Purification, characterization and utilization of bacterial rennet from Bacillus subtilis K-36 for cheese manufacture
SUMMARY

1. The crude enzyme obtained from Bacillus subtilis K-26 was purified by different biochemical techniques. Using Sephadex G-25, the enzyme was purified to 3.9-fold with 116% recovery of enzyme activity. Further purification was done by gel filtration through Sephadex G-100, resulting in 7.8-fold purity with 106% recovery of enzyme activity. When subjected to DEAE-Sephadex A-50 column chromatography, the enzyme had a specific activity which was 24 times greater than that of the initial solution with 80% recovery of enzyme activity.

2. SDS polyacrylamide gel electrophoretic analysis of the purified B. subtilis K-26 enzyme showed a single band, thereby confirming the homogeneity of the enzyme.

3. Analysis of purified B. subtilis K-26 enzyme by sedimentation velocity technique on an analytical ultracentrifuge showed a single peak, further confirming the homogeneity of enzyme.

4. Isoelectric focussing of purified B. subtilis K-26 enzyme revealed one band of protein at the anode. The isoelectric point of enzyme was found to be 4.6.

5. A steady increase in activity with decrease in milk pH up to 5.0 was noted with purified B. subtilis K-26 enzyme.
6. Purified *B. subtilis* K-35 enzyme was most stable at pH 7.5 and at 37°C for 30 minutes. However, the enzyme retained 90% of its activity after exposure to pH ranging between 6.5 and 8.0.

7. The maximum activity of the purified *B. subtilis* K-35 enzyme was noticed at 60°C.

8. The purified *B. subtilis* K-35 enzyme was stable at 40°C for 30 minutes, whereas at 60°C the enzyme retained only 10% of its original activity.

9. Purified *B. subtilis* K-35 enzyme lost 97.5% of its activity on storage for 10 days at 37°C, whereas the loss in enzyme activity was 81.5% at 23°C and 45% at 5°C. No loss in enzyme activity was observed on storage at -19°C even after 10 days. Further, the enzyme stored at the above temperature showed no loss in milk clotting activity after 30 days.

10. A linear relationship between enzyme activity and concentration of enzyme was observed in the range of 15 to 80 μg/ml.

11. The Michaelis constant of purified *B. subtilis* K-35 enzyme was found to be 2.77 mg of k-casein/ml.

12. The enzyme peaked a maximum potency of milk clotting activity at 40 x 10^{-3}M CaCl₂. At still higher concentrations, no stimulation in enzyme activity was noted.
13. Metal ions like Hg^{++}, Pb^{++}, Cu^{++} and Ni^{++} inactivated the milk clotting activity appreciably, while Ag^{+} and Mn^{++} exhibited no inhibitory effect on milk clotting activity.

14. Purified *B. subtilis* K-35 enzyme was irreversibly inhibited by EDTA. Complete inhibition was also noted with 1,10-phenanthroline. The enzyme could, however, be reactivated to 14% of the original activity by adding Zn-acetate.

15. Purified *B. subtilis* K-35 enzyme was not affected by the presence of DFP, suggesting the probable absence of a serine residue at the active centre of the enzyme.

16. Sulphydryl reagents and thiol compounds either failed to influence or only partially modified the activity of purified *B. subtilis* K-35 enzyme. The above results indicate that a sulphydryl group may not be essential for the enzyme activity.

17. The molecular weight of the purified *B. subtilis* K-35 enzyme preparation was 27,000 by gel filtration technique.

18. All the amino acids normally present in a protein hydrolysate were present in purified *B. subtilis* K-35 enzyme. Aspartic acid was present in highest amount as compared to other amino acids. Valine and leucine were present in minimum amounts.
19. Purified *B. subtilis* K-35 enzyme preferentially hydrolyzed casein and different fractions of casein in the following order: whole casein, \( \alpha \)-casein, \( \kappa \)-casein and \( \beta \)-casein.

20. During cheese manufacture, acid development was relatively faster when *B. subtilis* K-35 milk clotting enzyme was used either singly or in combination with calf rennet or swine pepsin in 50:50 ratio.

21. Protein and fat losses in whey were slightly higher in the case of cheese made with *B. subtilis* K-35 crude milk clotting enzyme, resulting in lower cheese yields as compared to other combinations of enzymes.

22. The yield of Cheddar cheese prepared using a combination of purified *B. subtilis* K-35 enzyme and calf rennet in 50:50 ratio, was similar to that obtained with calf rennet alone.

23. The initial moisture retention in *B. subtilis* K-35 enzyme cheese was lesser as compared to calf rennet cheese.

24. The rise in pH values during ripening was observed from 30 days to 6 months of ripening in all the five types of cheese made with five different systems of milk clotting enzymes.
25. Higher maturity index values were observed in case of Cheddar cheese made with *B. subtilis* K-35 milk clotting enzyme when used either singly or in combination with other milk clotting enzymes. Cheese made with a mixed coagulant consisting of purified bacterial milk clotting enzyme and swine pepsin showed lower value of maturity index when compared to either crude or purified *B. subtilis* K-35 enzyme cheese.

26. Among fractions of casein, $\alpha$- and $\beta$-fractions were degraded more rapidly in cheese made with *B. subtilis* K-35 enzyme, as compared to cheese made with calf rennet.

27. Organoleptic evaluation of Cheddar cheese prepared by using purified *B. subtilis* K-35 enzyme and swine pepsin in 50:50 ratio, gave higher scores than the cheese made with crude *B. subtilis* K-35 enzyme.

28. Organoleptic evaluation of the cheeses made with five different enzyme systems indicated that cheese prepared by using a mixture of purified *B. subtilis* K-35 enzyme and calf rennet was more acceptable than cheese made with calf rennet alone.

29. On the basis of the above findings it was clear that purified milk clotting enzyme from *B. subtilis* K-35 can be a satisfactory rennet substitute when used in combination with calf rennet in 50:50 ratio for preparation of Cheddar cheese.