FEBS Letters 382 (1996) 186–188 FEBS 16788

Plant chitinases use two different hydrolytic mechanisms

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Received 27 November 1995; revised version received 3 February 1996

Abstract Bacterial, fungal, animal, and some plant chitinases form family 18 of glycosyl hydrolases. Most plant chitinases form the family 19. While some chitinases also have lysozyme activity, animal lysozymes belong to different families. For glycosyl hydrolases, two reaction mechanisms are possible, leading to either retention or inversion of the anomeric configuration. We analyzed by HPLC the stereochemical outcome of the hydrolysis catalyzed by cucumber and bean chitinases, belonging to families 18 and 19, respectively. Cucumber chitinase used the retaining mechanism as known for bacterial chitinases and hen egg white lysozyme for which the mechanism has been determined. In contrast, bean chitinase catalyzed the hydrolysis of chitooligosaccharides with overall inversion of anomeric configuration.

Key words: Chitinase; Lysozyme; Catalytic mechanism; Anomeric configuration

1. Introduction

Chitinases (EC 3.2.1.14) are enzymes that catalyze the hydrolysis of ß-1,4 linkages in chitin, a homopolymer of *N*-acetyl-D-glucosamine [1]. The substrates of chitinases and lysozymes are very similar. Indeed, some chitinases also hydrolyse bacterial cell walls [1,2].

Plant chitinases are a structurally diverse group with respect to their physical properties, enzymatic activities and localization [3]. Some chitinases, especially in combination with β-1,3 glucanases, inhibit fungal growth in vitro [4], substantiating the idea that these enzymes play an important role in plant defense against pathogens. Chitinases could also release elicitors from fungal cell walls [5]. Recently it was shown that root chitinases can differentially inactivate Nod-factors produced by certain *Rhizobium* strains and therefore may determine the specificity of the bacterium–host plant interaction as well as modulate the activity of Nod-factors [6]. Chitinase may also have an important function in development, as demonstrated by the ability of a chitinase to overcome a block in embryogenesis in a mutant cell line of carrot [7]. Plant chitinases have been divided into several classes based on amino acid sequence [8]. In the classification system of glycosyl hydrolases, all chitinases are grouped into two families [9]. Class I, II, IV and V chitinases form the family 19, that is only known in plants. Family 18 includes all fungal, animal and bacterial chitinases as well as plant chitinases of classes III and VI.

The hydrolytic action of glycosyl hydrolases can take place with either retention or inversion of the anomeric configuration. The hydrolytic mechanism of but a few chitinases has been analyzed. Two bacterial chitinases and hevamine, a plant chitinase/lysozyme displayed a retaining mechanism [10,11]. Hen egg white lysozyme (family 22) [12] is also a retaining enzyme, while T4 lysozyme (family 24) was recently found to invert the anomeric configuration [13]. Holm and Sander [14] recently proposed that a family 19 chitinase and several lysozymes descend from a common ancestor, based on a weak folding similarity and the similar function. Goose lysozyme (family 23) would be structurally more related to barley endochitinase than to T4 lysozyme. We determined the mechanism of two plant chitinases belonging to the two different families. Our results demonstrate a major difference between plant chitinases and hen egg white lysozyme.

2. Materials and methods

2.1. Purification of chitinases

Ethylene-treated bean leaves were homogenized in 100 mM Na-acetate buffer, pH 5.5. Extraction and all subsequent steps were performed as previously described for tobacco chitinase [15]. Intercellular fluid of tobacco necrosis virus (TNV)-infected cucumber leaves was extracted as described [16]. After dialysis, isoelectric focusing was performed twice with a liquid IEF Rotorfor system (Biorad) according to the supplier's protocol using a Bio-lyte range 3/5 ampholyte. Fractions containing the activity were combined, dialyzed and further purified by anion-exchange FPLC using a Mono-Q HR 5/5-column (Pharmacia). The column was equilibrated with 20 mM bisTris buffer, pH 6. After sample application the column was washed with 4 volumes of the same buffer. A lincar salt gradient from 0 to 1 M NaCl was applied, fractions with the highest chitinase activity were collected, dialyzed and lyophilized.

2.2. Preparation of chitooligosaccharides

Chitooligosaccharides (DP 2–5) were obtained by acid hydrolysis of chitin as described previously [10]. The sodium borohydride reduction of chitotetraose and chitopentaose into chitotetraitol and chitopentaitol was conducted in a conventional fashion [10].

2.3. HPLC analysis of the enzymatic hydrolysis

The cleavage sites and stereochemistry of hydrolysis of bean chitinase on chitotetraose, chitotetraitol and chitopentaitol were determined as follows: 20 ml of reduced oligosaccharides (1 mg/ml in water) were incubated with 10 ml of chitinase (1 mg/ml in water) at 37°C. For chitotetraose, 40 ml substrate (1 mg/ml in water) were incubated with 10 ml bean chitinase (1 mg/ml in water) at the same temperature. The cucumber chitinase (10 ml of a 0.5 mg/ml solution in water) was added to 30 ml of chitotetraose (0.5 mg/ml in water) and 10 ml of 25 mM citrate-phosphate buffer pH 3.0. After the indicated times, all the reaction mixtures were immediately analyzed by reverse-phase HPLC using a C18 nucleosil column (5 mm, Interchim) eluted with distilled water as described [10].

3. Results

3.1. Purification of chitinases

Chitinases were purified from ethylene-induced leaves of

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Abbreviations: ICF, interceilular fluid; TNV, tobacco necrosis virus.

SSDI S0014-5793(96)00174-3

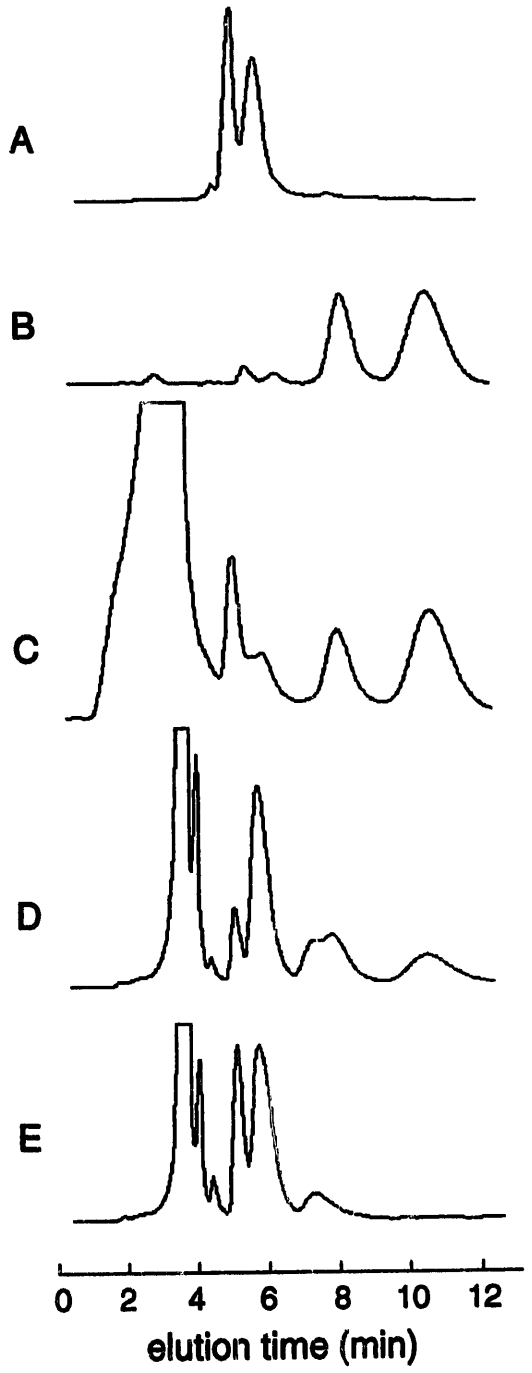

Fig. 1. HPLC separation of chitobiose anomers produced during hydrolysis of chitotetraose by plant chitinases. (A) Control: chitobiose, β- and α-anomers. (B) Substrate: chitotetraose, β- and α-anomers. (C) HPLC profile obtained after 3 min hydrolysis by cucumber chitinase. (D) Profile obtained after 5 min hydrolysis by bean chitinase. (E) Profile obtained after 1 h hydrolysis by bean chitinase.

bean and from TNV-infected cucumber plants. Bean chitinase was purified by affinity chromatography and gel-filtration. The chitinase eluted as a single peak consisting of the pure class I chitinase of 32 kDa as confirmed by SDS-PAGE [17], silver-stained according to Blum et al. [18]. Cucumber chitinase was purified by IEF and anion exchange chromatography. The chitinase eluted as a single peak consisting of the pure class III chitinase of 27 kDa as confirmed by SDS-PAGE.

3.2. Substrate specificity and stereochemical analysis

In the case of chitooligosaccharides, mutarotation is slow enough to distinguish each anomer on a HPLC profile. For instance, an equilibrated mixture of α- and β-chitobiose appears as two peaks on the chromatogram shown in Fig. 1A. It has been previously demonstrated that the peak with the shortest retention time corresponds to the β-anomer [10]. The stereochemical course of the enzymatic hydrolysis of chitooligosaccharides can therefore be studied using HPLC. Reduced oligosaccharide substrates can help to discriminate between the newly formed reducing ends and those originating from the preexisting reducing end of natural oligosaccharides. Bean chitinase was found to have virtually no action on chitotetraitol while it hydrolyzed chitopentaitol at two different sites releasing a mixture of chitobiose, chitobiitol, chitotriose and chitotriitol which was too complex to unambiguously infer the stereochemistry of hydrolysis (data not shown). Chitotetraose, on the other hand, was found to be hydrolyzed by bean chitinase at only one site, releasing exclusively chitobiose. In addition to the two peaks corresponding to unreacted chitotetraose, the chromatogram recorded after 5 min hydrolysis (Fig. 1D) shows two peaks corresponding to the two anomers of chitobiose. The peak corresponding to the α-chitobiose is 5 times larger than that of β-chitobiose, indicating that bean chitinase proceeds by inversion of configuration at the hydrolysis site. The chromatogram recorded after 1 h hydrolysis (Fig. 1E) shows that all the substrate has been hydrolyzed and that mutarotation has almost reached equilibrium with an α/β ratio of about 2.

The stereochemical course of the hydrolysis of chitotetraose by the cucumber chitinase was studied similarly. The chromatogram recorded after 3 min reaction shows that about 30% of the substrate has been hydrolyzed and that the main product is β-chitobiose (Fig. 1C). Since the mutarotation equilibrium normally gives a predominance of the α-anomer with an α/β ratio of about 2 (Fig. 1A,E), this demonstrates that the cucumber chitinase operates with overall retention of the anomeric configuration. Comparison of Fig. 1C and 1D clearly shows that the bean and cucumber enzymes operate by different mechanisms giving rise to opposite stereochemical outcomes.

4. Discussion

We have determined the hydrolytic mechanism of cucumber class III chitinase (family 18) and bean class I chitinase (family 19). Cucumber chitinase retained the anomeric configuration, as recently determined with another class III chitinase, hevamine [11]. Plant class III chitinases share with bacterial chitinases of the same family 18 the same mechanism and a very similar three-dimensional structure [10,19]. Surprisingly, the class I chitinase was found to operate with inversion of the anomeric configuration. Our results are in agreement with the recent observation that a yam chitinase (family 19?) also performs an inversion of the anomeric configuration [20]. It is therefore likely that all family 19 chitinases share the same active site structure, catalytic machinery and stereochemical outcome. The three-dimensional structure of a family 19 chitinase from barley has been reported [21] and was used to

propose a model for substrate binding and mechanism of action. The authors suggested that "the most likely mechanism is a double displacement similar to that of HEWL", despite the fact that they then show the structure to be more compatible with a single displacement mechanism. Our results suggest that the *H. vulgare* chitinase operates indeed with overall inversion of configuration. The difference in their respective mechanisms renders more unlikely the proposed superfamily of lysozymes and family 19 chitinases, based on a limited structural similarity [14]. The catalytic residues of most glycosyl hydrolases are submitted to a conservation pressure so intense that they can be virtually the only invariant residues in distantly related enzymes [22]. The evolution of an inverting into a retaining glycosyl hydrolase (or vice versa) via a change in the position of the catalytic base is, however, possible. T4 lysozyme could be converted from an inverting into a conserving enzyme by a single amino acid exchange, albeit at the cost of most of the catalytic efficiency [13]. In the case of enzymes hydrolysing *N*-acetylglucosaminic bonds by retention of configuration, however, the catalytic base can be replaced by the C-2 acetamido group [11,23]. This could reduce the conservation pressure on the catalytic base of retaining lysozymes and chitinases.

There have been two independent evolutions of chitinases in plants. Some belong to the ancient chitinase family 18, present in all kingdoms, from bacteria to animals and fungi. A few of them also have lysozyme activity. The double displacement mechanism may allow transglycosylation reactions which could be used for the building and degradation of cell walls containing chitin [24,25]. Plants, having no chitin in their cell wall, used this chitinase for defense. The possible additional lysozyme activity could also protect plants against bacterial pathogens.

On the other hand, the chitinase family 19 has so far only been found in higher plants. As mentioned, they may have developed from the same ancestor as animal lysozymes, but the evidence is weak, as these chitinases do not share the same hydrolytic mechanism, despite the fact that they can also function as lysozymes [1]. Alternatively, these catalytic domains may have evolved as part of an *N*-acetylglucosamine-binding protein, together with the binding domain also found in lectins and Win-proteins [26]. We have shown that the lectin domain of tobacco chitinase is not necessary for function but improves the antifungal activity of the enzyme [16]. The primary substrate may have been an endogenous oligosaccharide signal similar to the rhizobial Nod-factors involved in nodule formation [7]. Indeed, family 19 chitinases were found to inactivate Nod-factors [6]. They may have been recruted later to complement the other chitinases for defense against fungal or bacterial pathogens. The inverting mechanism could actually allow the hydrolysis of additional substrates with other substitutions in position 2, e.g. chitosan. While this paper was being reviewed, the mechanism of hydrolysis of chitosan by a bacterial chitosanase was reported and indeed found to be inverting [27].

Upon infection, plants produce several chitinases of both families that differ in localization, activity, chitin-binding property and, as shown here, catalytic mechanism. This variety of chitinases allows the plant to match many different requirements. Further investigations are needed to obtain more information about the substrate specificity of various chitinases as well as their possible involvement in the control of plant development.

Acknowledgements: This work was supported by the Swiss National Science Foundation, grant 3100-26492.89

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