Profiles of Proteinases in Gouda-Type Cheese

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ABSTRACT

The pH of buffers used for extraction of proteinases from Gouda-type cheese affected the kinds of extractable proteinases. Proteinases in the fractions extracted with pH 3.0, 4.0, and 6.0 buffers were separated by CM-Sephadex or diethylaminoethyl-cellulose. Two (F_3-I and F_3-II), three (F_4-I, F_4-II, and F_4-III), and one (F_6) proteinases were separated from the pH 3.0, 4.0, and 6.0 extracts. The F_3-I was completely inhibited by pepstatin. The F_3-I degradated α_{s1}- and β-casein into products with the same mobilities as α_{s1}-casein(f24 to 199) and β-casein(f1 to 189) peptide, respectively. The F_3-II was strongly inhibited by diisopropyl-fluorophosphate and soybean trypsin inhibitor, whereas F_3-II produced fragments with mobilities equal to those of γ-caseins, that is, β-casein(f29 to 209), β-casein(f106 to 209), and β-casein (f109 to 209). The F_4-I was completely inhibited by pepstatin as well as F_3-I, but changes in the polyacrylamide gel electrophoresis pattern of casein by F_4-I were different from those by F_3-I. Properties of F_4-II and F_4-III were similar to those of F_3-I and F_3-II; therefore, F_4-II and F_4-III are considered to be the same enzymes as F_3-I and F_3-II, respectively. The F_6 was strongly inhibited by diisopropyl-fluorophosphate and ethylenediaminetetraacetic acid. In Gouda-type cheese, there are at least four proteinases, two of them being serine proteinases and the other two acid proteinases.

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INTRODUCTION

Nonprotein nitrogen compounds increase during cheese ripening, and this increase is due to casein degradation by the protease systems in cheese. Proteolysis is related to the development of texture and flavor in the ripened cheese. Such compounds as peptides and amino acids, resulting from the proteolysis, are important constituents that affect the cheese quality. Therefore, it is necessary to elucidate the mechanism of proteolysis during cheese ripening before attempting to improve the flavor and texture of cheese. Understanding the mechanism of proteolysis during ripening will also give valuable information for the development of new systems of cheese manufacture, especially those involving accelerated cheese ripening. However, proteolysis during cheese ripening has not yet been clarified in detail. A few reports (1, 10, 11, 12) have been presented concerning proteases from cheese, but no information is available about the purification and characterization of these enzymes.

In this paper, we first examined the effect of the pH of the buffer used for extracting proteinases from Gouda-type cheese. The results showed that proteinases with different pH optima could be extracted from cheese by using buffers with different pH (pH 3.0 to 7.0). Thereafter, the proteinases in each fraction extracted with buffers of pH 3.0, 4.0, and 6.0 were separated by chromatography and their properties investigated. The kind and origin of proteinases in Gouda-type cheese are discussed.

MATERIALS AND METHODS

Materials

The CM-Sephadex and diethylaminoethyl (DEAE)-cellulose were purchased from Seikagaku Kogyo Ltd., Tokyo. Soybean trypsin inhibitor (STI), diisopropyl-fluorophosphate (DFP), and hemoglobin were obtained from Sigma Chemical Co. Chymostatin, antinpain,

leupeptin, and pepstatin were the kind gifts of H. Umezawa and T. Aoyagi of the Institute of Microbial Chemistry, Tokyo. All other reagents were of guaranteed grade.

Cheese

Gouda-type cheese ripened for 5 mo and similar cheese before ripening (5), termed unripe cheese in this paper, each presented by Snow Brand Milk Products Co. Ltd., were used for the experiments. The cheese was produced from high temperature, short time (HTST)-pasteurized milk. Calf rennet was the coagulant.

Casein

Casein was precipitated from fresh raw skimmed milk at pH 4.6 by dropwise addition of 1 N HCl at room temperature. The precipitate was washed with distilled water and lyophilized.

Measurement of Proteinase Activity

Proteolytic activity was measured using hemoglobin and casein as substrates. Hemoglobin was dissolved in .4 *M* acetate buffer (pH 4.0) and casein in .4 *M* phosphate buffer (pH 6.5) or .4 *M* Tris-HCl buffer (pH 8.0). The casein solution was heated at 80°C for 10 min before use to inactivate milk protease. Final concentration of the substrate was 1%. One milliliter of an enzyme solution was added to an equal volume of the substrate solution, and the reaction mixture was incubated at 30°C for 24 h with two drops of toluene as a preservative. (It was confirmed in the preliminary experiment that the rate of proteolytic reaction was linear over 24 h). After incubation, 1 ml of trichloroacetic acid (12%) was added. The mixture was passed through a filter paper and the amount of the proteolytic products in the filtrate was measured using Folin's reagent (3). Proteolytic activity is expressed by increase in optical density (OD) while incubating the reaction mixture for 24 h.

Polyacrylamide Gel Electrophoresis

Changes in casein by the enzyme were examined by polyacrylamide gel electrophoresis (PAGE). The reaction mixtures for these experiments were prepared in the same way as for proteolytic activity measurements, except that the pH of each buffer solution was changed. After incubating for 24 h, the mixture was added to an equal volume of .13 *M* Tris-HCl buffer (pH 6.8) containing 8 *M* urea, 20% glycerol, and 10% (vol/vol) 2-mercaptoethanol, and then heated in boiling water for 5 min to inactivate the enzyme. Sample solutions were kept in a refrigerator until slab-PAGE analysis was started. Slab-PAGE was performed according to the procedure of O'Farrell (9) using 7.5% polyacrylamide gel containing 4.5 *M* urea and Tris-glycine (pH 8.6) as the electrode buffer.

Extraction of Proteinase Fractions from Cheese with Buffers of Different pH

Fractions containing proteinases were extracted from cheese as follows: cheese (20 g) was mixed with 100 ml each of .05 *M* phosphate buffer, pH 6.0 and 7.0, and .05 *M* acetate buffer, pH 3.0 to 5.0. Each mixture was homogenized with a Polytron PT 20 (Kinematica, Switzerland) for 2 min. After the homogenate had stood for 5 min, the cream layer was removed. The partially defatted homogenate was adjusted to the desired pH with 1 N HCl or 1 N NaOH, shaken for 1 h, and centrifuged at 8000 rpm for 30 min. The supernatant under the layer of residual fat was filtered through absorbent cotton, dialyzed against distilled water overnight, and then lyophilized. By assaying the proteolytic activity in each of these lyophilized preparations (termed extract fractions), the effect of pH at extraction on the kind of extractable proteinases was surveyed. All treatments described were carried out at 4°C.

Chromatographic Separation of Proteinase Extracts

Separation of Proteinases from the pH 3.0, Extracted Fraction. The fraction (450 ml) extracted with the pH 3.0 acetate buffer was dialyzed against a .05 *M* acetate buffer at pH 4.0 for 24 h. Insoluble materials were removed by centrifugation, then the supernatant was concentrated by ultrafiltration and applied to a column of CM-Sephadex that had been equilibrated with a .05 *M* acetate buffer at pH 4.0. Elution was by a linear concentration gradient 0 to 1.5 *M* NaCl; aliquots collected were 20 ml. Hemoglobin dissolved in .4 *M* acetate buffer