

# Multiple dextranases from the yeast *Lipomyces starkeyi*

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**Abstract** The soil yeast *Lipomyces starkeyi* (NCYC 1436) secretes dextranase activity into the growth medium. Resolution of a dextranase-active protein fraction by SDS-PAGE produced three protein bands, of 66 kDa, 68 kDa and 78 kDa, and isoelectric focusing of the same fraction resulted in seven protein bands, of pIs 3.50, 3.85, 4.20, 4.80, 4.85, 5.00 and 5.30. Dextranase activity was demonstrated for all the isoelectric forms, and for the 78 kDa species in the presence of SDS. Amino acid compositions of the 66 kDa, 68 kDa and 78 kDa protein bands were determined, and the N-termini of the 66 kDa and 78 kDa protein bands were sequenced: the first two amino acids at the N-terminus of each protein were alanine and valine, respectively; an alanine-valine pair is seen early in the N-terminal coding sequences of the dextranases and the isopullulanase produced by the phylogenetically disparate organisms contributing to glycosyl hydrolase family 49.

**Keywords** Dextranase · GH family 49 · Isoforms · *Lipomyces starkeyi* · N-terminal sequences · Yeast

## Introduction

Dextranases cleave the  $\alpha$ 1,6 glucosidic linkages of dextran to yield either glucose or isomaltose (exodextranases) or isomalto-oligosaccharides (endodextranases), and are only produced by a small number of bacteria and fungi, including yeasts, and perhaps some higher eukaryotes (Khalikova et al. 2005). Dextran is an  $\alpha$ 1,6 glucan produced by microorganisms such as *Leuconostoc mesenteroides*, which can be used commercially for clinical purposes such as drug delivery (Sinha and Kumria 2001), and—by cross-linking—for the production of the well known chromatographic matrix Sephadex. However, dextran formation can impede sugar-refining processes, and dextranase can have a role in mitigating this significant economic problem (Roca et al. 1996; Eggleston and Monge 2005). Dextranolytic enzymes are also being used in the synthesis of potentially valuable oligosaccharides (Goulas et al. 2004) and as possible mouthwash ingredients (Kim et al. 2002). Furthermore, a cloned *Penicillium minioluteum* dextranase has recently been used to engineer a mycotoxin-detecting yeast-based bioassay (Li et al. 2006).

The only yeasts reported to produce dextranases are members of the *Lipomycetaceae* family (Barnett et al. 1990), and of these, only *Lipomyces kononenkoae* (Zinchenko et al. 1989) and *Lipomyces starkeyi* dextranases have been characterised. *L. starkeyi* (IGC 4047), when grown on dextran as a sole carbon source, produced a dextranase that was

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able to hydrolyse blue dextran and Sephadex G-100; the molecular weight, determined by gel filtration, was 23 kDa, and the isoelectric point was 5.4 (Webb and Spencer-Martins 1983). The dextranase of *L. starkeyi* (ATCC 20825) was further studied by Koenig and Day (1988, 1989a 1989b): dextranase purified from this strain and analysed by SDS-PAGE produced four bands, of molecular weights 65 kDa, 68 kDa, 71 kDa and 78 kDa. This enzyme occurred as multiple isoelectric species, with pIs of 5.61, 5.73, 5.80, 5.95 and 6.06. Also, a single protein of 100 kDa with both dextranase and amylase activities has been reported to be produced by *L. starkeyi* KSM 22 (Lee et al. 2003); a dextranase gene has recently been cloned from this yeast strain, and expressed in *Saccharomyces cerevisiae* (Kang et al. 2005).

We have used a rapid and simple technique to isolate the extracellular dextranase of *L. starkeyi* NCYC 1436 (previously karyotyped in our laboratory—Bignell et al. 1996) and have found that for our strain the enzyme occurs as three molecular weight species and seven isoelectric forms. We have also analysed amino acid compositions of the three dextranases, and undertaken N-terminal sequencing of the smallest and largest species.

## Materials and methods

### Chemicals

Unless otherwise stated, all chemicals and reagents were from Sigma-Aldrich Chemical Co. (Poole, UK). Dextranase used in assays and electrophoresis experiments was from *Penicillium* sp. (Sigma, catalogue number D4668).

### Isolation of dextranase

*L. starkeyi* NCYC 1436 was inoculated into 500 ml liquid YPDx (2% w/v bacto-peptone, 1% w/v yeast extract (Becton, Dickinson and Co., Sparks, USA), 2% w/v dextran (average molecular weight 100,000–200,000)) and incubated at 30°C with gentle shaking (150 rpm), until an  $A_{600}$  of 2.0 was reached.

The cells were harvested by centrifuging at 5,000 g for 10 min, at 4°, and the supernatant was removed and adjusted to a final concentration of 70% w/v ammonium sulphate. Precipitated protein was

recovered by centrifugation at 10,000 g for 10 min at 4°, and the pellet was re-dissolved in 5 mM phosphate buffer (pH 5.0), and dialysed overnight against several volumes and changes of the buffer at 4°, with gentle stirring. The dialysed solution was concentrated by ultrafiltration using a 30 kDa cut-off centrifugal filter (Millipore, Watford, UK), until the volume was approximately 0.5 ml.

### Gel electrophoresis and gel-blotting

SDS-PAGE was performed as described by Laemmli (1970), using both mini-gel and Protean II electrophoresis systems (BioRad Ltd, Hemel Hempstead, UK), and stained using Coomassie Blue, as described in the BioRad manual. Molecular weight markers were also from BioRad Ltd., and were used to construct a calibration curve, from which dextranase molecular weights were determined.

Native gel electrophoresis was performed as described for SDS-PAGE (BioRad manual), except that the loading buffer and gel lacked SDS and  $\beta$ -mercaptoethanol and the samples were not heated prior to loading on the gel.

Pre-cast IEF Ready-Gels (pH range 3–10) and IEF standards from BioRad were run on mini-gel systems, and stained using Coomassie Blue plus Crocein Scarlet (Sigma-Aldrich), as described in the BioRad ready-gel manual. Gel images were captured and analysed using a gel documentation system from UVP Ltd. (Cambridge, UK). A calibration curve of pI versus Rf was linear for the protein standards, and was used to determine the pIs of the dextranase peaks.

To analyse the relationship between isoelectric species and dextranase activity, an IEF gel (pH 3–10) was loaded with six lanes of isolated *L. starkeyi* NCYC 1436 dextranase along with one lane of IEF standards. After electrophoresis, the gel was cut longitudinally into two sections. One section, containing two dextranase-loaded lanes and the lane of IEF standards, was stained (Coomassie Blue/Crocein Scarlet), and the image captured (by a gel documentation system from UVP Ltd); analysis of the gel image using GelPro software (Media Cybernetics Ltd.) generated longitudinal plots of the stain density in the tracks of the dextranase and IEF standard samples. The other section contained four dextranase-loaded lanes, and was sectioned transversely into

forty equal (2 mm) sections. These 2 mm sections were placed into individual 1.5 ml microfuge tubes containing 100  $\mu$ l of 5 mM phosphate buffer (pH 5.0), vortexed for 2 min, then centrifuged at 12,000 g for 5 min. The supernatants (80  $\mu$ l) were assayed for dextranase activity using the fluorescein-dextran method (see below). The stain density scan for one of the two dextranase tracks (in arbitrary units) was arithmetically divided into forty sections—corresponding to the gel slices—and average stain densities per slice determined.

Blotting from electrophoresis gels onto PVDF membranes (BioRad Ltd) was performed using a Trans Blot Mini Cell (BioRad Ltd), as described in the BioRad Trans Blot manual. Blotted proteins were visualised by staining the membrane with Coomassie Blue (0.1% w/v Coomassie Blue in 50% v/v methanol, 10% v/v acetic acid) for 5 min, and destaining with several washes of 50% v/v methanol. Blot images were captured as above.

#### Dextranase Assay

The assay of dextranase activity in solution was a variation on that reported by Zhou et al. (1998).

One hundred  $\mu$ l of 40  $\mu$ g/ml fluorescein-dextran (Molecular Probes Inc, Oregon, USA) were mixed with 100  $\mu$ l of dextranase sample (diluted, if required, in 100 mM sodium acetate, pH 6.0) and incubated in the dark at room temperature for one hour. The fluorescence of the samples was measured (Fluorimeter 6200, Jenway Ltd, Leeds, UK), and the result compared to a series of standards of *Penicillium* sp. dextranase, in order to determine enzyme activities: fluorescence versus dextranase concentration yielded a linear log response up to 10 units per ml.

Dextranase activity in SDS gels was estimated, without extraction, by a plate assay modified from the method of Lawman and Bleiweis (1991). Two sample sets of dextranase proteins and pre-stained protein markers were electrophoresed on a 10% w/v SDS-PAGE gel. The gel was then cut in half, and one half was stained with Coomassie Blue and the image of the gel captured (see above). The other half of the gel was electroblotted onto a PVDF membrane, washed twice in sterile distilled water, twice more in 5 mM phosphate buffer (pH 5.0), and then placed protein-side down onto the surface of a blue dextran plate (0.5% w/v blue dextran, 1% w/v agarose). The membrane-blue dextran plate was incubated at 37°

until any clearings were visible, and the image of the membrane was recorded using the gel doc system (see above).

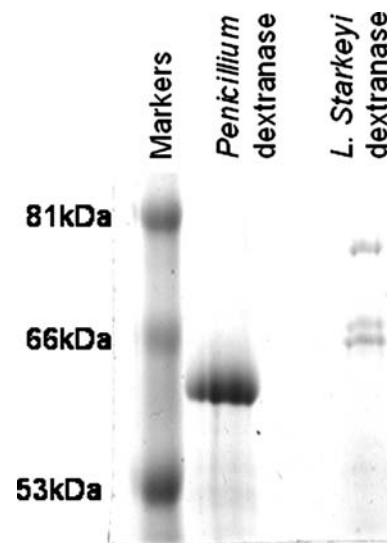
N-terminal amino acid sequencing, and total amino acid composition of SDS-PAGE resolved protein bands

Protein electroblots of SDS-PAGE gels were sent to the Protein and Nucleic Acid Chemistry facility at Cambridge University (Cambridge, UK (<http://www.bioc.cam.ac.uk/pnac>)) for N-terminal amino acid sequencing, and determination of the total amino acid compositions of protein bands.

## Results

### Dextranase molecular weights

A preparation of *L. starkeyi* NCYC 1436 dextranase was electrophoresed on an SDS-polyacrylamide (8% w/v) gel, together with pre-stained protein molecular weight markers. Three clear protein bands were evident after Coomassie Blue staining (Fig. 1). The molecular weights of these proteins were determined

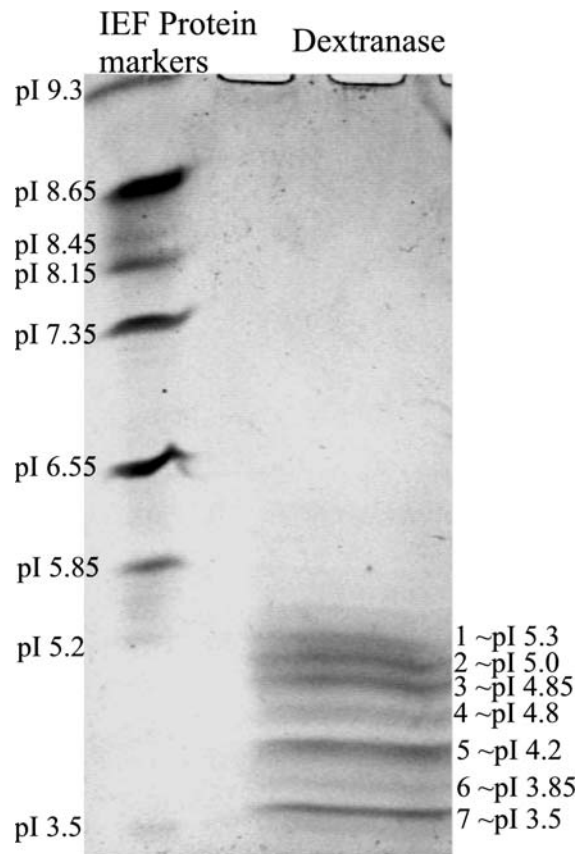


**Fig. 1** *L. starkeyi* NCYC 1436 and *Penicillium* sp. dextranase samples analysed on a 10% SDS-PAGE gel. Purified *L. starkeyi* dextranase was analysed on a 10% SDS-PAGE gel, together with a sample of *Penicillium* sp. dextranase (Sigma D4668). Molecular weights were calculated from the molecular size marker run on the same gel

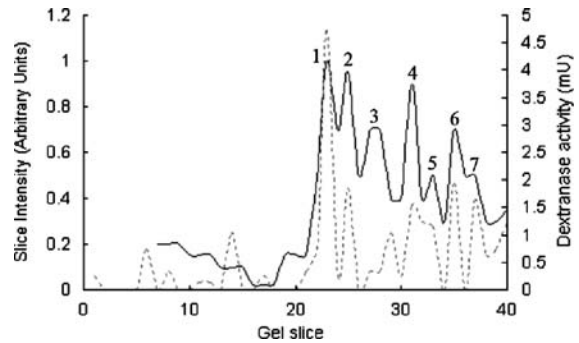
as 66 kDa, 68 kDa and 78 kDa. Assay of a blot of the three proteins from comparable (unstained) SDS gels using the blue dextran plate assay showed dextranase activity for the 78 kDa protein—the halo was clearly visible after 2 h incubation—but no detectable activity for the other two proteins after 18 h incubation (results not shown). Dextranase from *Penicillium* sp. analysed in the same way was found to be a single 62 kDa species (Fig. 1), and the blot assay showed no detectable dextranase activity after 18 h incubation.

#### Analysis of dextranase preparations by IEF

Isoelectric focusing of the same dextranase preparation analysed by SDS-PAGE showed seven visibly distinct species, with pIs of 3.50, 3.85, 4.20, 4.80, 4.85, 5.00 and 5.30 (Fig. 2). Analysis of IEF gels by



**Fig. 2** *L. starkeyi* NCYC 1436 dextranase analysed on an IEF gel (pH 3–10). Lane 1 contains IEF marker proteins, and lane 2 shows that the *L. starkeyi* dextranase preparation (identical to that used in Fig. 1) has been resolved into seven different isoelectric forms



**Fig. 3** Dextranase activities of isolectric forms separated on an IEF gel. As described in the Materials and Methods, an IEF gel (pH 3–10) was loaded with isolated *L. starkeyi* NCYC 1436 dextranase and IEF standard samples, and after electrophoresis the gel was analysed for protein peaks and dextranase activity, in stained (dextranase and standard) and unstained (dextranase only) tracks, respectively. The average stain density of each slice is plotted as a dashed grey line, and the dextranase activities (in mU) of the corresponding (unstained) slices are plotted with a solid black line

sectioning/dextranase assay experiments showed a clear overall correlation between protein stain absorbance peaks and dextranase activity peaks (Fig. 3), consistent with the existence of seven distinct isoelectric forms, all possessing dextranase activity. IEF of the *Penicillium* sp. dextranase showed two isoelectric forms, with pIs of 4.70 and 4.85.

#### Amino acid sequencing and analysis

The 78 kDa band was N-terminally sequenced three times, and the 66 kDa band was sequenced twice, from different protein blots. The consensus N-terminal sequences were: AVNDNDEISSSQ/PDGNAN-TVGE (78 kDa), and AVVLPID/GIVS/-V (66 kDa); the solidus (as in Q/P) refers to the possibility that either of the named amino acids might occur at that position, and the hyphen refers to a residue of unknown identity.

It was not possible to recover enough of the 68 kDa band for sequencing.

#### Discussion

The simple isolation technique reported here has produced a preparation of extracellular dextranase from *L. starkeyi* NCYC 1436, consisting of three

species of molecular weights 66 kDa, 68 kDa and 78 kDa. This result contrasts markedly with the Webb and Spencer-Martins (1983) report on purification of *L. starkeyi* dextranase, which yielded a single, 23 kDa, species, an exceptionally low molecular weight for any dextranase, though the dialysis membrane concentration technique used here would not have retained species smaller than 30 kDa. However, isopropanol-precipitation of culture supernatants from growth of our strain, NCYC 1436, as used by Webb and Spencer-Martins, resulted in only a single polypeptide species of molecular weight of 78 kDa—corresponding to the largest of the three species observed in this study (results not shown). Koenig and Day (1989), using carboxymethyl-Sepharose chromatography followed by agarose gel-filtration chromatography to purify dextranase from *L. starkeyi* ATCC 20825, resolved four polypeptides by SDS-PAGE, of molecular weights 65 kDa, 68 kDa, 71 kDa and 74 kDa, a result closer to our finding, and possibly reflecting strain differences. The *L. starkeyi* KSM22 dextranase gene (*lsd1*) recently cloned by Kang et al. (2005) gives a predicted size of 63 729 Da for the mature protein (after post-translational cleavage between G<sup>32</sup> and A<sup>33</sup> of the 608 amino acid precursor), which is near the estimated molecular weights of our two smaller dextranases. No evidence was found of a 100 kDa species with dextranolytic activity, as reported for *L. starkeyi* KSM 22 (Lee et al. 2003).

In order to confirm that the three polypeptides seen here were dextranases, a membrane blot of an SDS gel was assayed for dextranase activity, but only the largest (78 kDa) species showed activity—indicating a degree of tolerance to a strong detergent denaturant; this enzyme contrasts with the commercially available *Penicillium* sp. dextranase, which showed no activity after the same SDS treatment. It appears that the 66 kDa and 68 kDa *L. starkeyi* putative dextranase species are also intolerant of SDS exposure. The latter two proteins must indeed be dextranases, since all detectable proteins resolved by IEF of the same preparations which yielded the three (66 kDa, 68 kDa and 78 kDa) SDS-PAGE species, had dextranase activity (see Fig. 3). Dextranase preparations ran as smears on native PAGE, no discrete bands being detectable (result not shown); this was also found by Koenig and Day (1989b), and probably indicates that these extracellular enzymes are substantially glycosylated.

The isoelectric focussing analyses of *L. starkeyi* NCYC1436 dextranase resolved seven species (pIs ranging from 3.5 to 5.3, see Fig. 2), contrasting with the five isoelectric species reported by Koenig and Day (1989), with pIs from 5.6 to 6.1; the mature *lsd1*-encoded dextranase was predicted to have a pI of 5.78 (Kang et al. 2005).

The physiological significance of the array of isoelectric species seen here is unclear, but the approximate congruence of the activity and protein profiles of the IEF gels (Fig. 3) indicates that the specific activities of the enzymes are not grossly dissimilar, though it is possible that, as in the case of four *Arabidopsis* xyloglucan endotransglycosylase isoenzymes, for example, there are differences in temperature optima and substrate preferences (Campbell and Braam 1999). Certainly, multiple forms of an enzyme occur widely in microorganisms, and are likely to confer physiological advantages (Naessens and Vandamme 2003).

The amino acid compositions of the three SDS-PAGE-separated dextranases (not shown) displayed similarities and differences, and no particular idiosyncrasies. High aspartate + asparagine and glutamate + glutamine and low arginine + lysine contents were noted—consistent with the proteins being acidic, as shown by their pIs. All three proteins also contain substantial amounts of serine and threonine, providing ample sites for O-glycosylation, which could contribute to the poor resolution seen on native PAGE. The three dextranases show both similarities to (e.g. glycine: 9.5–10.9% w/w here, and 8.72 % w/w for LSD1) and differences from (e.g. isoleucine: 3.8–4.8% w/w here, and 8.06 % w/w for LSD1) the mature dextranase predicted from the gene cloned by Kang et al. (2005).

The glycoside hydrolase (GH) classification based on amino acid sequence similarity developed by Henrissat and colleagues (Henrissat and Gideon 2000, and web site: <http://afmb.cnrs-mrs.fr/CAZY/>) places the most fully characterised fungal dextranase, from *Penicillium minioluteum* (SWISS-PROT accession number PF03718), in glycoside hydrolase family 49 (GH 49). The *L. starkeyi* *lsd1* dextranase is also likely to belong to GH 49 (Kang et al. 2005). There is a similarity between the N-terminus of our 66 kDa dextranase (AVVLPID/GIVS/-V) and that of predicted, mature LSD1 (AAVLPRDNRV); three of the relevant codon changes require only single

nucleotide substitutions. Our N-terminal sequence data also show that residues 1 and 2 of both the 66 kDa and 78 kDa dextranase species from *Lipomyces starkeyi* NCYC 1436 are alanine and valine, respectively. Interestingly, in GH Family 49, all current members have an alanine–valine pair at similar positions within the first 35 amino acid residues from the initial methionine of the coding sequence: an AV pair has been shown to be at the N-terminus of the mature, secreted isopullulanase of *Aspergillus niger* (Aoki et al. 1996) and is thought to be at the N-terminus of the pro-form of the *P. minioluteum* endodextranase (Garcia et al. 1996), while an AV pair occurs next to the predicted N-terminal amino acid of the mature *lsdI*-encoded dextranase (Kang et al. 2005).

The molecular data we have presented on the dextranases of NCYC 1436, and the previously published data on other strains of *L. starkeyi*, discussed above, emphasize the complexity of the dextranolytic system of this potentially biotechnologically interesting yeast.

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