RUMEN FUNGAL Β-GLUCANASE AND XYLANASE GENES: POTENTIAL FOR GENETICALLY ENGINEERED CEREAL CROPS

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Summary. Microbial non-starch polysaccharide hydrolases have been used to improve the utilisation of cereal grains in brewing, feed and food industries. Two types of these hydrolases (β-glucanases and xylanases) were cloned from a highly lignocellulolytic rumen fungus, *Neocallimastix patriciarum*. The *N. patriciarum* β-glucanases, CELA and CELD, were found to possess high activity on barley β-glucan and cellulose through hydrolysis of 1,4-β-glucosidic linkages. Some of these fungal hydrolases have higher thermostability profiles than analogous enzymes from germinating barley. The genes encoding these hydrolases have potential value for the production of genetically engineered cereal crops to overcome the problems associated with non-starch polysaccharides in grains.

INTRODUCTION

Polysaccharides other than starch, such as β-glucans and pentosans are present in significant amounts in various cereal grains. The β-glucans constitute 2-10% of barley grain weight and the predominant form of cereal β-glucans is 1,3-1,4-β-glucan (8). In wheat and oats, arabinoxylan (pentosan) is the major form of non-starch polysaccharide. These polysaccharides often present a problem in utilisation of cereals. The presence of viscous solubilised β-glucans in wort causes a slow rate of filtration and a low extraction yield, which can be improved by the addition of microbial β-glucanases (4). Cereal grains are commonly used as animal feeds. Many recent studies have demonstrated anti-nutritive activity of non-starch polysaccharides in monogastric animals due to the increased viscosity of these solubilised polymers, which reduces the rates of digestion and absorption of nutrients in the small intestine and also causes sticky droppings in chickens (6). Addition of xylanase and β-glucanase to feeds has been shown to improve the growth rate and feed conversion efficiency of chickens (2). It has also been shown that removal of xylan by the addition of xylanase to flour can improve the loaf volume and the texture of bread (9, 14). Although addition of β-glucanases and xylanases to the above-mentioned processes has proven to be effective, the cost of the enzyme production is high and this often restricts industrial adoption of these technologies. An alternative approach to overcoming the noted difficulties is to transfer β-glucanase and xylanase genes to cereal crops for expression in grains.

β-glucanases and xylanases are produced from many lignocellulolytic microorganisms. Generally, lignocellulolytic fungi are excellent producers of these polysaccharide hydrolases and produce these enzymes with high activity. In this communication, we report the enzymic properties of cloned β-glucanases and xylanase from a rumen fungus, *Neocallimastix patriciarum* and discuss their potential use for genetic improvement of cereal crops.

METHODS

DNA manipulation techniques

General recombinant DNA techniques were performed essentially according to the procedures described by Sambrook et al. (15). CELA, CELD or XYNA cDNAs from a rumen fungus, *N. patriciarum,* were constructed in the pBTac2 vector (Boehringer). The cloned fungal enzymes were prepared from recombinant *E. coli* as described previously (21).

Assays for β-glucanases and xylanase

β-glucanase and xylanase activities were measured, based on the amount of reducing sugars released from substrates, as described previously (19, 21). One unit of enzyme activity is defined as 1 µmole of reducing sugars released from polysaccharide substrates per minute.

RESULTS AND DISCUSSION

Rumen anaerobic fungi have been shown to degrade plant fibre very actively and produce various plant polysaccharide hydrolases (18). A number of genes encoding β-glucanases and xylanases from the rumen fungus, *N. patriciarum*, have been cloned (19-21). Of these β-glucanases, CELA and CELD were found to be highly active enzymes against various substrates (Table 1). Besides their ability to hydrolyse cellulose (1,4-β-glucan), they are most active in hydrolysis of 1,3-1,4-β-glucans, such as lichenan and the predominant form of barley β-glucan. These β-glucanases had no activity on laminarin which contains about 90% or more 1,3-β-glucosidic linkages, thus indicating that these enzymes hydrolyse only 1,4-β-linkages. We also isolated a xylanase gene (XYNA) encoding an enzyme possessing high activity in hydrolysis of oat spelt xylan (Table 1).

Table 1. Activity of crude β-glucanases and xylanase cloned from *N. patriciarum.*

Substrate	Specific activity (U/mg protein)		
	CELA	CELD	XYNA
CM-cellulose	0.47	5.92	0
Crystalline cellulose (Avicel)	0.20	0.18	0
Lichenan	13.60	14.31	0
Barley β-glucan	3.51	5.36	0
Laminarin	0	0	0
Oat spelt xylan	0	0.12	2.7

In an attempt to improve enzyme activities and reduce their molecular size, the cloned fungal hydrolases have been modified by manipulation of the genes encoding them. We also tagged these enzymes with an antigenic epitope (Flag peptide) to simplify purification and detection procedures of the recombinant enzymes. The specific activities of the genetically modified hydrolases are shown in Table 2.

Table 2. Specific activity of genetically modified *N. patriciarum* enzymes.

Substrate	Specific activity (U/mg protein of purified enzyme)		
	CELA*	CELD Domain II*	XYNA Domain II

Barley β-glucan	3838	458	0
Oat spelt xylan	0	4	5500

* Flag peptide-tagged at the N-terminus of these enzymes.

The molecular mass of the modified CELD and XYNA was reduced from 91 kDa and 53 kDa to 44 kDa and 26 kDa, respectively. In order to assess effectiveness of these hydrolases in various environmental conditions, pH and temperature profiles were determined. As shown in Table 3, all three enzymes exhibited maximum activity at acid pH and had substantial activity in a pH range from 4.5 to 7 except xylanase which was able to work even at mildly alkaline pH. The optimal temperature of these fungal hydrolases is basically similar to the physiological temperature in the rumen. The cloned β-glucanases in a non-glycosylated form produced in *E. coli* were almost completely inactive at 60°C. The fungal xylanase had slightly higher thermostability than the β-glucanases (Table 3). Interestingly, our recent study showed that the cloned glycosylated CELA produced in *S. cerevisiae* exhibited increased thermostability. A significant portion of enzyme activity was retained after heating the enzyme solution at 70°C for 1 hr (Denman and Xue, unpublished data). The plant 1,3-1,4-β-glucanases from germinating barley have been shown to be thermo-unstable (5, 7), but have better resistance to heat inactivation in the kilning of barley malt when they are in a low-moisture condition and in the presence of reduced glutathione (5). It is possible that the fungal hydrolases, when protected, would have even higher thermostability.

Table 3. Molecular mass, pH and temperature profiles of cloned *N. patriciarum* enzymes produced in *E. coli*.

	CELA	CELD DomainII	XYNA DomainII
Molecular mass	44 kDa	44 kDa	26 kDa
pH range			
>40% of maximum	4.5-7	4.5-7	4.5-9
Optimal	5	5	6
Temperature range			
>40% of maximum	25-55°C	25-55°C	25-60°C
Optimal	40°C	40°C	50°C

It is of interest to introduce these fungal genes encoding highly active hydrolases into cereal crops for genetic improvement in respect to non-starch polysaccharide contents and β-glucanase and xylanase activity. With rapid advances in techniques of plant genetic manipulation, many fertile transgenic cereal crops such as barley, wheat, oats and sorghum have been produced using the particle bombardment method (16). The feasibility of producing transgenic crops with low non-starch polysaccharide contents or higher hydrolase activities in germinating seeds without damaging viability of the plants is supported by classical genetic studies. Some success has been reported in obtaining healthy mutants using

chemically-induced mutation of seeds, resulting in reduced non-starch polysaccharide contents of cereal grains or higher hydrolase activities (1, 12). Stable expression of microbial hydrolases (phytase and xylanase) in tobacco under the control of the constitutive CaMV 35S promoter has recently been demonstrated (10, 13). Aspegren et al. (3) have also obtained success in introducing a fungal β-glucanase into suspension-cultured barley cells using a constitutive promoter. However, if high levels of these enzymes are required (e.g. >1% total cellular protein), it might be desirable to restrict the expression of the hydrolase transgenes to seeds and express at an appropriate developmental time. Many studies towards an understanding of gene regulation in maturing and germinating seeds of cereal crops and characterisation of seed-specific promoters have been documented in a number of recent reviews (11, 16, 17). Further studies in this area will promote the production of desired transgenic crops with expression of these hydrolytic enzymes in cereal grains.

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