Isolation and characterization of a cDNA clone from *Lolium temulentum* L. encoding for a sucrose hydrolytic enzyme which shows alkaline/neutral invertase activity

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Abstract

A novel cDNA clone, functionally expressed in *E. coli*, was isolated from a *L. temulentum* L. cDNA library. The expressed protein hydrolysed sucrose with an apparent $K_m$ of approximately 18 mM, and produced equi-molar concentrations of glucose and fructose. Optimum activity was observed at pH 7–7.5; there was little or no activity at pH 5.5. The expressed protein did not hydrolyse raffinose, stachyose or maltose. The activity of the expressed protein was inhibited by fructose (50% at 15 mM) and TRIS (50% at 2.5 mM), but was not affected by MgCl$_2$, CaCl$_2$ or MnCl$_2$. These findings suggest that this cDNA clone encodes for an alkaline/neutral invertase. Sequence analysis revealed little homology with published sequences for acid invertase, however the invertase motif (NDPN) identified in other invertases was present. Expression studies show that the gene encoding for this enzyme is not regulated by sucrose accumulation in leaf tissue.

Key words: cDNA clone, *Lolium temulentum* L, sucrose hydrolysis, invertase activity.

Introduction

Sucrose is central to plant growth and development. It plays a key role in partitioning, transport, and regulation of metabolism and gene expression (Stitt, 1991; Pollock and Farrar, 1996; Koch, 1996). Consequently, sucrose metabolism has been the subject of much investigation. Sucrose is known to be catabolised in two ways: hydrolysis via the action of invertase (EC 3.2.1.26), and cleavage in the presence of UTP, leading to the production of UDP-glucose and fructose. This reaction is catalysed by sucrose synthase (EC 2.4.1.13) (Copeland, 1990).

Invertases can be divided into two classes, the acid invertases, with pH optima in the range 4–5.5, and the alkaline/neutral invertases with pH optima in the range 7–8. The acid invertases can be further divided into soluble and insoluble forms. The soluble acid invertases are believed to reside in the vacuole while the insoluble acid invertases are associated with the cell wall (Leigh et al., 1979; Faye et al., 1986; Lauriere et al., 1988; Fahrendorf and Beck, 1990; Walker and Pollock, 1993). Unlike acid invertases (Obenland et al., 1993; Walker and Pollock, 1993), alkaline/neutral invertases are not glycosylated (Strommel and Simon, 1990; Chen and Black, 1992; Walker et al., 1997), and are believed to reside in the cytoplasm (Ricardo and ap Rees, 1970; Copeland, 1990). Both acid and alkaline/neutral invertases can be found in the same tissue, and Zrenner et al. (1996) showed that altering the activity of acid invertase had little effect on the activity of alkaline invertase in the same tissue.

The two classes of invertases are believed to play different roles in plants. Acid invertases have been shown to constitute the majority of invertase activity within plant cells and their activity has been suggested to correlate negatively with sucrose concentration, high acid invertase activity being observed in tissues containing low sucrose concentrations (reviewed by ap Rees, 1974). High acid invertase activities are also found in plant tissues in which there is a high demand for hexose, such as rapidly dividing cells (Schaffer et al., 1987), young developing fruits and seeds (Yelle et al., 1991; Sung et al., 1994), or ripening tissue (Iwatsubo et al., 1976; Klann et al., 1993; Sato et al., 1993). Acid invertase activity has also been regulated by sucrose accumulation in leaf tissue.

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shown to be involved in phloem unloading in sink tissue (Ho, 1988) and futile cycling of sucrose in source tissue (Huber et al., 1992).

By contrast, alkaline/neutral invertases have been proposed as ‘maintenance’ enzymes involved in providing substrate for the TCA cycle in tissues where acid invertase or sucrose synthase activities are low (ap Rees, 1974). High alkaline/neutral invertase activity has been reported to be closely related to sucrose accumulation in mature roots of sugar beets (Masuda et al., 1987). Alkaline/neutral invertase has also been shown to decline with accumulation of sucrose in developing muskmelon (Cucumis melo) and peach fruit (Prunus persica) (Ranwala et al., 1991; Vizzotto et al., 1996). Walker et al. (1997) showed that activity of alkaline/neutral invertase in L. temulentum leaf blades was restricted to the lower, heterotrophic zones. However, Roitsch et al. (1995) found that pre-incubation of autotrophic tissue cultures of Chenopodium rubrum in sugars had no effect on the levels of alkaline/neutral invertase, and that there was no tissue-specific distribution of this enzyme activity. Although alkaline/neutral invertase has been studied at the enzyme level, little work has been carried out on the regulation of its expression or its homology with the acid invertases.

L. temulentum is known to contain multiple forms of invertase, including an alkaline/neutral form. To help understand the metabolic role of alkaline/neutral invertases a cDNA clone from mRNA of L. temulentum leaf tissue was isolated which, when expressed in E. coli, encodes for an enzyme that shows similar characteristics to alkaline/neutral invertases reported in the literature. This study looked at the regulation of this clone by sucrose and investigated its homology to acid invertases at both the DNA and amino acid levels. The characterization of this clone was also used to consider whether the alkaline/neutral invertases described in the literature represent either two distinct enzyme classes or modified versions of the same class.

Materials and methods

Plant material for mRNA extraction

L. temulentum L. (Ba 3081, summer annual) plants were grown from seed in a 16°C heated glasshouse in trays of John Innes No. 3 compost (Winters et al., 1995). Prior to sucrose feeding, plants at the 4th leaf stage were maintained at low irradiance to deplete the leaves of soluble carbohydrates (Cairns and Pollock, 1988). Leaves were subsequently detached and fed sucrose through the transpiration system by incubation in 200 mM sucrose solution in the dark for 24 h (Housley and Pollock, 1985). Leaves were removed from the sucrose solution at various time intervals, washed with distilled water to remove excess sucrose, the bottom 1 cm removed, and the remainder frozen in liquid N₂ and stored at −80°C.

Water-soluble carbohydrate analysis

Sucrose accumulation in the excised leaves following incubation in 200 mM sucrose in the dark was determined by HPLC (Cairns and Pollock, 1988). Values represent the mean of five determinations.

mRNA extraction and cDNA synthesis

Total RNA was extracted from the frozen leaves as described by Thomas (1990). Poly(A)⁺ RNA was purified with an mRNA purification kit (Dynabeads, product 610.01 Dynal LTD, Merseyside, UK) according to the manufacturer’s instructions. Equal quantities of mRNA, from leaf tissue harvested at different times after excision, were pooled and a cDNA library was constructed using a uni-directional lambda Zap II cDNA synthesis kit (Stratagene Cambridge, UK) according to the manufacturer’s instructions. The library was packaged using Gigapack II Gold packaging extract (Stratagene).

Screening

Plasmid isolation, ligation and transformation were carried out as described by Ausubel et al. (1992). The library was screened using functional activity. Phagemids (pBluescript) were excised from the lambda DNA in vitro as described in the manufacturer’s instructions (Stratagene) and used to transform E. coli (Solar cells–Stratagene). Transformed cells were plated on to M9 minimal media supplemented with ampicillin (50 mg l⁻¹), 1 mM IPTG and sucrose (2 g l⁻¹) as the sole carbon source (Ausubel et al., 1992). A full-length clone (Succ2) was isolated using the original clone as a probe to re-screen the library using the method of ‘plaque lifts’ as described by Winters et al. (1995).

Northern analysis

Total RNA (10 μg) was denatured and separated on 1.5% agarose gels containing 17.2% formaldehyde. After separation, RNA was transferred to Hybond-N⁺ membrane (Amerham International plc, Buckinghamshire, UK) by capillary transfer and cross-linked by UV light. Pre-hybridizations and hybridizations were carried out as described by Winters et al. (1995). cDNA probes were labelled with ³²P-dATP using random-primed DNA labelling kit (Stratagene) in accordance with the manufacturer’s instructions. Final washes were carried out in 0.1 x SSC, 0.1% (w/v) SDS at 45°C.

cDNA sequencing

Plasmid DNA was isolated using the modified alkaline-lysis method (sequencing protocol—Perkin Elmer, Beaconsfield, UK) and the cDNA was sequenced with a Perkin Elmer Model 480 (Applied Biosystems, Warrington, UK) using Taq Dydeoxy Terminator Cycle Sequencing Kit (Applied Biosystems). Sequences were compiled using the instrument software (version 1.2.1) and compared with other sequences in the EMBO database using FastA, Tiastra, and Blast (BBSRC Computing Center 4600).

Induction and extraction of protein from E. coli

E. coli cultures were incubated overnight in LB media (Ausubel et al., 1992) supplemented with ampicillin (50 mg l⁻¹) in a shaking incubator at 37°C. Cultures were diluted 1:100 with fresh LB supplemented with ampicillin and incubated at 30°C to OD₆₀₀=0.5. Cultures were induced by the addition of IPTG (1 mM final concentration) to the culture media and returned to the incubator for a further 2 h. Cultures were then removed and spun for 1 min at 10,000 g at 4°C. The supernatants were removed and the pellets washed twice and resuspended in imidazole buffer (pH 7.0) (1/5 the culture volume). Cells were lysed by sonication (MSE sonicator, Fisher Scientific, Loughborough, UK) on ice for 3 x 15 s separated by a 30 s
interval. Cell debris was removed by centrifugation (15 min at 10,000 g) at 4°C. Bacterial extracts were desalted on a Bio-gel P6-DG column (Bio-Rad, Hemel Hempstead, UK).

**Extraction of leaf proteins**

Leaves of *L. temulentum* were ground in 100 mM phosphate/citric acid buffer (pH 7.4) containing 1 mM EDTA and 20 mM β-mercaptoethanol at 4°C. The extracts were spun (10,000 g for 10 min) and the supernatant removed. Proteins were precipitated between 30% and 80% ammonium sulphate, spun as above and the pellet resuspended in 50 mM phosphate/citric acid buffer. The extract was desalted by centrifugation through a 10 ml bed of Bio-Gel equilibrated with the same buffer.

**Enzyme activity**

Enzyme activity was measured either by continuous or stopped reactions. Stopped reactions were terminated by placing the samples in a boiling water bath for 2 min, microfuging at top speed for 5 min, and assaying the supernatant. For determination of sucrose hydrolytic activity, the NADP-coupled assay was used as described by Gordon *et al.* (1987), and each value represents the mean of five determinations.

**Activity of initial bacterial isolates**

Bacterial cell extracts transformed with pBluescript containing cDNA inserts were incubated in 50 mM imidazole pH 7, 50 mM sucrose for 1 h at 30°C, and the products determined by the NADP coupled assay. Control reactions contained a similar extract from a strain harboring the pBluescript plasmid, but without the Suc22 insert.

**HPLC analysis**

Bacterial extracts were incubated in 50 mM imidazole buffer (pH 7) containing 200 mM sucrose in a 1 ml reaction volume at 30°C. Aliquots were removed at various time intervals, placed in a boiling water bath for 2 min, passed through ion-exchange resin, and analysed by HPLC as described by Cairns and Pollock (1988).

**Substrate specificity**

Bacterial extracts were incubated in 50 mM imidazole buffer (pH 7) containing 100 mM substrate for 2 h at 30°C. The reactions were terminated and the products determined using the NADP coupled enzyme assay.

**Inhibition by fructose**

Bacterial extracts were incubated in a solution of 50 mM sucrose, containing varying concentrations of fructose (0–20 mM) for 10 min at 30°C. Reactions containing extract and varying fructose concentrations, but in the absence of sucrose were used as controls. The reactions were terminated and assayed by the NADP coupled assay.

**Inhibition by TRIS**

A series of TRIS solutions was made up and adjusted to pH 7 using HCl. Bacterial extracts were incubated for 1 h at 30°C in a reaction mixture containing 50 mM imidazole, 200 mM sucrose and varying concentration of TRIS (0–20 mM). The reactions were terminated and the release of glucose determined using the NADP coupled assay.

**Other inhibitors**

Bacterial extracts were incubated in 50 mM imidazole buffer (pH 7) containing 100 mM sucrose, supplemented with inhibitor (20 mM) for 30 min at 30°C. The reactions were terminated and products determined using the NADP coupled assay, modified to contain similar concentrations of inhibitor.

**Determination of pH profile**

A stock solution containing equi-molar concentrations of bicine, MES and MOPS was made up, aliquots adjusted to varying pH using NaOH and the solutions made up to a final buffer concentration of 25 mM (Gordon and James, 1997). *L. temulentum* and bacterial extracts were incubated in a reaction mixture containing 100 mM sucrose, and 20 mM buffer in a 300 µl volume. Similar solutions containing boiled extracts were used as controls. The reactions were incubated for 2 h at 30°C. At the end of the reaction, 1 ml of 200 mM imidazole buffer (pH 7) was added, and the reaction terminated as previously described. The release of glucose was determined using the NADP coupled assay.

**Results and discussion**

Initial screenings of *E. coli* cells (Solar) transformed with the *L. temulentum* cDNA library yielded five clones capable of growth on minimal media supplemented with ampicillin and sucrose as a sole carbon source. Table 1 shows the rate of glucose and fructose accumulation (expressed as changes in NADPH concentration) when cell extracts of these clones were incubated in 50 mM imidazole (pH 7) and 50 mM sucrose. Cell extracts from cells transformed with pBluescript, containing no insert, were used as a control. These results show that cell extracts from all five clones cleave sucrose to produce glucose and fructose. Analysis by HPLC (Table 2) and TLC (data not shown) showed that glucose and fructose were the only products of the incubation. Extracts from control cells showed no ability to hydrolyse sucrose.

Sequence analysis showed all five clones to be identical, although differing in length. The longest of these clones was used as a probe to rescreen the library and to isolate a full length clone (Suc22). All the results described below were obtained using Suc22. Confirmation that the clone inserts were responsible for the sucrose hydrolytic activity was obtained by showing that extracts from DH5α *E. coli* cells transformed with the plasmids from only the sucrose-utilizing cells, hydrolysed sucrose (results not presented).

**Characterization of expressed protein**

**pH profile.** A pH profile for the sucrose hydrolytic activity of the Suc22 gene product was determined (Fig. 1). The pH optimum was found to be in the range pH 7–7.5, with little or no activity at pH 5.5 or lower. A similar pH

**Table 1. Sucrose hydrolytic activity in bacterial cell extracts**

<table>
<thead>
<tr>
<th>Bacterial isolates</th>
<th>Control</th>
<th>Suc1</th>
<th>Suc2</th>
<th>Suc3</th>
<th>Suc4</th>
<th>Suc5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>0.71</td>
<td>6.76</td>
<td>5.14</td>
<td>8.65</td>
<td>5.96</td>
<td>3.72</td>
</tr>
<tr>
<td>Fructose</td>
<td>0.33</td>
<td>6.59</td>
<td>5.31</td>
<td>7.87</td>
<td>5.57</td>
<td>3.38</td>
</tr>
</tbody>
</table>

Extracts from bacterial cell cultures were incubated in 50 mM sucrose at 30°C and the products determined using the NADP-coupled assay, and expressed as µmol product h⁻¹ ml⁻¹ of extract.
Table 2. HPLC analysis of the products of Suc22 cell extracts following incubation in 200 mM sucrose

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Suc22</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sucrose</td>
<td>Glucose</td>
</tr>
<tr>
<td>0</td>
<td>97.8</td>
<td>0.28</td>
</tr>
<tr>
<td>30</td>
<td>89.17</td>
<td>3.91</td>
</tr>
<tr>
<td>60</td>
<td>82.58</td>
<td>7.5</td>
</tr>
<tr>
<td>120</td>
<td>69.58</td>
<td>14.95</td>
</tr>
</tbody>
</table>

Bacterial cell extracts were incubated in 200 mM sucrose at 30 °C. At varying time intervals samples were removed and analysed by HPLC. Results are presented as % of each compound in the sample.
Alkaline/neutral invertase

by the inclusion of MgCl$_2$, CaCl$_2$ or MnCl$_2$ in the reaction. This is similar to the findings of Van den Ende and Van Laere (1995) (alkaline/neutral invertase) and Lee and Sturm (1996) (neutral invertase), but differs from the findings of Morell and Copeland (1984) (alkaline/neutral invertase) and Lee and Sturm (1996) (acid invertase).

**Sequence and expression analysis**

Suc22 was sequenced and shown to consist of a 1.95 kb coding sequence (Fig. 4), encoding a protein of 571 amino acids with an estimated molecular weight of approximately 63 kDa. This is consistent with the published molecular weights for purified alkaline/neutral invertases as determined by SDS–PAGE (Chen and Black, 1992; Van den Ende and Van Laere, 1995; Lee and Sturm, 1996; Ross et al., 1996), although it differs from the value reported for the carrot alkaline invertase (Lee and Sturm, 1996). The sequence shows little homology with published sequences for acid invertases, but it does contain the invertase motif as specified by Sturm and Chrispeels (1990) which is underlined in the sequence. The lack of glycosylation sites is consistent with the findings of Chen and Black (1992) and Walker et al. (1997) and suggests that this enzyme resides in the cytoplasm (Ricardo and ap Rees, 1970; Copeland, 1990).

Suc22 was used to probe Northern blots of *L. temulentum* total leaf RNA. The RNA was isolated from excised leaves incubated for various times in the dark in 200 mM sucrose. A single band of approximately 2 kb was observed at each time point (Fig. 5). Although sucrose accumulates in the leaf tissue over time, there is little change in the level of Suc22 message. This suggests that the clone represents a gene which is not regulated by sucrose or related metabolite accumulation. This is in contrast to other sucrose-hydrolysing enzymes which showed altered patterns of expression in response to varying carbohydrate levels (Koch, 1996).

**Conclusions**

A novel cDNA clone has been isolated from leaves of *L. temulentum*. The sequence shows little homology with the published sequences for acid invertases, and it is proposed that it represents the first sequenced alkaline/neutral invertase. This cDNA was expressed in *E. coli*. Characterization of the expressed protein revealed that it is an alkaline/neutral invertase, of the type reported by Chen and Black (1992), Van den Ende and Van Laere (1995), and Ross et al. (1996). It differs from the alkaline invertase purified from carrot (Lee and Sturm, 1996) which showed a more alkaline pH optimum, a different molecular weight, and different inhibition characteristics. However, Suc22 and the carrot enzyme showed similar
substrate specificity and apparent $K_m$. Lee and Sturm (1996) also described a neutral invertase from carrot which unlike the other alkaline/neutral invertases, had $\beta$-fructofuranosidase activity. Additionally, Morell and Copeland (1984) described an alkaline/neutral invertase from soybean nodules which displayed a mixture of characteristics i.e. $\beta$-fructofuranosidase activity, and $\text{CaCl}_2$, $\text{MgCl}_2$, and $\text{MnCl}_2$ inhibition. There appears, therefore, to be more than one class of enzyme within the umbrella of alkaline/neutral invertase. These classes need to be defined in relation to substrate specificity and effect of inhibitors, rather than on their pH optimum alone. As more of these enzymes are isolated and sequenced, it should be clearer whether these enzyme classes are encoded by a similar gene, post-transcriptionally modified to display slightly different characteristics as suggested by Lee and Sturm (1996), or as seems more likely, are encoded by different genes.

Understanding their regulation and tissue distribution will help determine their contribution to sucrose metabolism, and the Suc22 clone described in this report will be valuable in such studies.

Acknowledgement

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