Isolation and Characterization of Two Forms of an Acidic Bromelain Stem Proteinase

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Two forms of an acidic bromelain proteinase isolated from crude bromelain, an extract from pineapple stem, were found by a two-step FPLC purification procedure. The basic main components were removed by cation exchange chromatography and the breakthrough fraction was further resolved by anion exchange chromatography into 15 protein fractions, only two of which, called SBA/a and SBA/b, were proteolytically active. These components were characterized by electrospray mass spectroscopy (ESMS), isoelectric focusing, N-terminal amino acid sequence analysis, monosaccharide analysis, and enzymatic parameters. The molecular masses of SBA/a and SBA/b were determined by ESMS to be 23,550 and 23,560, respectively. The isoelectric points (pI) of the two bands of SBA/a were 4.8 and 4.9; SBA/b focused as a single band at pI = 4.8. Partial N-terminal amino acid sequences (11 residues) were identical to SBA/a and SBA/b and identical with those of stem bromelain, the basic main proteinase of the pineapple stem, and fruit bromelain, the acidic main proteinase of the pineapple fruit. Both components are highly glycosylated; hydrolysis of SBA/a yielded about twofold more monosaccharide per protein than SBA/b. The comparison of the catalytic properties of SBA/a with those of SBA/b revealed no relevant differences in the hydrolysis of three peptidyl-NH-Mec substrates and in the inhibition profiles using chicken cystatin and E-64, indicating that these components can be considered as two forms of a single enzyme. Both forms are scarcely inhibited by chicken cystatin and slowly inactivated by E-64, hence are nontypical cysteine proteinases of the papain superfamily.

1. INTRODUCTION

The crude bromelain extract from pineapple stem (Ananas comosus) is an unusual complex mixture of different cysteine proteinases and their peptide inhibitors, in addition to some other noncharacterized components. Crude bromelain has shown under in vitro and in vivo conditions antiproliferative, antimetastatic, and tumor cell growth inhibitory properties in different tumor models (Batkin et al., 1988; Munzig et al., 1994; Garbin et al., 1994; Harrach et al., 1994; Grabowska et al., 1997). However, it is still unclear which of the components of the crude bromelain extract were responsible for the observed effects.
Using high-resolution FPLC chromatography on a Mono S column, two main basic glycosylated proteinases were found in crude bromelain (Rowan et al., 1988; Harrach et al., 1995). On the basis of enzyme kinetic and structural parameters, both main components were considered as two forms of a single protein called stem bromelain, EC 3.4.22.32 (Rowan et al., 1988; Harrach et al., 1995). This major basic proteinase has been sequenced. On the basis of its amino acid sequence similarity it was shown to be a member of the papain superfamily (Ritonja et al., 1989). Further purification using different cation-exchange chromatography procedures yielded a minor basic nonglycosylated proteinase called ananain, EC 3.4.22.31 (Rowan et al., 1988), or bromelain F9 (Harrach et al., 1995). On the basis of molecular masses, N-terminal amino acid sequences, and enzymatic activity both preparations of the minor proteinases seemed to be identical, but were found to be distinct from the main basic proteinases (Napper et al., 1994; Harrach et al., 1995). Very recently the complete amino acid sequence of ananain/F9 was reported (Lee et al., 1997). In addition, from the ananain side fraction, comosain was isolated and partially characterized (Rowan et al., 1990; Napper et al., 1994). Both ananain and comosain appeared in separable and characterizable multiple enzyme forms. The three basic stem enzymes, stem bromelain, ananain, and comosain, have been considered to be genetically distinct cysteine proteinases (Napper et al., 1994).

However, DEAE-Sephadex ion-exchange chromatography of the pineapple stem extract eluted from CM-Sephadex yielded a minor acidic proteinase with an \( M_r \) of 23,000. This proteinase has been characterized by amino acid and carbohydrate composition, as well as by its capacity to hydrolyze synthetic substrates (Ota et al., 1985). On the basis of these parameters, the glycosylated acidic proteinase from pineapple stem was found to be very similar to the main proteinase of pineapple fruit, which is an acidic protein, except for differences of the amino-terminal sequence and of the activity toward casein and some synthetic substrates (Ota et al., 1985). Furthermore, Yamada et al. (1976) purified by cellulose AP and ion-exchange chromatography the acidic (pI 4.6), nonglycosylated 31,000-Da main proteinase from pineapple fruit, called FA2. Its amino acid composition was not markedly different from that of basic stem bromelain. In addition, the N-terminal amino acid sequence of FA2 was found to be identical with that of stem bromelain.

Fruit bromelain has been isolated by cation-exchange and affinity chromatography of an acetone fraction obtained from pineapple fruit juice. The kinetic parameters of the hydrolysis of some peptidyl-NH-Mec substrates have been determined: chicken cystatin did not, whereas E-64 only scarcely inhibited the fruit bromelain proteinase (Rowan et al., 1990).

Thus it seemed necessary to purify and characterize the acidic proteinase(s) from crude pineapple stem extract in order to sort out this discrepancy and provide a more detailed comparison between basic and acidic stem proteinases as well as between fruit and stem proteinases. In the present paper we describe the purification of two acidic proteinases from crude bromelain, which are presumably two forms of a single enzyme.

2. EXPERIMENTAL

2.1. Materials

Crude bromelain from pineapple stem was kindly provided by J. Houck (Seattle, WA) and used for enzyme purification. In addition, for analytical purposes, crude bromelain was also obtained from Sigma Chemicals (Deisenhofen, Germany, Cat. No. 2252). A further preparation for pharmaceutical use was obtained from Mucos Pharma GmbH (Geretsried, Germany). Coomassie blue G-250 was from Serva (Heidelberg, Germany). Bovine serum albumin, compound \( L\)-trans-epoxyacetyl-l-leucylamido(4-guanidino)-alanine (E-64), soybean trypsin inhibitor, and iodoacetamide were purchased from Sigma. \( L\)-Pyroglutamyl-l-phenylalanyl-l-leucine-p-nitroanilide (PFLNA) was supplied by Bachem Feinchemikalien AG (Bubendorf, Switzerland). The electrophoresis calibration kit (low-molecular-mass proteins), isoelectric focusing calibration kit (pH 2.5–6.5), and Immobiline were from Pharmacia (Freiburg, Germany). Monosaccharide stan-