials.

INTRODUCTION

In the dairy industry the term "rennet" is used to designate any crude enzyme preparation of animal, plant, or microbial origin which coagulates milk. It is universally employed in the manufacture of Cheddar and other varieties of natural cheese. Pure milk curdling enzyme is referred to as rennin. The commercial enzyme extract commonly employed in the manufacture of cheese is derived from the abomasum or fourth stomach of unweaned calves.

The decline of calf slaughter combined with increasing cheese production has resulted in a worldwide search for calf rennet substitutes. Besides, there is a demand for a suitable substitute for calf rennet due to the fact that a very substantial number of people in the world, for varying reasons, would prefer cheese manufactured with an enzyme from plant or microbial sources rather than animal sources.

Among plant sources, there are proteases from a large number of higher plant species such as the fig, papaya, pineapple and pumpkin etc. known to possess milk clotting properties. Babbar et al.(1965) in their review reported the development of bitter taste to varying degrees in cheese manufacturing trials, with various vegetable enzymes. Ilany-Feigenbaum and Netzer (1969) reported production of a suitable Cheddar-type cheese with

a mixture of animal and partially inactivated vegetable enzymes.

Among microbial sources a large number of bacterial and fungal species with milk clotting properties have been investigated. Sardinas(1972) in his review reported the availability of commercial preparations from three fungal species, namely Endothia parasitica, Mucor pusillus, and Mucor miehei. He reported them to be suitable for the manufacture of different cheese varieties as a partial substitute when used with modified cheese-making techniques.

No coagulant is reported to be on the market from bacterial sources. In spite of some seemingly satisfactory cheese trials with some bacterial enzymes, a number of difficulties such as bitterness, off-flavor, and hard texture etc. have been reported to prevail(Sardinas,1972).

From the taxonomical classification point of view, Tendler and Burkholder in 1961 surveyed about 1000 bacterial isolates and found that Thermoactinomyces species possessed the milk clotting property.

This study was initiated to explore the potential of T. vulgaris, for producing a suitable substitute for calf rennet for the manufacture of Cheddar cheese.

REVIEW OF LITERATURE

General Considerations

1. Mechanism Of Milk Coagulation

Milk from a variety of animals is used for cheese production throughout the world. However, cow's milk is predominantly used for the major portion of cheese produced on a commercial scale(Sardinas,1972).

In the cheese manufacturing process, following the ripening of milk by addition of a starter culture, rennet is added to cause coagulation of casein and consequent curd formation. The resultant curd is then subjected to varying processing techniques depending upon the type of cheese desired(Sardinas,1972).

The nitrogenous composition of cow's milk consists of 78% casein, 5.1% alpha-lactalbumin, 8.5% beta-lactalbumin, 1.7% immune globulin, 1.7% peptone, and 5% non-proteinaceous substances(Reed,1966). Extensive investigations and hypotheses, have attempted to explain the mechanism of milk coagulation and curd formation(Berridge, 1954; Dyachenko and Slavyanova,1962; Hill and Wake,1969; Reed, 1966; Sardinas,1972).

In his review Reed(1966) suggested that rennin converts κ-casein into para-κ-casein which precipitates in the presence of calcium ions. He further summarized the following two theories pertaining to the mechanism of milk

coagulation:

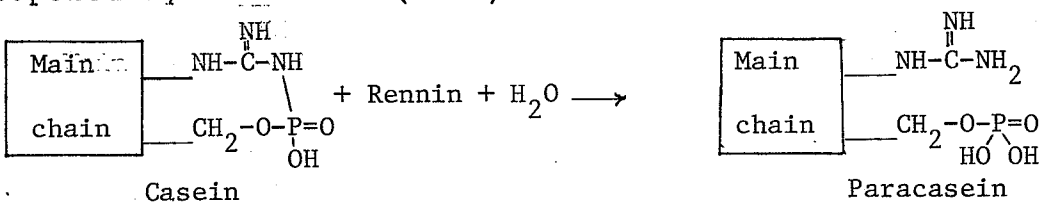
(i) Casein is present in milk in soluble form and in the form of micelles of 400-2800 Angstroms in diameter. These micelles are in equilibrium with soluble casein. In a micelle, one molecule of κ-casein, whose calcium salt is soluble, is linked with three molecules of alpha-casein whose calcium salt is insoluble. It is suggested that κ-casein is a phosphoprotein. It contains between 6-10% carbohydrates, and has a minimum molecular weight of 30,000. As such it does not undergo precipitation in the presence of calcium ions.

The enzymatic action of rennin on κ-casein results in the formation of a soluble fraction containing 20-30% carbohydrates, and an insoluble fraction termed para-κ-casein or para-casein. Although the nature of linkage between glycoprotein and para-κ-casein in a κ-casein molecule has not been determined with certainty, it is assumed to be a peptide or ester linkage. Regardless of its nature, the linkage is considered to be specifically split by rennin action (Delfour et al., 1965; Alais and Jolles, 1964; Reed, 1966).

Following the hydrolysis of κ-casein into a glycoprotein fraction and para-κ-casein by rennin action, the para-κ-casein fraction precipitates in the presence of calcium ions and leads to the precipitation of other casein fractions which have been exposed to calcium ions

as a result of K-casein hydrolysis. Removal of calcium or addition of oxalate, citrate or phosphate limits the tendency towards coagulation. In conclusion, it is suggested that K-casein acts as a protective colloid thereby stabilizing the entire casein micelle, and its hydrolysis results in the precipitation of all the casein fractions if calcium ions are present(Waugh and von Hippel,1956; Beeby,1963; Beeby and Nitschmann,1963; Reed,1966). The calcium content of cow's milk is 0.125%(Jenness and Patton,1959).

(ii) According to an alternate theory proposed by D'Iachenko(1963), the milk clotting process is attributed to the phosphoamidase activity of rennin. It is believed that rennin exerts phosphoamidase activity and can split -N-P-N- bonds through which cross-linkages within the casein molecule are presumed to be established. This is supported by the evidence that rennet is capable of liberating about 20% of the phosphate groups chemically linked in a casein molecule(Aiyar and Wallace,1964). The notion that phosphoamidase activity is directly responsible for the curdling effect of rennin can be explained on the basis of the following mechanism as proposed by D'Iachenko(1963):



Casein Paracasein

Both of the above theories suggest that the milk clotting reaction is a two step process, namely: (a) formation of para-κ-casein as a result of enzymatic hydrolysis of κ-casein, and (b) the precipitation of para-κ-casein in the presence of calcium ions. The first step, involving enzymatic hydrolysis, can be carried out at a very low temperature such as 2°C and thus separated from the second step of precipitation of para-κ-casein which occurs only at higher temperatures such as 40° C (Reed, 1966). While the above ambiguity remains unresolved, the investigations by by Jolles et al. (1968) have indicated that the specific action of rennin splits a peptide linkage between the phenyl alanine and methionine residues of κ-casein (cow's). Splitting of this peptide linkage results in formation of para-κ-casein (with phenyl alanine at C terminal) and κ-caseino-glycopeptide (with methionine at N terminal). They further reported that para-κ-casein precipitates even in the absence of Ca^{++} ions.

2. Assay Procedures

A. Milk Clotting Activity:

A number of methods for determining the milk clotting potency of rennets have been described in the past. Babbar et al. (1965), Foltman (1970), and Sardinas (1972) have offered comprehensive reviews on the subject. There is considerable variation among various methods and

no universally accepted standard procedure exists. The variation in the milk clotting assay procedure results from the differences in the substrate composition and its pH, testing temperature, mixing techniques, and the end point determinations. Nevertheless, all the methods underline the concept that for any fixed volumes of substrate and enzyme employed in the test, the concentration of enzyme must be appropriately adjusted so that the clotting time falls within the range in which it is inversely proportional to the enzyme concentration.

Various types of substrates employed in milk clotting tests include raw or pasteurized whole or skim milk, or reconstituted solutions from solids of whole or non-fat skim milk powder. Special casein preparations have also been employed(Babbar et al., 1965; Salati,1953; Sardinas, 1972).

Other variations in substrate composition result from the use or non-use of buffering agents and cofactors such as calcium. Buffering with acetate buffer and use of 0.01M calcium chloride have been employed by many workers(Berridge, 1955; Balls and Hoover, 1937). Temperature and pH parameters in the test vary between 30-45°C and pH 5-6.7 respectively(Sardinas, 1972).

Great diversity in mixing techniques and end point determinations results from the use of a variety of manual, semi-mechanical, and mechanical devices, in which

the milk clotting end point, as indicated by the appearance of discrete casein particles, is detected visually or mechanically. The frequently quoted method of Berridge(1955), employs a device in which a test tube containing enzyme and milk substrate is sloped at an angle of 30° to the horizontal and rotated along its axis. A thin film of milk is formed on the glass vertically above the surface of the liquid. The appearance of discrete casein particles in this thin film of milk substrate is visually detected as an end point. For automatic detection of milk clot in the mixture of rennet and milk substrate, the use of blood clot timing machines has been reported by deMan and Batra(1964), Sardinas(1972), and Wolfe(1971).

Viscometric techniques which measure viscosity increase at coagulation have been employed for milk clotting assays by Scott Blair and Oosthuizen(1962), and Richardson et al.(1971).

Everson and Winder(1968) reported the use of an instrument for the continuous measurement of sound velocity in liquids, for determination of milk coagulation time by rennet. At coagulation, an increase in the sound velocity was reported to correspond to the end point of the test and this was automatically recorded.

Since the milk clotting assay procedures are so diverse, reported enzyme potencies are understood to be only relative, and animal rennet or rennin is generally