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Expression, activation and processing of a novel plant milk-clotting aspartic protease in *Pichia pastoris.*

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Highlights

- Cloning and expression of a plant aspartic protease (preprogaline B) in *Pichia pastoris*.
- Obtention of the preprogaline B either active or inactive form by change of pH medium.
- Description of activation process in the culture medium.
- Preprogaline B has milk clotting activity
- Preprogaline B shows the same pattern of hydrolysis on κ-casein than chymosin, but stronger proteolytic action on α- and β-casein.

Abstract

Galium verum, also known as Lady's Bedstraw or Cheese Rennet, is a herbaceous perennial plant traditionally used in cheese-making. We used RACE PCR to isolate novel enzymes from *Galium verum* with the ability to clot milk. This approach generated two cDNA sequences (named preprogaline A and B) encoding proteins displaying the typical plant aspartic protease primary structure. Preprogaline B was expressed in the yeast *Pichia pastoris*, after deleting and replacing its original signal peptide with the yeast α-factor signal peptide from *Saccharomyces cerevisiae*. The secreted recombinant protein was obtained by growing *P. pastoris* in YPD medium and had the ability to clot milk. The mature form of progaline B is a heterodimeric glycosylated enzyme, with a molecular weight of approximately 48 kDa, that contains a heavy (30.7 kDa) and a light (13.5 kDa) polypeptide chains linked by disulfide bonds. Western blot analysis revealed that progaline B is activated by the acidification of the yeast culture medium and that enzymatic activation requires two steps. First the precursor protein is cleaved into two polypeptide chains by partial removal of the plant-specific insert (PSI) present in plant aspartic proteases; this is later followed by propeptide removal. By altering the pH of the *P. pastoris* culture medium, we