Review

Amylolytic enzymes from the yeast *Lipomyces kononenkoae*

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Abstract: The *Lipomyces kononenkoae* α-amylases LKA1 and LKA2 belong to the glycoside hydrolase family 13 and exhibit specificity towards α-1,4 and α-1,6 linkages in starch and related substrates. LKA1 exhibits specificity towards α-1,4 and α-1,6 linkages and large amounts of reducing sugars are liberated from highly branched amylopectin and glycogen and linear amylose. LKA2, on the other hand, shows high reactivity towards lintner starch, dextrin and amylase, although only small amounts of reducing sugars are liberated from branched substrates, such as amylopectin and glycogen. These enzymes share the four conserved segments of the catalytic domain found in other members of the family, but have some major variant amino acids within these segments. In addition, LKA1 consists of an N-terminal starch-binding domain (SBD). This is the only α-amylase known to possess this N-terminal domain and it exhibits homology to the N-terminal SBD of *Rhizopus oryzae* glucoamylase. It shares no homology with the C-terminal starch-binding domains present in the cyclodextrin glucanotransferases, glucoamylases or α-amylases. The evolutionary tree based on the sequence alignment of SBDs reveals that the N-terminal SBDs are separated from the C-terminal SBDs.

Key words: *Lipomyces kononenkoae* α-amylases, sequence analyses, substrate specificity.

Abbreviations: CGTase, cyclodextrin glucanotransferase; GH, glycoside hydrolase; LKA1, *Lipomyces kononenkoae* α-amylase 1; LKA2, *Lipomyces kononenkoae* α-amylase 2; SBD, starch-binding domain.

Introduction

Starch is a major renewable resource consisting of two types of molecules, amylose, which generally makes up 20-30%, and amylopectin, which generally constitutes 70–80%. Both these types of molecules consist of polymers of α-D-glucose units in the $^4C_1$ conformation. In amylose, these are linked α-(1,4), with the ring oxygen atoms all on the same side, whereas in amylopectin, about one residue in every twenty or so is also linked α-(1,6), forming branch points (Immel et al., 2000). The use of amylolytic enzymes to break down starch to soluble sugars has become increasingly important in the production of various commercial products, such as fuel ethanol, alcoholic beverages and sweeteners. α-Amylases were originally recognised as a group of starch hydrolases and related enzymes that exhibit clear sequence similarities and a predicted common super secondary fold, a parallel $(\beta/\alpha)_8$ barrel (Farber & Petsko, 1990; Farber, 1993). The α-amylase family is the largest glycoside hydrolase (GH) family comprising over 30 different specificities from hydrolases, transferases and isomerases. Currently, the α-amylase family constitutes the clan GH-H of families 13, 70 and 77, and the enzymes of these different families can operate on α-1,1-, α-1,2-, α-1,3- and α-1,5-linkages, as well as on α-1,4- and α-1,6-glucosidic linkages (Van Der Maarel et al., 2002). In nature, there are more than 150 starch-assimilating yeasts, but only a few of them secrete a combination of enzymes that can cleave the α-1,4 and α-1,6 linkages of the complex starch molecule. Several yeasts, such as *Saccharomycopsis fibuligera* (Itoh et al., 1987), *Shizosaccharomyces pombe* (Wood et al., 2002), *Cryptococcus neoformans* (Iefuji et al., 1996) produce α-amylases; the yeast species *Schwanniomyces occidentalis* secretes two α-amylases SWA1 and SWA2, which were expressed in *Saccharomyces cerevisiae*. SWA1 and SWA2 secreted from *S. cerevisiae* showed a significant modification of their properties when these were compared with those of the wild-type enzymes (Abarca et al., 1989; 1991; Yanez et al., 1998). The ability of the yeast species *Lipomyces kononenkoae* to degrade raw starch completely has been well documented (Spencer Martins & Van Uden, 1979; Wilson & Ingledew, 1982; De Mot et al., 1984) Spencer Martins & Van Uden (1979) eval-

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uated 81 raw starch-assimilating yeasts, representing 59 species, and reported that the highest biomass production on starch media was obtained with strains of *Lipomyces kononenkoae* and *Lipomyces starkeyi*. These two yeast species secrete a set of starch-hydrolysing enzymes of which the genes encoding for some of these have been cloned and expressed in simpler systems, such as *Saccharomyces cerevisiae* and *Escherichia coli* (Steyn & Pretorius, 1995; Bignell et al., 2000; Eksteen et al., 2003a; Kang et al., 2004). The genes encoding two novel α-amylases, LKA1 and LKA2, were isolated from *L. kononenkoae* strain IGC4052B, cloned and expressed in *S. cerevisiae* (Steyn & Pretorius, 1995; Eksteen et al., 2003b). These α-amylases exhibit different substrate specificities towards starch and related substrates, acting on their α-1,4 and α-1,6 linkages. In this review, we summarise the sequence analyses and properties of *L. kononenkoae* α-amylases LKA1 and LKA2.

Amylolytic system of *Lipomyces kononenkoae*

All the starch-assimilating yeasts necessarily produce one or more extracellular amylolytic enzymes. The crude concentrates of culture fluids of *Lipomyces* strains are able to extensively hydrolyse a wide variety of polysaccharides, including starch, and two strains of *L. kononenkoae* were identified to produce a different extracellular amylolytic system capable of total starch hydrolysis (Spencer Martins & Van Uden, 1979). However, due to their complex genetics, only a few genes encoding these enzymes have been identified and further characterised. The culture filtrate from *L. kononenkoae* strain CBS5608 consists of three different enzymes: α-amylase, glucoamylase, and a third enzyme with both debranching activity on amylopectin and transferase activity hydrolysing α-1,6 chains in panose. *L. kononenkoae* strain IGC4051 was reported to secrete an extracellular isoamylase, which increases the amylolysis of amylopectin and glycogen, completely hydrolysing these substrates into maltose when combined with a β-amylase. This enzyme does not have any action on pullulan or dextrin (Spencer Martins, 1982).

The strain *L. kononenkoae* IGC4052B, which is a catabolite-derepressed mutant of IGC4052, secretes two enzymes with activity on starch, namely a novel raw starch-degrading α-amylase, LKA1, and a second α-amylase, LKA2. LKA1 α-amylase acts on glucose polymers containing α-1,4 and α-1,6 bonds by endohydrolysis, producing maltose, maltotriose and maltotetraose. LKA2 exhibits properties of α-amylase, but also possesses side activity towards dextrin. The genes encoding these enzymes have been identified, cloned and expressed in *Saccharomyces cerevisiae* (Steyn & Pretorius, 1995; Eksteen et al., 2003a). The expression of LKA1 in *S. cerevisiae* in conjunction with LKA2 results in higher ethanol yields as a result of the synergistic action of these enzymes (Eksteen et al., 2003b).

Biochemical properties of *L. kononenkoae* α-amylases

LKA1 α-amylase has a theoretical molecular mass of approximately 76 kDa (Steyn & Pretorius, 1995). The enzyme exhibited properties of a glycoprotein, indicated by DIF-glycan detection. The temperature optimum of LKA1 ranged from 40 to 50℃ (Steyn & Pretorius, 1995); and this is within the range of optimum temperatures reported for other yeast α-amylases (De Mot, 1990; Iefuji et al., 1996). The stability of the enzyme is maintained between 20 and 50℃, however, and it retains 83% of its activity at 55℃. The optimal pH range of 3-8 was similar to the values reported for *L. starkeyi* α-amylase (Kelly et al., 1985) and other yeast amylases (De Mot, 1990).

LKA1 α-amylase activity was partially inhibited by Cu^{2+} and Zn^{2+}, whereas other metal ions, such as Co^{2+} and Mg^{2+}, had a slight stimulating effect. Calcium, which stabilises most α-amylases, had no effect on the activity of LKA1. Enzymatic activity was not affected by EDTA or EGTA, but was stimulated by SDS and low concentrations of urea, and was sensitive to acetone.

The second α-amylase expressed in *S. cerevisiae*, LKA2, had a temperature optimum of 60℃ and maintained its maximum activity in the temperature range of 50-65℃. The optimum pH for activity was estimated to be 3.5. The molecular weight of this protein was around 55 kDa and, similar to the LKA1 α-amylase, neither Ca^{2+} nor EDTA had any effect on enzymatic activity (Steyn & Pretorius, 1995; Eksteen et al., 2003a).

LKA1 exhibited specificity towards α-1,4 and α-1,6 linkages and large amounts of reducing sugars were liberated from the highly branched amylopectin and glycogen and amylose. The k_m values for lintner starch and amylopectin were 2.31 and 1.97 mg/mL respectively. LKA1 also exhibited a side activity on pullulan. LKA2, however, showed high reactivity towards lintner starch, dextrin and amylose, but only small amounts of reducing sugars were liberated from branched substrates, such as amylopectin and glycogen (Eksteen et al., 2003a). The relative activities of the LKA1 and

Table 1. Activity of *Lipomyces kononenkoae* α-amylases on starch and related substrates (Steyn & Pretorius, 1995; Eksteen et al., 2003a).

Glucose polymer	Relative enzyme activity (%)	
	LKA1	LKA2
Amylopectin (potato)	100	17
Starch (lintner)	95	100
Glycogen (oyster)	75	9
Dextrin	65	91
Pullulan	2	2

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AMYA   21  AEWRSQSIYF LLTDRFARTD NSTTAECDTS .AKYCGGTWQ GIINQLDYIQ   70
ALP1   31  DKWRSQSIYQ IVTDRFARTD GDTSASCNTE DRLYCGGSFQ GIIKKLDYIK   81
SWA2   36  AAWRSESIYQ LVTDRFARTD GSTSATCNTG DRVYCGGTFQ GIIDKLDYIQ   86
LKA1  151  ANWRGRSIYQ VVTDRFARTD GSITYSCDVT DRVYCGGSYR GIINMLDYIQ  200
AMY2   29  HGWRKQSIYS LLTDRFASTN ...PKPCNPE DREYCGGNWR GIIDKLDYIQ   76
LKA2   27  AEWKELSIYQ VITDRFATTN ...LTAPDCW IRAYCGGTWK GLERKLDYIQ   74


AMYA   71  GMGFTAIWIT PVTANLEDGQ .HGEAYHGYW QQDIYALNPH FGTQDDLRAL  120
ALP1   82  DMGFTAIWIS PVVENIPDNT AYGYAYHGYW MKNIYKINEN FGTADDLKSL  132
SWA2   87  GMGFTAIWIS PVVEQIPDDT GYGYAYHGYW MKDIYAINSN FGTADDLKNL  137
LKA1  201  GMGFTAIWIS PIVENIPDDT GYGYAYHGYW MKDIFALNTN FGGADDLIAL  251
AMY2   77  GMGFTAIWIS PIIKNIEGRT KYGEAYHGYW PQDLYTLNPH FGTEQDLIDL  127
LKA2   75  NMGFDAVWIS PVIHNIEVNT TWGFAFHGYW GDDPYRLNEH FGTAADLKSL  125
                                 ** **
                      Region 1
AMYA  121  SDALHDRGMY LMVDVVANHF GYDAPAASVD YSAFNP.FNS ADYFHTPCDI  170
ALP1  133  AQELHDRDML LMVDIVTNHY GSDGSGDSID YSEYTP.FND QKYFHNYCLI  182
SWA2  138  SNELHKRNMK LMVDIVTNHY AWNGAGSSVA YSNYNP.FNQ QSYFHDYCLI  187
LKA1  252  ATELHNRGMY LMVDIVVNHF AFSGNHADVD YSEYFP.YSS QDYFHSFCWI  301
AMY2  128  ADALHDRGMY LMVDTVVNHM GSSDPR.NID YGIYRP.FNQ SSHYHPMCPI  176
LKA2  126  SDSLHARGMS LMVDVVINHL ASYTLPQDVD YSLYPAPFNT SSAFHQPCPI  176
                      °   °*

AMYA  171  TDYDNQTQVE DCWLYTD.AV SLPDVDTTNE EVKEIWYDWV GDLVSDYSID  220
ALP1  183  SNYDDQAQVQ SCWEGDS.SV ALPDLRTEDS DVASVFNSWV KDFVGNYSID  232
SWA2  188  TNYDDQTNVE DCWEGDN.TV SLPDLRTEDS DVSSIFNLWV AELVSNYSID  237
LKA1  302  TDYSNQTNVE ECWLGDD.SV PLVDVNTQLD TVKSEYQSWV KQLIANYSID  351
AMY2  177  E.QDKPLSLE QCWIGTE.DM TLPDIDTENP QIIETLYNFI HDQVKQFKID  225
LKA2  177  D.FSNQSSIE DCWLVTEPAP ALVDLKNEDQ VILDALINSV VDLVETYDID  226
            *          °          * °
      Region 2                 Region 3
AMYA  221  GLRIDTARHV QKDFWRDYND AAGVYCVGEV FQGDPDYTCG YQEVMDGVLN  271
ALP1  233  GLRIDSAKHV DQGFFPDFVS ASGVYSVGEV FQGDPAYTCP YQNYIPGVSN  283
SWA2  238  GLRIDSAKHV DESFYPSFQS AAGVYLLGEV YDGDPAYTCP YQNYMSGVTN  288
LKA1  352  GLRIDTVKHV QMDFWAPFQE AAGIYTVGEV FDGDPSYTCP YQENLDGVLN  402
AMY2  226  GLRVDATKHV RRTFWPGFCE SAGVYCQGEE WTGQADLFCE WQEYMDGLHN  276
LKA2  227  GIRLDTARHV PKPSLAKFQE KVGVFVTGEA LNQSVPYVAQ YQGPLNSAIN  277
             ▲  *°                    *▲* *
                                                  Region 4
AMYA  272  YPIYYPLLRA FSSTSG.SLS DLANMIETVK YTCSDATLLG NFIENHDNPR  321
ALP1  284  YPLYYPTTRF FKTTDS.SSS ELTQMISSVA SSCSDPTLLT NFVENHDNER  333
SWA2  289  YPLYYPMLRF FQGTSN.SVD ELNAMISSLE SDCKDITLLG NFIENHDQPR  338
LKA1  403  YPVYYPVVSA FQRVGG.SIS SLVDMIDTLK SECIDTTLLG SFLENQDNPR  452
AMY2  277  FPVQGVAAES VIPLNDRALR KTAIAMNLVA HHCKDSTLLG LFLESQDAPR  327
LKA2  278  YPLWYALVDS FMGRTT..FD YLESVVKSEQ ATFSDAHALT NFLDNQDQPR  326
           *                                          * *▲

AMYA  322  FASYTDDIS. ....LAKNVA AFVILSDGIP IIYAGQEQHY SGAGDPANRE  367
ALP1  334  FASMTSDQS. ....LISNAI AFVLLGDGIP VIYYGQEQGL SGKSDPNNRE  379
SWA2  339  LPSYTSDSA. ....LIKNAI AFNLMSDGIP IIYYGQEQGY SGSSDPNNRE  384
LKA1  453  FPSYTSDES. ....LIKNAI AFTILSDGIP IIYYGQEQGL NGGNDPYNRE  498
AMY2  328  LAALNNDYT. ....VLKNAM TLNLMSDGIP IVFYGQEQMF NGSHDPVNRP  373
LKA2  327  FASYLGDGNG DDVLRDENAA TFLFFVSGIP VIYYGFEQRF DGGFDPVNRE  377
                                                    *   *

AMYA  368  ATWLSGYDST SELYQFISKT NQIRNHAIWQ NET..YLSYK NYAIYNENNV  416
ALP1  380  ALWLSGYNKE SDYYKLIAKA NAARNAAVYQ DSS..YATSQ LSVIFSNDHV  428
SWA2  385  ALWLSGYSTS NGYYKLISSV NQIRNQAIYK DSK..YTTYW SDVLYASGHV  433
LKA1  499  ALWPTGYSTT STFYEYIASL NQIRNHAIYI DDT..YLTYQ NWVISYSDSTT 547
AMY2  374  ALWDQGYNTD GPLYQYTSKV NKIRRDLINS EDGEIYIRSI THAIMIGDHV  424
LKA2  378  PMWTSGYNTS TPLYNYLARL NAIRKYAASI TGTQVFYSDD TVFLGSGVSH  428

AMYA  417  LAMRKGFDGS QIITILTNAG ADAGS.STVS VPNTGFTAGA AVTEIYTCED  466
ALP1  429  IATKRGS... .VVSVFNNLG SSGSS..DVT ISNTGYSSGE DLVEVLTCST  474
SWA2  434  IALQRGADDQ RIVSVFNNLG SSGS...QTV TFSTKYSGGE KVVDVLTCQT  481
LKA1  548  IAMRKGFTGN QIITVLSNLG SSGSS.YTLT LSNTGYTASS VVYEILTCTA  597
AMY2  425  MVMYKGP... .VITFITNYG AVDK...EYL IK...MPGSE TMIDLLTCTL  465
LKA2  429  MAMQRGP... .LVIVLTNVG QHIIDNTGYT VTGSQFSAGD SLTDLVSCTK  475

AMYA  467  ITVSGSG.EV SVPMESGLPR VLYPKAKLEG SGICGL                 502
ALP1  475  VSGSSD...L QVSIQGGQPQ IFVPAKYASD ICS                    505
SWA2  482  SYANSDS.TL TVSISGGAPR IYAPASLIAN SGICNF                 517
LKA1  598  VTVDLSG.NL AVPMSGGLPR VFYPESQLVG SGICSM                 633
AMY2  466  IEVEGEV..M RTSIKKGEPK ILYPYQLAFR DGFCQEQITL QEIDDVFMGR 514
LKA2  476  VKVVGANGTF TSPSNGGKAR IWIKSKYAGK FCS                    509
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Fig. 1. Alignment of the amino acid sequences of several .α-amylases. The sequences of the mature proteins from *Aspergillus nidulans* (AMYA; SwissProt: Q9UV07), *Saccharomycopsis fibuligera* (ALP1; Itoh et al., 1987), *Debaromyces occidentalis* (SWA2; Claros et al., 1993), *Lipomyces kononenkoae* (LKA1; Steyn et al., 1995), *Schizosaccharomyces pombe* (AMY2; Wood et al., 2002), and *Lipomyces kononenkoae* (LKA2; Eksteen et al., 2003a) have been aligned by introducing gaps (.) to maximize the similarity. Regions 1, 2, 3, and 4 indicate the conserved regions in the amylolytic enzymes. Bold letters represent identical amino acids or conservative replacements. ▲, residues implicated in catalysis; *, residues involved in substrate binding; ○, residues for Ca^{2+} binding (Eksteen et al., 2003a).

Table 2. Variations between and similarity within the conserved regions of the catalytic domain of *Lipomyces kononenkoae* amylases.

Enzymes	Source	Region 1	Region 2	Region 3	Region 4
α-Amylase	*Aspergillus oryzae*	117 DVVANH	202 GLRIDTVKH	230 EVLD	292 FVENHD
Pullulanase	*Klebsiella aerogenes*	600 DVVYNH	671 GFRFDLMGY	704 EGWD	827 YVSKHD
Neopullulanase	*Bacillus stearothermophilus*	242 DAVFNH	324 GWRLDVANE	357 EIWH	419 LLGSHD
Amylopullulanase	*Clostridium thermohydrosulfuricum*	488 DGVFNH	594 GWRLDVANE	627 ENWN	699 LLGSHD
CGTase	*Bacillus macerans*	135 DFAPNH	225 GIRFDAVKH	258 EWFL	324 FIDNHD
Amylomaltase	*Streptococcus pneumoniae*	224 DMWAND	291 IVRIDHFRG	332 EELG	391 YTGTHD
Isoamylase	*Pseudomonas amyloderamosa*	292 DVVYNH	371 GFRFDLASV	435 EPWA	505 FIDVHD
LKA1	*Lipomyces kononenkoae*	264 DIVVNH	248 GLRIDTVKH	376 EVFD	439 FLENQD
LKA2	*Lipomyces kononenkoae*	137 DVVINH	223 GIRLDTARH	251 EALN	312 FLDNQD
LSA	*Lipomyces starkeyi*	287 DIVVNH	371 GLRIDTVKH	400 EVFD	462 FLENQD

LKA2 enzymes on different glucose polymers are summarised in Table 1.

Sequence alignment of *L. kononenkoae* α-amylases

The analysis of sequences of LKA1 and LKA2 reveals homology to various yeast and plant α-amylases, namely to the α-amylases of *Aspergillus nidulans*, *Debaryomyces occidentalis*, *Saccharomycopsis fibuligera* and *Schizosaccharomyces pombe*, cyclodextrin glucanotranferases (CGTases), pullulanases, α-glucosidase, and β-amylase from *Bacillus polymyxa* (TAKIZAWA & MUROOKA, 1985; KAWAZU et al., 1987; KANEKO et al., 1998). The sequence comparison of α-amylases from different yeasts is presented in Figure 1 (EKSTEEN et al., 2003a). LKA1 α-amylase exhibits a high homology of around 78% to the α-amylase from *L. starkeyi*, whereas LKA2 has a homology of about 36% to it (KANG et al., 2004). The three-dimensional structure of *Aspergillus oryzae* TAKA amylase (MATSUURA et al., 1984) was used as a reference model for the structure interpretation of both the α-amylases, LKA1 and LKA2. The analyses of primary structures of LKA1 and LKA2 with that of TAKA amylase revealed corresponding similarities for the catalytic domain and the C-terminal stabilising domain. The sequence comparison suggested that the amino acid sequences of both the LKA1 and LKA2 included the four conserved regions comprising the strands β_3, β_4, β_5, and β_7, containing the catalytically important residues His122, Asp206, Glu230, and Asp297 respectively (HENRISSAT, 1991; TAKATA et al., 1992; SVENSSON, 1994), as previously identified in the α-amylase family of enzymes (KURIKI et al., 1991). Further, six amino acid residues, which are strictly conserved in the four conserved regions of the α-amylase family, are also completely preserved in LKA1 and LKA2. It has been suggested earlier (JANECEK, 1997) that conservations in the catalytic regions of α-amylases might be related to the maintenance of structure and variations within the segments may be characteristic of certain enzyme specificities.

Variations in the conserved segments of the catalytic domain

The sequence analyses showed some amino acid residues that are variable in the conserved segments of *L. kononenkoae* α-amylases, as shown in Table 2. Region IV of LKA1, LKA2 and LSA contains an uncharged Gln in the place of the highly conserved, charged His, which is involved in substrate binding in other α-amylases. The importance of the histidine has been well demonstrated through structural, kinetic and mutagenesis approaches. The mutation of His296 to Asn led to a 100-fold decrease in the K_{cat} values in barley α-amylase (SØGAARD et al., 1993); a similar mutation led to a loss of 50% of the original activity in CGTase from a *Bacillus*, and similar results have been observed for human pancreatic α-amylase (ISHIKAWA et al., 1993). In *Bacillus stearothermophilus* neopullulanase, the mutation of the corresponding His to Glu led to a 70% decrease in its activity towards α-1,4 linkages, leading to a lower maltose release and higher panose production (KURIKI et al., 1991). Inhibitor-binding studies conducted on barley α-amylase suggest that the imidazole group of the His residue forms a hydrogen bond with the OH of the glycone residue at the bond cleavage site. Thus, the mutation of this His296 greatly diminishes the ability of the enzyme to stabilise the transition state, as indicated by the decreases in $K_{\mathrm{cat}}/\mathrm{K_m}$ for the substrates used (SØGAARD et al., 1993). These research findings reiterate the importance of the invariant histidine (corresponding with His296 of the barley α-amylase) in this family of enzymes.

The uncharged Gln in the place of His296 in the fourth conserved sequence region is unique to the *Lipomyces* α-amylases cloned and characterised thus far. This is clear from the sequence comparison of these enzymes with other members of the α-amylase family. The significance of Gln at this position in LKA1 α-amylase is currently under investigation (N. RAMACHANDRAN et al., unpublished data). It is worth mentioning that understanding the role of variant amino acids in such highly conserved regions of these enzymes might broaden our knowledge on the charac-

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LKA1   ESVTGSNHVQLASYEMCGSTLSASLYVYNDDYDKIVTLYYLTSSG---TTG-ST 50
LSA    ESVTSSNHVQLASHEMCDSTLSASLYIYNDDYDKIVTLYYLTSSG---TTG-SV 50
Rg     ASIPSSASVQLDSYNYDGSTFSGKIYVKNIAYSKKVTVIYADGSDNWNNNGNTI 54
Aa     NSPPDDKAVALSSYSYCGGYLSASAFVKNLSYDKLVTLYWTNADNKSTPLNAGS 54
        *      * * *:      :*   :: *  * * **: :

LKA1   LALILPVWSN-NWELWTL--SAIAAGAVEITGASYVDSDTSVTYTTS------  94
LSA    TASYSSSLSN-NWELWSL--SAPAADAVEITGASYVDSDASATYATSFDIPL-  99
Rg     AASYSAPISGSNYEYWTF--SASINGIKEF----YIKYEVSG-----------  90
Aa     LDYVKAASDDQSWELWSLNVTTVPDGVDALLNITYVAASIGKTNSQQLNVQVE 107
             :* *::  ::       :    *:
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Fig. 2. Sequence alignment of the N-terminal SBDs of LKA1 (*Lipomyces kononenkoae* α-amylase; Steyn et al., 1995) and Rg (*Rhizopus oryzae* glucoamylase; Ashikari et al., 1986) with the N-terminal regions of Aa (*Arxula adeninivorans* glucoamylase; Bui et al., 1996) and LSA (*Lipomyces starkeyi* α-amylase; Kang et al., 2004). Asterisks and colons signify the identical and similar residues, respectively.

teristic properties of the individual enzymes. The significance of variations in the *LKA2*-encoded α-amylase: Leu in region 2, Ala and Asn in region 3, and Asp and Gln in region 4, is yet to be explored. In addition, LKA2 α-amylase contains an Arg residue in the place of the Lys121 (TAKA amylase numbering), which is well conserved in the α-amylase family. Previous reports on mutational analyses of Lys to Arg/Asn in *Saccharomycopsis fibuligera* showed a three-fold increase in activity towards shorter substrates (Matsui et al., 1992).

Starch binding and raw starch hydrolysis

In some distinct members, the catalytic domain is preceded by an extra sequence, a domain whose role is uncertain (MacGregor et al., 2001). The N-terminal region of the LKA1 α-amylase preceding the catalytic domain consists of an extension of 132 amino acid residues that exhibits significant homology to the N-terminal regions of *L. starkeyi* α-amylase, and 36% similarity to those of the glucoamylases from *Rhizopus oryzae* and *Arxula adeninivorans* (Fig. 2). Predictions of a hypothetical secondary structure using the predict protein tools (http://www.expasy.ch/) suggest the presence of six β-strands. This region in *R. oryzae* has been reported to be the starch-binding domain (SBD) (Ashikari et al., 1986). Deletion of the N-terminal region (1-132) in LKA1 resulted in a loss of the binding function of this enzyme to raw starch (N. Ramachandran et al., manuscript submitted). It is also known, that the presence of SBD may confer thermostability properties to enzymes (Iefuji et al., 1996). LKA2 α-amylase, on the other hand, possesses a short extension of 19 amino acid residues, which does not exhibit any significant homology to any known α-amylase sequences. Whether this region functions as an extension of the catalytic domain or is involved in any protein function is yet to be understood.

On the basis of the knowledge of different SBDs present in this family, they have been classified into the carbohydrate-binding module (CBM) families 20, 21, 25, 26, 34 and 41 (Coutinho & Henrissat, 1999; Rodriguez-Sanoja et al., 2005). In particular, the SBDs from lactobacilli classified as CBM26 have been described by Santiago et al. (2005). The SBDs present at the C-terminal end of the enzymes have been classified under CBM20, along with the SBDs from CGTases (EC 2.4.1.19, domain E), α-amylases (EC 3.2.1.1), β-amylases (EC 3.2.1.2), maltotetraose-forming exoamylases (EC 3.2.1.60), maltogenic α-amylases (EC 3.2.1.133) and other hydrolases. The tertiary structures of nine of the family CBM20 SBDs are known thus far (Cornett et al., 2003). The *Rhizopus oryzae* and *Arxula adeninivorans* SBDs are currently classified as the family CBM21. The sequence homology of LKA1 to the other N-terminal SBDs is shown in Figure 2. However, no three-dimensional structures are available for this family of SBDs, which limits the knowledge of the interactions of the SBD with the other domains on a structural level. It is evident, however, that the N-terminal SBDs from *R. oryzae* glucoamylase and LKA1 α-amylase are very distantly related to all the other known C-terminal SBDs. The differences in the sequences of the C- and N-terminal SBDs belonging to CBM20 and CBM21, respectively, may reflect the possibility that, during their evolution, the *Rhizopus* and *Aspergillus* glucoamylases obtained their SBDs independently (Tanaka et al., 1986). While most amylases possess their binding domains at the C terminus, it can be speculated that multiple domain organisations existed in ancestral fungi and that these enzymes did not follow phylogenetic lines. This might explain why *R. oryzae* and *A. adeninivorans* possess a putatively newer type of SBD at the N terminus, even though Zygomycetes like *Rhizopus* diverged from *Ascomycetes* before the appearance of yeasts (Coutinho & Reilly, 1997). Moreover, the presence of SBD in an amylolytic enzyme is closely connected to the enzyme origin (Janecek et al., 2003). The presence of the N-terminal region in the *LKA1*-encoded α-amylase homologous to the SBD of *R. oryzae* glucoamylases might suggest the independent evolutionary behaviour of SBDs in glycoside hydrolases. Bork et al. (1998) further reported alignment of the SBDs of bacterial and fungal amylases and a bacterial CGTase with some mammalian glycogen-targeting subunits. Strikingly, the amino acids Gly146, Asn152, Lys157, Asp189, Phe193, Tyr213 and Asn224 of these mammalian polysaccharide-binding domains are well-conserved only in the N-terminal SBD of *R. oryzae* and LKA1. No similarity was noted in any of the C-terminal SBDs examined.

The differences between the C-terminal SBDs and the N-terminal SBDs are clearly reflected in their evolutionary tree (Fig. 3), which was calculated using the amino acid sequences (Table 3) according to Janecek et al. (2003); in the current study we have included the *Lipomyces* amylases and enzymes with N-terminal starch SBDs to this evolutionary tree. The C-

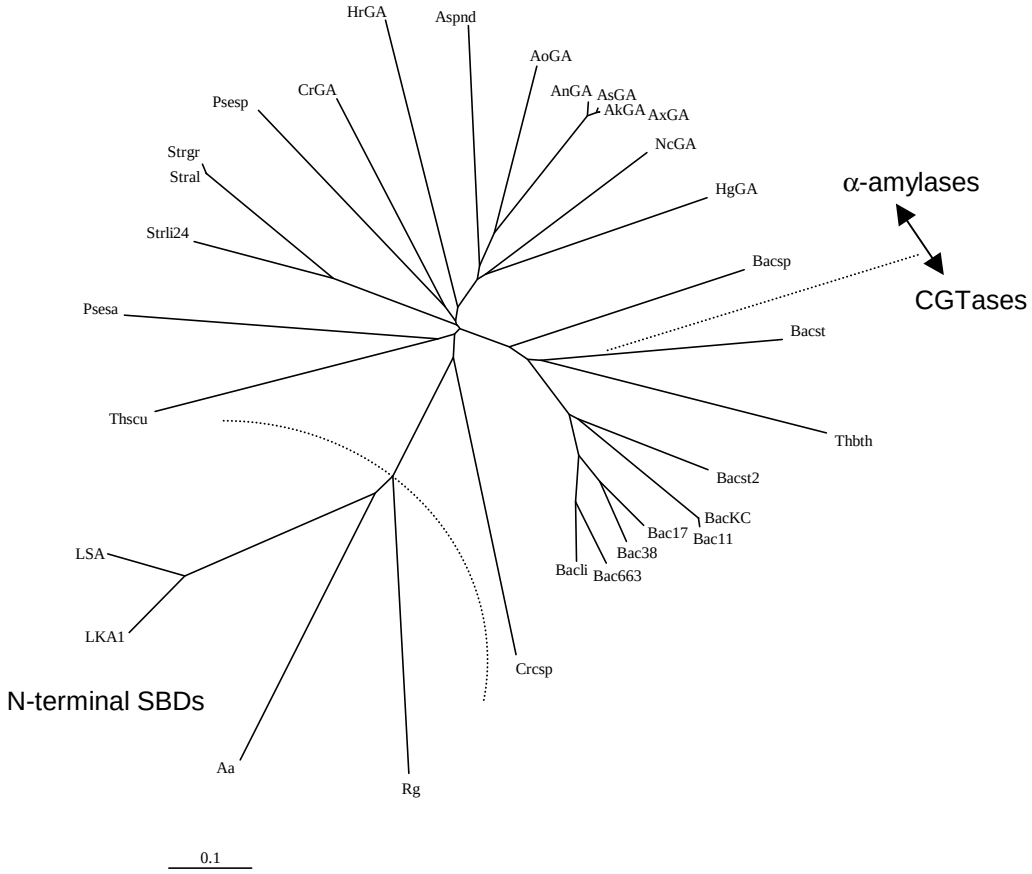

Fig. 3. Evolutionary tree of SBDs of α-amylases, glucoamylases and CGTases.

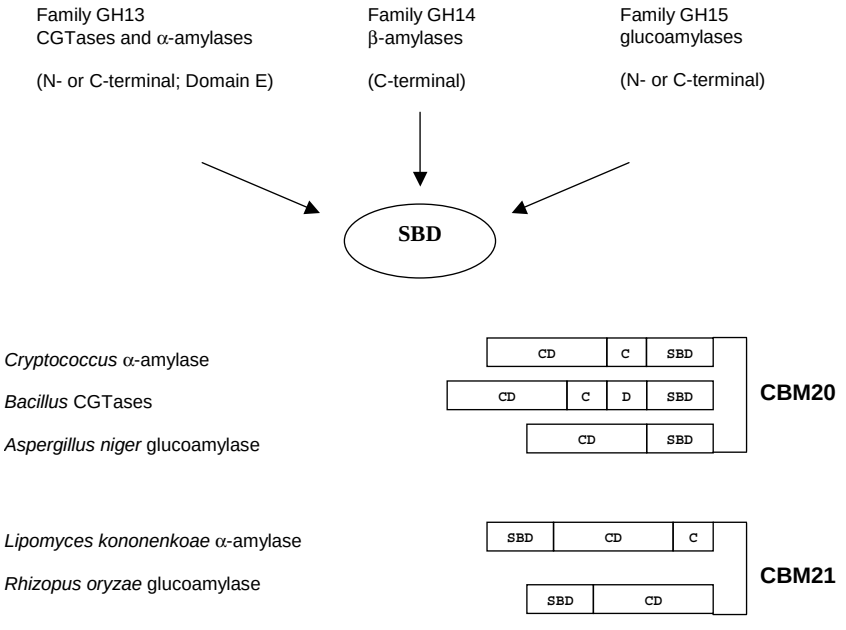

Fig. 4. Location of the starch binding domains in glucosyl hydrolase members. **CD**, catalytic domain; **SBD**, starch binding domain; **C**, domain C; **D**, domain D.

terminal SBDs of α-amylases and glucoamylases cluster together, while the CGTases are on the branch adjacent to the α-amylase-glucoamylase group. The *Lipomyces* enzymes, LKA1 and LSA α-amylases, are on the same branch as that of the *R. oryzae* glucoamylase SBD. The fact that the SBD occurs in GH13, GH14 and GH15 (JANECEK & SEVCIK, 1999) supports the idea that there has been a separate evolution of this domain. The different organisations found in the α-amylase family (JESPERSEN et al., 1991; JUGE et al., 2002) with regard to the SBDs are illustrated in Figure 4.

Table 3. Amino acid sequences used for comparative analyses and construction of evolutionary tree.[a]

Enzyme	Source	Abbr.	SwissProt	Reference
C-terminal SBDs				
α-Amylases	*Aspergillus nidulans*	Aspnd	Q9UV09	Unpublished
	Aspergillus kawachii	Aspka	P13296	Kaneko et al. (1996)
	Cryptococcus sp. S2	Crcsp	Q92394	Iefuji et al. (1996)
	Streptomyces albidoflavus	Stral	P09794	Long et al. (1987)
	Streptomyces griseus	Strgr	P30270	Vigal et al. (1991)
	Streptomyces lividans TK21	Strli21	O86876	Unpublished
	Thermomonospora curvata	Thscu	P29750	Petricek et al. (1992)
Maltotetraohydrolase	*Pseudomonas saccharophila*	Psesa	P22963	Zhou et al. (1989)
Maltopentaohydrolase	*Pseudomonas* sp.KO-8940	Psesp	Q52516	Shida et al. (1992)
Maltogenic α-amylase	*Bacillus stearothermophilus*	Bacst	P19531	Diderichsen & Christiansen (1988)
CGTases	*Bacillus* sp. 1-1	Bac11	P31746	Schmid et al. (1988)
	Bacillus sp. 17-1	Bac17	P30921	Kaneko et al. (1989)
	Bacillus sp.38-2	Bac38	P09121	Kaneko et al. (1988)
	Bacillus sp.6.6.3	Bac663	P31747	Unpublished
	Bacillus licheniformis	Bacli	P14014	Hill et al. (1990)
	Bacillus stearothermophilus No 2	Bacst2	P31797	Fujiwara et al. (1992)
	Thermoanaerobacter thermosulfurogenes	Thbth	P26827	Bahl et al. (1991)
	Thermococcus sp. B1001	Thcsp	Q9UWN2	Yamomoto et al. (1989)
Glucoamylases	*Aspergillus* sp. X-100	Ax	Q12537	Unpublished
	Aspergillus kawachi	Ak	P23176	Hayashida et al. (1989)
	Aspergillus niger	An	P04064	Boel et al. (1984)
	Aspergillus oryzae	Ao	P36914	Hata et al. (1991)
	Neurospora crassa	Nc	P14804	Stone et al. (1993)
	Humicola grisea	Hg	Q12623	Unpublished
	Hormoconis resinae	Ho	Q03045	Joutsjoki et al. (1992)
	Corticium rolfsii	Cr	Q12596	Nagasaka et al. (1995
N-terminal SBDs				
$\alpha-$Amylases	*Lipomyces kononenkoae*	LKA1	Q01117	Steyn et al. (1995)
	Lipomyces starkeyi	LSA	Q6YF33	Kang et al. (2004)
Glucoamylases	*Arxula adeninivorans*	Aa	P42042	Bui et al. (1996)
	Rhizopus oryzae	Rg	P07683	Ashikari et al. (1986)

[a] Based on the study by Janecek et al. (2003).

Future prospects

The *L. konoenkoae* α-amylases and their unique specificity properties support the rationale for structure-function relationship studies in the α-amylase family. The unique modular organisation of LKA1 also invites the construction of novel enzymes using *L. kononenkoae* α-amylases with advantageous combinations of properties, such as raw starch adsorption and activity towards a wide range of substrates. Furthermore, such studies would provide insight into various structure-function implications and would assist in unravelling how the extra domains interact with the catalytic domain or how the variant amino acids influence catalysis and specificity towards different substrates. The positioning of the N-terminal SBD, insight into cooperation between the SBD and the catalytic domain, and the investigation of the reaction mechanism and substrate specificity are among the major questions that need to be addressed in relation to the basic knowledge about these proteins.

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