Milk Clotting using Peptidases of Basidiomycetes

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Hiermit erkläre ich, dass ich die vorliegende Arbeit selbstständig verfasst und die benutzten Hilfsmittel und Quellen sowie gegebenenfalls die zu Hilfeleistungen Institutionen vollständig angegeben habe.

Die dissertation wurde nicht schon als Masterarbeit, Diplomarbeit oder andere Prüfungsarbeit verwendet.

Hannover, den 13.07.2011 Hassan Abd El-Baky

Preliminary notes

This work was carried out between 2008 and 2011 at Institute of Food Chemistry of the Faculty of Natural Science of Gottfried Wilhelm Leibniz Universität Hannover in cooperation with Department of Dairy Science and Technology, Faculty of Agriculture, Fayoum University through the frame of the Egyptian-German Channel program.

Publication list

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4- **Abd El-Baky**, **H.**; Linke, D.; Nimtz, M.; Metry, W.; El-Demerdash, O.; Berger, R.G. (2011b) The extracellular aspartic peptidase of basidiomycete *Wolfiporia cocos* is a highly efficient milk clotting enzyme. *Milchwissenscahft* (Article in press).

Poster

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Zusammenfassung

In dieser Arbeit wurden essbare Basidiomyceten auf die Sekretion von Milch dicklegenden Aktivitäten (MCA) untersucht, wobei sie in Submerskulturen mit verschiedenen pflanzlichen Proteinen oder Hefeextrakt induziert wurden. *Wolfiporia cocos* und *Piptoporus soleniensis* zeigten die höchsten MCA/peptidolytischen Aktivitäts-Verhältnisse. Der konzentrierte Kulturüberstand von *Wolfiporia cocos* wurde zur Herstellung von Frischkäse und einem gereiften Hartkäse vom Gouda-Typ eingesetzt, und die resultierenden Käse zeigten sehr ähnliche sensorische Charakteristika wie die mit Lab hergestellten Produkte.

Die verantwortliche Aktivität wurde aus dem Kulturüberstand von *Wolfiporia cocos* über eine Einstufen-Reinigung mittels präparativer Native-PAGE isoliert. Durch Vergleich der durch ESI-MS/MS generierten Partialsequenzen wurde das Enzym als Aspartat-Peptidase identifiziert, und die Charakterisierung mittels SDS-PAGE ergab ein Homotrimer mit 17 kDa Untereinheiten. Ihre spezifische Milch dicklegende Aktivität lag deutlich über der von technischen Rennin-Präparationen. Desweiteren zeigte das Enzym diverse Vorteile für den Einsatz in der Käserei: partielle Inaktivierung bei Temperaturen ab 40 ˚C, mit der Koagulation mittels Lab vergleichbare Proteinkonzentrationen in der Molke, eine nur einmalige Spaltung der κ-Caseinkette und die daraus folgernde Vermeidung von Bitterpeptiden.

Piptoporus soloniensis sekretierte nach Hefeextrakt-Induktion eine Peptidase, welche nach fraktionierter Ammoniumsulfatfällung und anschließender präparativer Native-PAGE bis zur elektrophoretischen Reinheit isoliert wurde. ESI-tandem-MS Untersuchung tryptischer Peptide identifizierte das Enzym eindeutig als Aspartat-Peptidase. Homologievergleiche der Partialsequenzen zeigten größere Ähnlichkeiten zu Chymosin aus Säugetieren als aus mikrobiellen Organismen. Biochemische Untersuchungen des Enzyms wiesen Charakteristika analog zum Kälberrennin auf. Deutlich hervorzuheben ist hier der Vorteil, dass das pilzliche Enzym anders als Lab β-Casein nur begrenzt gespaltete, was sich in einer verminderten Produktion an Bitterpeptiden positiv auswirken wird. κ-Casein hingegen wurde in gleicher Weise wie mit Lab nur an einer Stelle gespalten, und SDS-PAGE Analysen verifizierten die Bildung von nur einem Hauptfragment.

Die Nutzung von Basidiomyceten als Quelle von Enzymen für das Dicklegen von Milch stellt eine vielversprechende Anwendung der Weiße Biotechnologie zur Herstellung von verbesserten Käserei-Produkten dar. Ohne weitere Optimierung erreichen einige der Peptidasen ein Eigenschaftsprofil vergleichbar dem der marktüblichen Chymosinsubstitute.

Stichwörter: Basidiomyceten, *Wolfiporia cocos*, *Piptoporus soloniensis*, präparative native PAGE, Milchdicklegung, Chymosin, Käse, β- Casein, κ-Casein

Summary

Basidiomycetes, as a potential source of a wide diversity of enzymes, such as peptidases, are currently of interest for different areas of industrial applications, especially in the dairy field. Screening for new milk clotting enzymes from edible basidiomycetes, a food character source, using different media revealed that submerged cultivated basidiomycetes secreted milk clotting enzymes after addition of different inducers. *Wolfiporia cocos* and *Piptoporus soloniensis* showed the highest milk clotting to peptidolytic activity (MCA/PA) ratio. Sensory analysis of fresh and ripened cheeses (Gouda-type) produced by the peptidases mixture of *Wolfiporia cocos* revealed that the organoleptic characteristics of the basidiomycetous cheeses were nearly the same as those produced by calf chymosin.

A highly efficient milk clotting aspartic peptidase (51 kDa, with 17 kDa subunits indicating a homotrimer) from *Wolfiporia cocos* was harvested during the submerged cultivation in standard nutrient medium and was purified in one single purification step using preparative native-PAGE with high activity recovery of 47%. The enzyme showed a specific MCA higher than the commercial microbial rennet. The enzyme exhibited a number of technological advantages: thermal inactivation by moderate temperatures (above 40 ˚C), equivalent residual protein concentration in the whey (4.5 mg/ml) after coagulation compared with the commercial rennet, and the production of large peptides after the hydrolysis of *β*-casein and, therefore, no formation of bitter taste in the resultant cheese. Partial amino acid sequence identified the *Wco* peptidase as a member of the aspartic peptidase A1 family, similar to an aspartic peptidase from *Laccaria bicolor*.

An aspartic peptidase (a monomer with 38 kDa) of *Piptoporus soloniensis* (PsoP1) was purified to electrophoretical homogeneity from the culture supernatant using fractionated precipitation with ammonium sulfate and preparative native-PAGE. ESI-tandem MS sequencing revealed an amino acid partial sequence of PsoP1 which was more homologous to mammalian milk clotting peptidases than to the commercial rennet substitute from *Mucor miehei*. The enzyme was similar to calf chymosin in its biochemical characteristics, except of a beneficial limited hydrolysis of *β*-casein compared to calf chymosin or to rennet substitutes. SDS-PAGE patterns showed a cleavage action on *κ*-casein resulting in the formation of only one major fragment in a way similar to chymosin.

The use of basidiomycetes as a natural source of milk clotting enzymes represents a promising application of white biotechnology in the dairy industry. Without any further optimization, some of the new peptidases compete favourably with established substitutes of rennet.

Keywords: Basidiomycetes, *Wolfiporia cocos*, *Piptoporus soloniensis***,** preparative native-PAGE, milk clotting, chymosin, cheese, β-casein, κ-casein.

Abbreviations list

BSA	Bovine serum albumin
CBS	Centraalbureau voor Schimmelcultures
CMP	Caseinomacropeptide
DSMZ	Deutsche Sammlung für Mikroorganismen und Zellkulturen
DTT	1,4-dithiothreitol
EDTA	Ethylenediamine tetraacetic acid
ESI-MS	Electrospray ionization mass spectrometry
FAO	Food and Agriculture Organization
Ffo	*Fomes fomentarius*
Fve	*Flammulina velutipes*
MCA	Milk clotting activity
MCE	Milk clotting enzyme
MES	Morpholine ethanesulfonic acid
Msc	*Marasmius scorodonius*
MWCO	Molecular weight cut off
PA	Peptidolytic activity
PAGE	Polyacrylamide gel electrophoresis
p*I*	Isoelectric point
PMSF	Phenylmethysulphonylfluoride
Pso	*Piptoporus soloniensis*
PsoP1	*Piptoporus soloniensis* milk clotting peptidase
SDS	Sodium dodecyl sulphate
SNL	Standard nutrient media
TCA	Trichloroacetic acid
TEMED	*Tetramethylethylenediamine*
Tve	*Trametes versicolor*
Wco	*Wolfiporia cocos*

Table of Contents

1 Introduction

Enzymes have been used for many years in a wide number of applications like food, pharmaceutics, detergents, leather, paper, textiles and in ecological bioremediation processes. Peptidases represent one of the three largest groups of the industrial enzymes (peptidases, lipases and amylases) and account for about 60% of the total worldwide sale of enzymes. Peptidases (E.C. 3.4.) are a complex group of enzymes which, by definition, hydrolyze the peptide bond in a protein molecule(Sandhya et al., 2006).

Peptidases are grouped on the basis of three major criteria: (i) catalyzed reaction type, (ii) the nature of the active site, and (iii) structure similarity. Peptidases are may also be roughly subdivided into two major groups depending on their site of action (Rao et al., 1998):

1- Exopeptidases: they are characterized by acting only to the ends of polypeptide chains. Based on their site of action at the N or C terminus, they are classified as amino- and carboxypeptidases, respectively.

2- Endopeptidases which are characterized by their preferential action at the peptide bonds in the inner regions of the polypeptide chain. Endopeptidases are divided into five subgroups based on their catalytic mechanism:

- Cysteine peptidases: the activity of most of cysteine peptidases depends on a catalytic dyad consisting of cysteine and histidine; Papain is the best known plant cysteine peptidase (Rao et al., 1998).
- Serine peptidases: this group is characterized by the presence of a serine group in their active site and it is originally distinguished by the presence of the Asp-His-Ser “charge relay” system or “catalytic triad”, like chymotrypsin and subtilisin (Hedstrom, 2002).
- Metallo peptidases: are characterized by the requirement for a divalent metal ion for their activity, e.g. thermolysin and collagenase.
- Threonine peptidases: they are characterized by the presence of a threonine residue within the active site. Penicillin acylase is considered as an ideal example of this group (Barrett et al., 2004).
- Aspartic peptidases: aspartic peptidases contain two aspartyl residues (Asp32 and Asp215 for pepsin) at the active site. Most of aspartic peptidases exhibit maximum activity at low pH values (~3–5) (Crappe, 2004), and they are characterized by a

complete inhibition by pepstatin A, a hexapeptide naturally produced by *Streptomyces* strains that contains two statin residues, an unusual amino acid.

Chymosin (EC 3.4.23.4) is the conventional milk clotting enzyme (MCE), which belongs to aspartic peptidases, obtained from the fourth stomach of the young calves. The natural function of chymosin is to hydrolyze κ-casein once the milk is in the calf's stomach, resulting in a formation of a gel that can be easily digested.

1.1 Biochemical characteristics of chymosin (rennin)

Chymosin (35.6 kDa with 323 amino acids) is the main coagulant used in cheese production. It is synthesized first as preprochymosin (42.1 kDa with 381 amino acids) by the gastric mucosa. Preprochymosin is secreted as inactive form named prochymosin with a molecular mass of 40.8 kDa and 365 amino acids, produced by the cleavage of the N-terminal peptide. Prochymosin is activated in the acidic environment of the stomach by autocatalytic removal of the 42-amino acid prolob (Claverie-Martín & Vega-Hernández, 2007). The isoelectric point of chymosin is reported to be 4.5 with an optimum pH in the acidic range depending on the nature of the substrate (whole casein, bovine serum albumin, β-, κ- and α_s-caseins) and it shows its maximum activity in a temperature range of 30 to 40 ˚C. Chymosin exhibits its maximum stability at pH values between 5.3 and 6.3. At acidic and alkaline pH values, chymosin loses its activity because of auto-degradation and irreversible conformational changes, respectively. Below 50 ˚C, chymosin appears to be relatively stable (Crappe, 2004).

1.2 Milk protein

Cow milk contains 3-3.5% protein. About 20% of the total protein belongs to a group named whey or serum proteins. This group of protein is remained soluble after the precipitation of the casein either by acid or enzymatic precipitation. It is well known that whey proteins consist of two major groups called α-lactalbumin and β-lactoglobulin (which represent 70% of the total whey) and the rest of the whey protein are in the form of blood serum albumin, immunoglobulins, proteose peptones and non-protein nitrogen (Fox, 2003). About 80% of the total proteins in bovine milk exist in the form of casein micelles. These micelles represent four gene types: α_{s1}-, α_{s2}-, β- and κ-caseins which are found in the approximate proportions 4:1:4:1, respectively, (Davis & law, 1980) and which are all phosphorylated. κ-Casein represents

about15% of the total casein, which is located around the other casein fractions, while α_{s1}-, α_{s2}- and β-caseins are located in the core and represent 85%. In milk, more than 95% of the caseins exist naturally as large colloidal particles or micelles. Various models of casein micelle structure have been suggested. (Walstra, 1999) explained an accepted model of casein micelle structure which is based on some points; (1) the casein micelle is roughly spherical with a non-smooth surface, (2) it is built of sub-micelles consisting of two main types, α_s and β-caseins as well as α_s and κ-caseins, (3) the sub-micelles are linked together with clusters of calcium phosphate, (4) the sub-micelles aggregate to form a micelle and (5) the κ-casein protrudes from the micelle surface, forming a hairy layer around the other casein fractions (Figure 1).

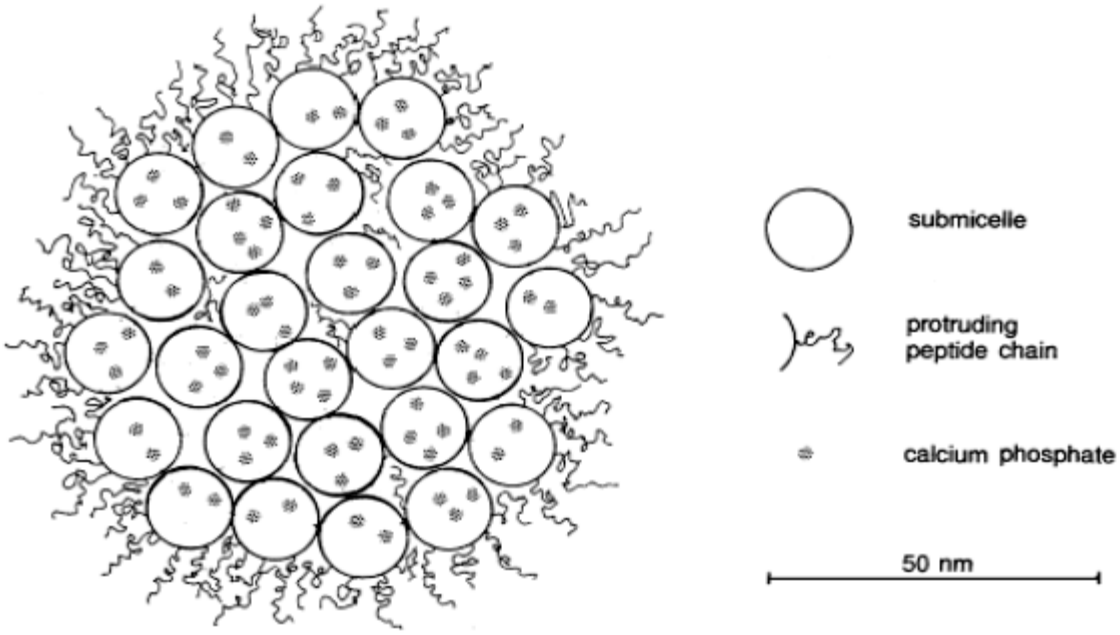


Fig. 1 Structure of a casein micelle (Walstra, 1999).

The casein micelle system is a standard example of a colloidal dispersion depending on two main concepts: (1) κ-casein is a calcium insensitive protein which acts as a protective layer around α_{s1}-, α_{s2}- and β- caseins, which otherwise would easily be precipitated by Ca^{2+}. Hence, κ-casein prevents the precipitation of the casein micelles in the milk, (2) casein micelles are negatively charged, resulting in a repulsive energy barrier which provides a steric stabilizing layer around casein micelles (Fox, 2003).

1.3 Milk coagulation by chymosin

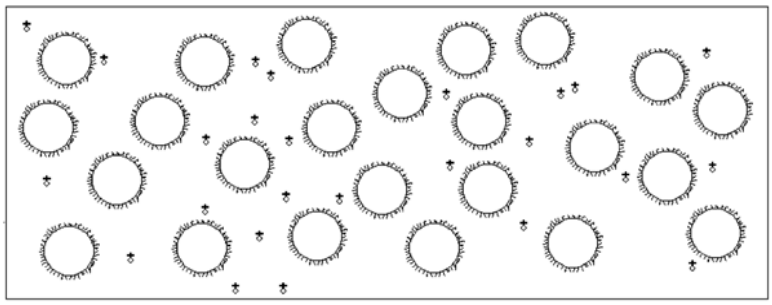
The main important step in the production of all cheese types is coagulation, and the vast majority of cheese varieties are produced by enzymatic (rennet) coagulation. As explained above, the caseins exist as micelles stabilized by a surface layer of *κ*-casein (Fox et al., 2000). The milk coagulation process is comprised of two main phases; (1) the primary enzymatic phase which is initiated by the cleavage of *κ*-casein molecules (169 amino acids) at the Phe_{105}-Met_{106} bond resulting in a reduction of both, the net negative charge and steric repulsion and (2) the secondary non-enzymatic phase in which the subsequently exposed micelle cores begin to aggregate in the presences of Ca^{2+} which is accompanied by the formation of para-*κ*-casein (fragment 1-105) and release of the hydrophilic caseinomacropeptide (CMP) into the whey (fragment 106-169) (Horne & Banks, 2004). The two phases of coagulation are overlapped in the way that the casein micelles start to aggregate before the enzymatic phase is finished. At the end, the aggregates form a three-dimensional gel network. The rennet gel formation and characteristics of the resultant gel are influenced by the coagulant type, the pH of the milk, calcium ion concentration as well as by a previous heat treatment of the milk. Rennet coagulation of milk is shown in Figure 2.

1.4 The role of chymosin in cheese ripening

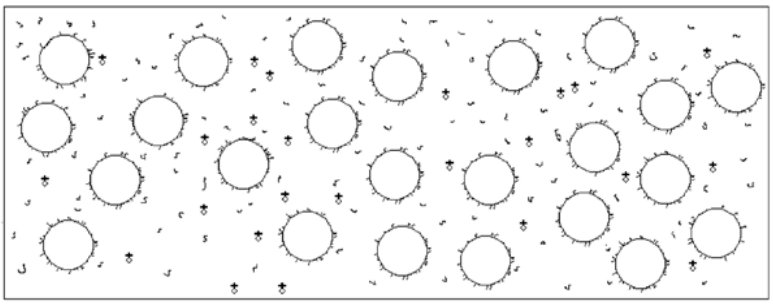
Some cheese varieties are consumed fresh without storage, such as Quark, creamy cheeses and Cottage cheese. On the other hand, most of the cheese varieties are stored for a maturation (ripening) period from 2 weeks (e.g., Mozzarella) to 2 years (e.g., Parmigiano-Reggiano or extra old Cheddar), and in between this period. The various types of cheese can be consumed depending on susceptibility of the consumers for taste and the economical status. During maturation, three main biochemical changes occur, glycolysis, lipolysis, and proteolysis (Upadhyay et al., 2004).

Proteolysis is considered as the most important and complicated change which occurs through the catalytic action of the following items; chymosin as a coagulant, enzymes from the milk, such as plasmin and lipoprotein lipase, enzymes from starter bacteria, and enzymes from secondary organisms (Fox & McSweeney, 2004).

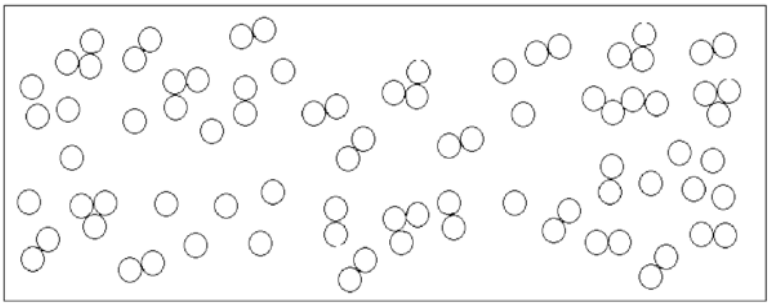
(A) Micelles (O) + enzyme



(B) Partially renneted micelles



(C) Aggregating micelles in small clusters



(D) Percolating clusters

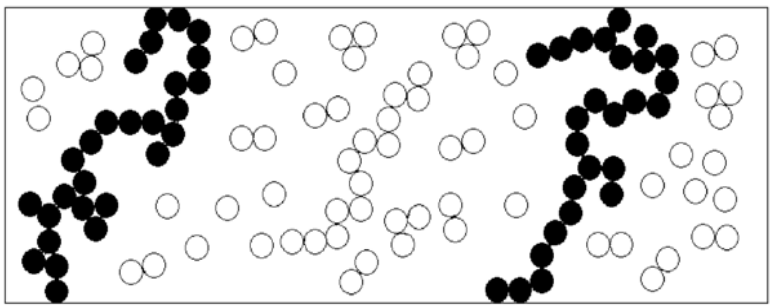


Fig. 2 A schematic description of the various stages envisaged in the enzymatic coagulation of milk, starting from the initial mixture of casein micelles and enzyme (A) and proceeding through proteolysis (B), initial aggregation into small clusters (C) and reaching a gel point at percolation (D) (Horne & Banks, 2004).

Normally, proteolysis is contributing to the development of the texture of the protein matrix of cheese, to flavour and perhaps to the off-flavour formation via the liberation of short hydrophobic peptides.

The residual activity remaining after cheese production at the point of sale relates to the characteristics of the resultant cheese after ripening and is considered as the sum of peptidolytic activities. After clotting, most of the added rennet is lost through the whey. 0-30% remains in the curd depending on chymosin type, cooking temperatures, pH value, and the moisture content of the curd (Upadhyay et al., 2004).

The action of chymosin on casein fractions (κ, α_s, and β) starts with the cleavage of κ-casein in Phe_{105}-Met_{106} bond resulting in the liberation of para-κ-casein and ending with the formation of a gel network. Rennet substitutes from microbial sources cause an extensive nonspecific hydrolysis of both, κ-casein and para-κ-casein, unlike chymosin (Shammet et al., 1992). β- and α_s-caseins are not hydrolyzed during the coagulation step but during the ripening process. Hydrolysis of casein fractions directly affects the cheese texture and flavour. On the other hand, the formed flavour could be the typical flavour of cheese type which is accepted by the consumer or, on the other side, may result from an extensive and nonspecific hydrolysis of caseins which leads to the accumulation of bitter taste. It was reported that the majority of potentially bitter peptides identified in cheeses originate from β- and α_{s1} caseins (Singh et al., 2005). Therefore, the right choice of the coagulant should consider the hydrolytic activity level, and the appropriate enzyme should have a high ratio of milk-clotting activity to peptidolytic activity (MCA/PA) (Crappe, 2004).

1.5 Sources of chymosin

Chymosin has been traditionally extracted from the fourth stomach of suckling young species of different mammals, especially calves. The motifs encouraging the search for rennet substitutes are diverse: an increase of global cheese production coupled with high prices and shortage of calf rennet production, ethical issues and religious considerations in several countries, a growing number of people following vegetarian life-style which do not accept cheeses made from animal origin sources, laws restricting the slaughter of small animals and a strong increasingly objection of a number of consumers against genetically engineered enzymes. Rennet has been isolated from different sources:

1.5.1 Animals:

Animal rennet is secreted in the abomasal mucosa of unweaned ruminants and other mammals like:

- calves: in former times the clotting enzyme was extracted from cleaned and air-dried stomachs and used in different types of cheeses, while the stomachs in modern rennet manufacture are frozen immediately after slaughtering the animals and then exposed to activation, clarification and purification to remove the nonspecific peptidases from the crude rennet. Recently, the ultrasonic extraction of chymosin was developed (Zhang & Wang, 2007).
- lamb: prochymosin and chymosin have been isolated from lamb (Baudys et al., 1988). Nowadays lamb rennets are used in production of traditional sheep milk cheeses in Spain and Italy (Jacob et al., 2011).
- pig: pig chymosin and pepsin have been isolated and used as coagulants, they exhibited higher MCA towards porcine milk than to bovine milk (Foltmann et al., 1981).
- camel: several trails were carried out to produce cheese from camel milk using either calf rennet or other rennet substitutes, but the obtained coagulum was soft and light. Few studies reported the use of camel chymosin to coagulate bovine or camel milks and characterized the enzyme (Elagamy, 2000).
- buffalo: although buffaloes are the main source of milk in some countries like India, the gelation of buffalo's milk requires higher amounts of calf rennet compared to bovine milk; only few trials have been made to obtain chymosin from buffalo (Elagamy, 2000; Mohanty et al., 2003) and they observed slight differences in stability and peptidolytic activity of buffalo chymosin compared to bovine chymosin. This suggested that buffalo chymosin might be the best choice to produce buffalo milk cheese.
- other animals have been used as a chymosin source like fish, chicken, seal, and kitten. Furthermore, chymosin from kid (young goat) was reported by few studies with characteristics which were very closed to bovine and ovine chymosin in terms of chromatographic behavior, molecular characteristic and technological properties (Moschopoulou et al., 2006).

Several factors limit the use of animal rennets: high costs, religious and ethical considerations, laws forbidding the slaughter of the small animals and the rejection of these enzymes by vegetarians.

1.5.2 Plants:

Several plant extracts have been used since decades instead of animal rennets. Latex of several species of genus *Ficus* (El-Shibiny et al., 1973), Pineapple (Cattaneo et al., 1994), Artichoke (Llorente et al. 2004; Sidrach et al., 2005) and *Solanum dubium* seeds (Ahmed et al., 2010) have been reported to exhibit milk-clotting activity. However, the vast majority of these extracts are not suitable for the cheese production because they exhibited extensive peptidolytic activity resulting in bitter taste of the cheeses. An exception are the *Cynara* spp. like *C. cardunculus* which have been used for centuries in Portugal and Spain to produce traditional artisanal cheeses made from ewe milk (Jacob et al., 2011). These enzymes are known now as cynarases 1, 2 and 3 (from dried flowers) and cardosin A, B, E, F, G and H (extracted from fresh flowers). Cheeses produced by *Cynara* spp. extracts are expensive because of the time-consuming cultivation of the plants and the subsequent enzyme preparation. Furthermore, these preparations contain several isoenzymes, they may contains impurities, such as bracts and remains of leaves, and the crude extracts have a poor microbiological quality (Fernández-Salguero et al., 2003).

1.5.3 Microorganisms:

Several milk clotting peptidases of microbial origin have been used as coagulants. Fungi are considered as the main sources for commercial rennet substitutes. (Yegin et al., 2011) divided milk clotting peptidases from fungi (ascomycetes) into two groups: (a) pepsin-like enzymes produced from *Aspergillus spp.* (Vishwanatha et al., 2009) and *Rhizopus spp.* (Kumar et al., 2005) and (b) rennin-like enzymes produced by *Mucor spp.* (Machalinski et al., 2006; Venera et al., 1997), *Rhizomucor spp.* (De Lima et al., 2008; Silveira et al., 2005), and *Endothia parasitica* (Sardinas, 1968). Out of these species, *Mucor miehei, Mucor pusillus and Endothia parasitica* have been established for large scale production and use in cheese making. In general, the use of microbial rennet substitutes in cheese production is associated with two main problems; first the non-specificity of microbial enzymes resulting in an extensive

cleavage of milk caseins which leads to loss of protein and yields as well as to the formation of bitter peptides. The second noticeable problem is the high thermostability of these enzymes which retain their activity after the cooking step resulting in undesirable flavours and bitter peptides from the ongoing hydrolysis.

1.5.4 Recombinant milk clotting peptidases:

Because the natural sources do not meet the high demand of milk clotting peptidases for the industrial application, attention has been directed towards genetically modified rennet substitutes. (Johnson & Lucey, 2006) reported that the recombinant chymosin comprises about 80% of the global rennet market. Until now recombinant *Bos taurus* chymosin is the most predominant genetically modified chymosin by cloning the prochymosin coding cDNA in a suitable host (*Escherichia coli, Bacillus subtilis, Lactococcus lactis, Saccharomyces cerevisiae Aspergillus, oryzae Kluyveromyces lactis* and *Aspergillus niger*). Bovine recombinant chymosin is now produced and used on an industrial scale. The main reason for its popularity is the high yield cheese due to the low amount of proteolysis (100% chymosin), whereas calf rennet preparation contains 10–20% pepsin which is much more peptidolytic and nonspecific resulting in the loss of some peptides into the whey (Kumar et al., 2010).

It is difficult to produce camel chymosin, because small camels are rarely slaughtered. (Kappeler et al., 2006) succeed in cloning and expressing camel chymosin in *Aspergillus niger*. Recombinant camel chymosin exhibited a 70% higher specific activity to bovine κ-casein, and at least a fourfold lower nonspecific activity, resulting in a sevenfold higher ratio of MCA/PA than calf chymosin. Additionally, the enzyme showed thermostability similar to that of calf chymosin.

Recombinant chymosins from other animals include deer, buffalo, antelope, giraffe, ovine, caprine, porcine and equidae species (Jacob et al., 2011) was studied but without any industrial applications. MCEs from *Cynara spp.* have been cloned and expressed successfully in bacterial and eukaryotic expression systems. Genetically modified milk clotting cynarase from *Cynara cardunculus L* was produced in *E. coli* and used in ewes' milk cheese compared to calf rennet (Fernández-Salguero et al., 2003). There were no differences between the two types of coagulants in most of the chemical parameters, while the proteolysis was more considerable and rapid in cheese containing recombinant cynarase.

Genetically modified milk clotting enzymes (MCEs) are now produced on a large scale by different companies using different hosts. Many cheese varieties have been manufactured using the recombinant MCEs and evaluated in comparison to cheeses which were produced by the wild type enzymes. No significant differences were detected regarding to cheese yield, texture, flavor, rate of proteolysis and the overall characteristics of the resultant cheese (Kumar et al., 2010).

The use of genetically engineered MCEs still faces some problems. Lots of consumers still have concerns about the use of these types of food. There are only few fragmentary data about the long term health effects of the cheeses produced by these enzymes; therefore, no conclusion could be made about the safety of the genetic engineered enzymes. Furthermore, there are restrictions and laws around the use of these enzymes.

In general, there are several constraints of using animal, microbial, plant and genetically modified MCEs, which have promoted the search for other natural source for MCEs. One of these food grade sources would be edible Basidiomycetes (Mushrooms).

1.6 Basidiomycetes

Basidiomycota and ascomycota comprise the subkingdom Dikarya which are known as *Higher Fungi* under the Kingdom Fungi (Carlile & Watkinson, 1994). The Basidiomycota includes about 30,000 well known and described species, and represent about 37% of the species of *true Fungi* (www.basidionet.de). The class Basidiomycetes contains some of the most common and familiar known fungi, including mushrooms, bracket fungi and puffballs. The majority of this class are saprotrophs on woody plant materials in the soil, in which the mycelium plays an important role to degrade plant residues and produce the humus or grow symbiotic with trees in form of mycorrhizae (Moore-Landecker, 1996).

There are two main features characterizing basidiomycetes at the microscopic level. The first feature is the presence of clamp connections apparently linking adjacent cells of the hyphae, and the second is that the basidiospores are usually ballistospores (Carlile & Watkinson, 1994). There are about 12,000 species of fungi considered as mushrooms, with at least 2000 edible. More than 200 species have been collected and used for different purposes. About 35 species are cultivated on an industrial scale, such as *Pleurotus spp.* (oyster mushroom), *Flammulina velutipes* (winter mushroom) and *Lentinus edodes* (shiitake).

According to the Food and Agriculture Organization (FAO) the total world production of mushrooms was more than 2 million metric tons in the year 2007 (Aida et al., 2009).

Basidiomycetes secrete a wide diversity of enzymes, in which peptidases represent a major group. Peptidases from basidiomycetes have been studied previously by a number of researchers. (Kawai & Mukai, 1970) screened the extracellular peptidolytic and clotting activities through 44 basidiomycetous strains cultivated in different liquid media. They reported that only few strains exhibited MCA and *Irpex lacteus* was the only strain which produced a ripened cheese without off-flavours. (Rudenskaia et al., 1980) purified a carboxylic peptidase from *Russula deccolorans* with milk clotting activity.

Two carboxy peptidases with MCA were purified from *Irpex lacteus* and the characterization of these enzymes showed high peptidolytic activity towards *β*-casein compared to calf rennet. On the other hand, it cleaved *κ*-casein at the same position as calf rennet, but additionally at other positions. Therefore, it exhibited a high proteolysis rate to *κ*-casein, resulting, like microbial rennets, in another fragment beside para-*κ*-casein (Kobayashi et al., 1983; Kobayashi et al., 1985b). Another screening for basidiomycetous milk clotting and peptidolytic activities was performed by (Kobayashi et al., 1994). Six strains showed high MCA and *Laetiporus sulphureus* was chosen, among the tested basidiomycetes, for further investigations because it was more heat labile than the other enzymes. (Okamura-Matsui et al., 2001) discovered that the basidiomycete *Schizophyllum commune* produced both lactate dehydrogenase and MCE. A cheese produced by the cell extract from the fruiting bodies contained about 0.58% *β*-D-glucan, which is supposed to have a preventive effect against cancer. It also exhibited thrombosis prevention activity. Purification and characterization of MCE from *Laetiporus sulphureus* was done (Kobayashi & Kim, 2003) and revealed an aspartic milk clotting peptidase with characteristics similar to calf rennet.

It was concluded that, after more than 40 years of work on basidiomycetes as a source for MCEs, only Irpex lacteus was suggested for cheese making, and that Laetiporus sulphureus exhibited good characteristics compared to other rennet substitutes. Due to the tremendous biochemical variety of basidiomycetes it was assumed that there were more potential enzymes with milk clotting activities with low peptidolytic activity towards casein proteins and with characteristics closer to calf rennet than to other microbial sources.

1.7 The aim of the work

The aim of the work was divided into three main parts:

a) Screening for milk clotting and peptidolytic activities in edible basidiomycetes

b) Feasibility of producing a semi-hard cheese (Gouda) and its sensory evaluation (Figure 3)

c) Purification and characterization of the most promising milk clotting peptidases

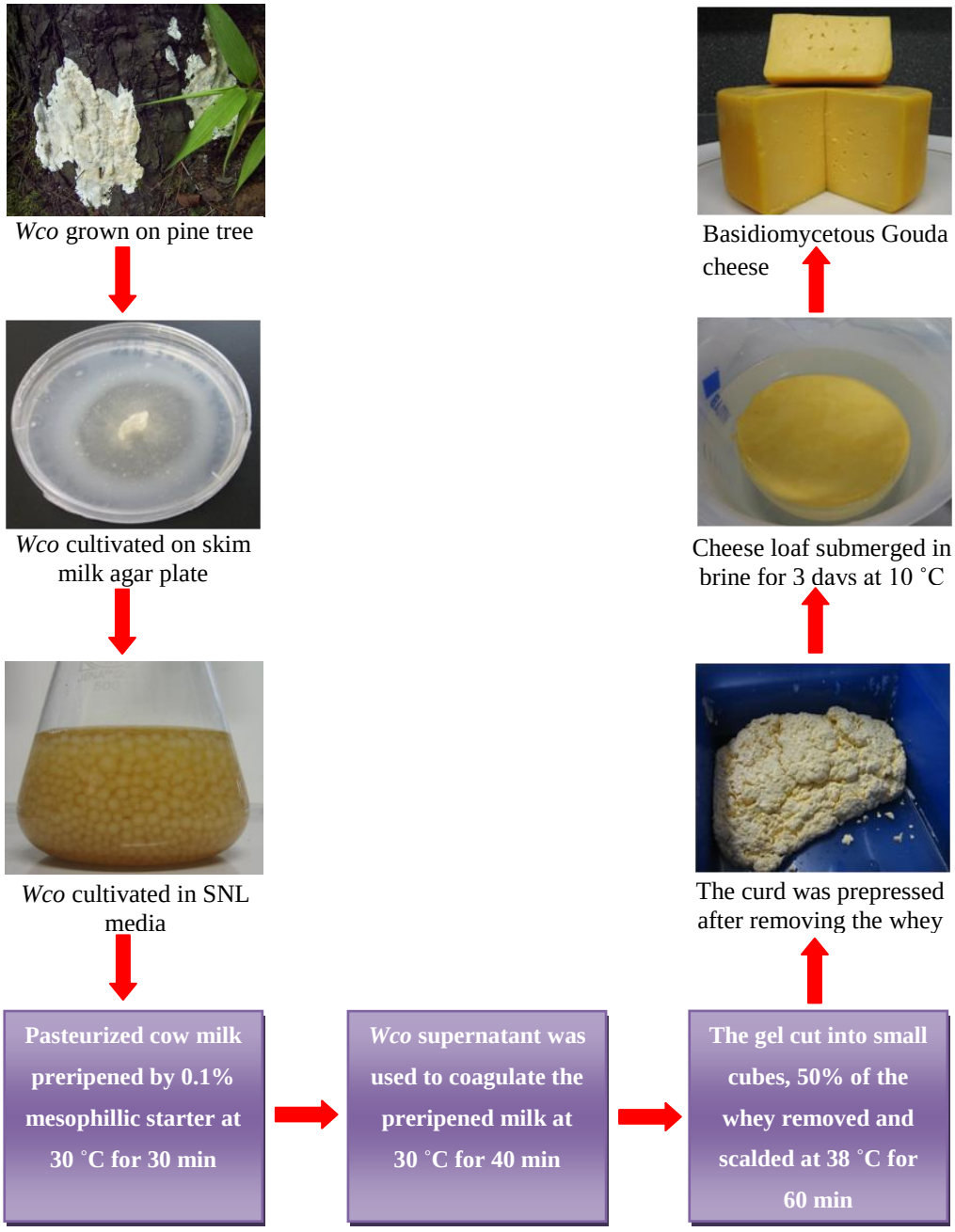


Fig. 3: Flow diagram of the manufacture of a semi-hard Gouda cheese by *Wco* culture supernatant

1.8 Preparative native-polyacrylamide gel electrophoresis (PAGE)

One methodical focus of this work was the purification of the enzymes by the use of a novel preparative SDS-PAGE system. To eliminate the effects of nonspecific enzymes in the rennet preparations, several purification steps were required to obtain the clotting enzyme in a pure form with minimal amounts of contaminants. Previously, MCEs were purified using several tedious and costly purification strategies. Table 1 summarizes different procedures used to purify rennet substitutes from different sources.

Table 1: purification procedures for MCEs from different sources

Purification procedures and yield %	Enzyme source	References
Amberlite CG50, Sephadex A-50 and Sephadex A-100 columns (DEAE) - 33.1%	*Mucor pusillus* var. *Lindt*	(Arima et al., 1968)
Dehydroacetylpepstatin gel, DEAE-cellulose and Isoelectric focusing - 23%	Basidiomycete, *Irpex lacteus*	(Kobayashi et al., 1985a)
Ammonium sulphate precipitation, Sephadex G-100, DEAE-Sepharose FF and Buttyl-Toyopearl - 36%	Basidiomycete, *Laetiporus sulphureus*	(Kobayashi & Kim, 2003)
Charcoal precipitation, DEAE-Sepharose and pepstatin-agarose - 33.3%	Globle Artichoke (*Cynara scolymus* L.)	(Llorente et al., 2004)
Ammonium sulphate precipitation, DEAE-cellulose and Sephadex G-100 - 26%	*Rhizopus oryzae*	(Kumar et al., 2005)
Preparative native PAGE - 46.8%	Basidiomycete, *Wolfiporia cocos*	(Abd El-Baky et al., 2011b)

Compared to the other strategies used previously, preparative PAGE is considered as a superior technique in terms of a simple and single step method giving a high recovery of activity. The principle of this technique is the same like SDS PAGE in which proteins are migrate according to their size. During the run, samples are electrophoresed through a cylindrical gel. As molecules migrate through the gel matrix, they separate into ring shaped bands. Individual bands migrate off the bottom of the gel where they pass directly into the elution chamber for collection (Figure 4). Preparative native-PAGE is a technique for high

yield purification of biologically active peptides. In this system, the sample constituents migrate depending on both their charge and molecular mass. The main advantages of this technique are the possibility of using different buffers systems (from pH 3.8 to 10.2) which keeps the enzyme active and the separated protein is recovered in the form of liquid fractions which facilitates the subsequent experiments with the purified enzyme. In this work, the implementation of a preparative native PAGE system was performed to separate complex protein samples of fungal supernatants produced in submerged cultures.

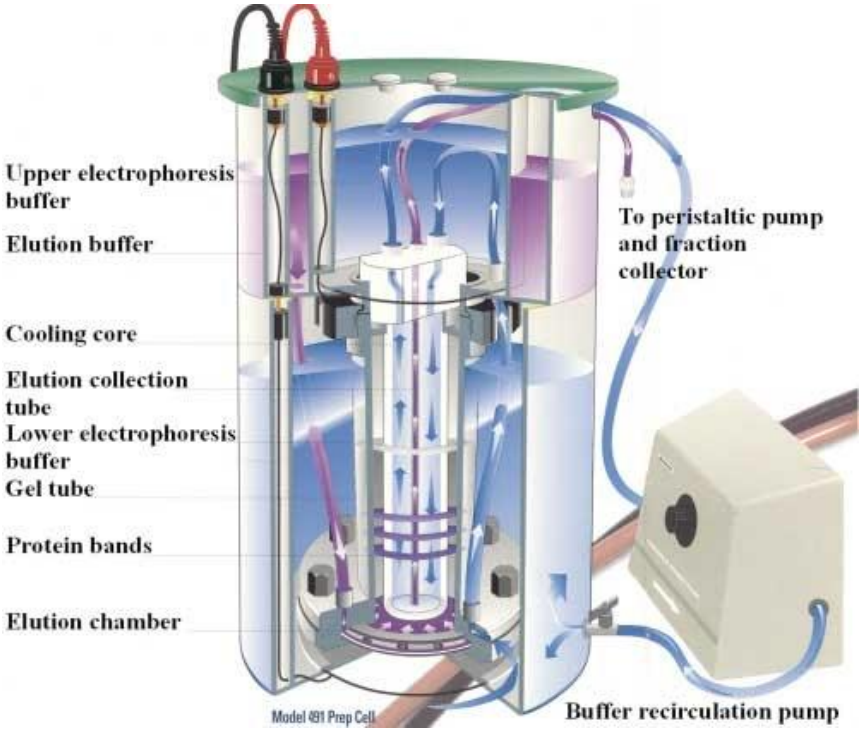


Fig. 4 preparative native-PAGE system

Bio-Rad Model 491 Prep-Cell

2 Submerged cultured basidiomycete fungi secrete peptidases with distinct milk clotting properties

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2.1 Abstract

An orientating study using five basidiomycetes which grew well in nutrient media containing skim milk showed that both milk clotting activity (MCA) and peptidolytic activity (PA) were present. Using wheat gluten, a rather tough peptidase substrate as an inducer, a high MCA/PA ratio of 9.9 was measured for *Wolfiporia cocos* (*Wco*), a wood-decaying fungus ("Indian bread"). The culture supernatants of *Wco* and *Trametes versicolor* (*Tve*, a species possessing a high PA) were used as rennet substitutes to produce a Feta-type cheese. While the *Tve* peptidases yielded a bitter product, the *Wco* peptidases gave a cheese with pleasant sensory quality, comparable to the standard rennet clotted product. A more extensive screening of 28 basidiomycete species showed that MCA was a common trait. *Piptoporus soloniensis* (*Pso*) gave highest MCA/PA values of 16.4 and 28 in gluten medium and in standard nutrient medium (SNL), respectively; *Wco* reached a maximum of 15.2 in SNL. SDS-PAGE showed that *Mucor* rennet and both basidiomycete peptidase mixtures formed completely different patterns of peptides when bovine *β*-casein was the substrate. A Gouda-type cheese was produced using *Wco* culture supernatant. After two months of ripening the organoleptic characteristics of the product were nearly the same as those of a commercial standard Gouda cheese.

Keywords: Peptidase, basidiomycotina, rennet substitution, Feta cheese, Gouda cheese, ß-casein

2.2 Introduction

Milk coagulation is the primary step in cheese making. Chymosin (rennin; EC 3.4.23.4) is a neonatal gastric aspartic proteinase widely used as a MCE (Crappe, 2004). The motifs stimulating the search for rennet substitutes are manifold: the increase of both world cheese production and of calf rennet's price, the increase of vegetarian life-styles, ethical considerations, laws restricting the slaughter of small animals, and the non-founded but strong objections of many people against genetically engineered rennets (Ahmed et al., 2010).

Milk clotting enzymes were found in plants, such as *Ficus carica* (El-Shibiny et al., 1973), *Cynara cardunculus* (Heimgartner et al., 1990), *Ananas comosus* (Cattaneo et al., 1994), *Cynara scolymus* (Sidrach et al., 2005) and *Bromelia hieronymi* (Bruno et al., 2010). Unfortunately, most of the plant origins produce bitter cheeses (except cheese varieties which are manufactured using Cynara extracts). Different trials were carried out to produce rennet substitutes from microorganisms, such as *Penicillium janthinellum* (Hofmann & Shaw, 1964), *Endothia parasitica* (Sardinas, 1968), *Mucor puillus* (Arima et al., 1970), *Rhizopus oryzae* (Kumar et al., 2005), *Basillus subtilis natto* (Shieh et al., 2009), and *Aspergillus oryzae* (Vishwanatha et al., 2009). Most of the microbial rennets (except the recombinant ones) are associated with two problems: high peptidolytic activity and high thermal stability causing uncontrollable proteolysis, in turn resulting in flavour imbalance and bitter taste.

Basidiomycotina are higher fungi commonly found inhabiting forest detritus, leaf litter, and fallen trees. Determined by environmental factors they may switch to a sexual stage, where typical fruiting bodies carrying basidiospores develop (www.basidionet.de). Some basidiomycetes form peptidases to mobilize nitrogen from wood proteins. Peptidases of basidiomycetes were studied, such as *Coriolis consors, Fomitopsis pinicola*, *Irpex lacteus* (Kawai, 1970; Kobayashi et al., 1983), *Russula deccolorans* (Rudenskaia et al., 1980), *Phellinus chrysoloma*, *Kuehneromyces mutabilis* and *Ganoderma aplanatum* (Nerud et al., 1989), *Schizophyllum commune* (Okamura-Matsui et al., 2001), *Laetiporus sulphureus* (Kobayashi & Kim, 2003) and *Pleurotus ostreatus* (Lebedeva & Proskuryakov, 2009). Out of all of these basidiomycetes, only *Irpex lacteus* and *Laetiporus sulphureus* were suggested for cheese making (Kobayashi & Kim, 2003; Kobayashi et al., 1985a and Kikuchi et al., 1988).

Fruiting bodies of *Schizophyllum commune* were thought to be useful in preparing a cheese-like food having health benefits (Okamura-Matsui et al., 2001).

The aim of this study was to provide substantiated proof for the usefulness of peptidases from edible basidiomycetes as rennet substitutes Induction of peptidase formation using protein rich substrates was the experimental starting point, and a resultant peptidase mixture should be used to produce and sensorially characterize Feta-type and Gouda-type cheeses for sensory evaluation.

2.3 Materials and methods

2.3.1 Chemicals

Agar-agar, ammonium persulphate, bromophenol blue, Chymosin, 1,4-dithiothreitol (DTT), glycerol, glycine, skim milk powder (~36% protein and ~50% lactose), Rotiphorese gel 40 (37, 5:1), Rotiphorese 40 (29:1), L-serine, sodium dodecyl sulphate ultra pure (SDS), tetramethylethylenediamine (TEMED), Tris, Tris-HCl and Triton X100 were purchased from Karl Roth (Karlsruhe, Germany). *β*-Casein from bovine milk, rennet from *Mucor miehei* type II, gelatin from porcine skin, ultra low range molecular mass marker M 3546 (M.W. 26.6 to 1,06 kDa) and azo-casein were from Sigma-Aldrich (Taufkirchen, Germany). Gluten was from Roquette (Lestrem, France). D-(+)-glucose monohydrate, L-asparagine-monohydrate, potassium dihydrogen phosphate, yeast extract, magnesium sulphate and coomassie brilliant blue R-250 were purchased from Merck (Darmstadt, Germany). Calcium chloride-2-hydrate was purchased from Riedel-de Haën (Seelze, Germany). All blue prestained standard was obtained from Bio-Rad (Hercules, USA). Direct vat set (DVS) freeze dried lactic cultures (Flora Danica mesophilic aromatic culture and mesophilic homofermentative culture R-704) were from Chr. Hansen (Hørsholm, Denmark). Pasteurized cow's milk was purchased from Hemme Milch, (Wedemark, Germany).

2.3.2 Cultures and culture conditions

Twenty eight basidiomycetes strains used in this study were purchased from the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ, Germany) and from the Centraalbureau voor Schimmelcultures (CBS), Netherlands and inoculated onto SNL agar,

prepared according to (Wu et al., 2007). Skim milk medium contained 40 g/l skim milk, 1.5 g/l potassium dihydrogen phosphate, 0.5 g/l magnesium sulphate and 1 ml/l trace element solution. Gluten media contained 40 g/l gluten (gluten powder was dry autoclaved at 140 ˚C / 8 hours before using), 1.5 g/l potassium dihydrogen phosphate, 0.5 g/l magnesium sulphate and 1 ml/l trace element solution (trace element solution: 80 mg/l iron (III) chloride hexahydrate, 90 mg/l zinc sulphate heptahydrate, 27 mg/l manganese sulphate monohydrate, 5 mg/l copper sulphate pentahydrate and 400 mg/l Titriplex III). The pH was adjusted to 6 using sodium hydroxide prior to sterilization at 120 ˚C for 20 min.

To prepare pre-cultures, 1 × 1 cm agar plugs from the leading mycelium edge were inoculated into a 300 ml Erlenmeyer flask containing 100 ml SNL and homogenized using an Ultra Turrax (Miccra Art, Germany). Cultures were kept for 7 days at 24 ˚C on a rotary shaker (INFORS, Bottingen, Switzerland) at 150 rpm, then 20 ml of the pre-cultures were transferred into a 500 ml Erlenmeyer flask containing 250 ml fresh SNL, or skim milk or gluten medium for the preparation of main cultures. With the skim milk and gluten main cultures, 20 ml of the pre-culture was centrifuged, the supernatant discarded, the pellets mixed with 20 ml of minimal media and inoculated in the main culture. The main cultures were cultivated for 20 days on 24 ˚C at 150 rpm. MCA and PA were checked daily in the culture supernatant after removing the mycelia by centrifugation.

2.3.3 Milk clotting activity (MCA)

MCA was determined according to (Arima et al., 1970) with a slight modification. Skim milk powder was reconstituted by dissolving 10 g in 100 ml of 10 mM $CaCl_2$ solution pH 6. Clotting assay: 5 ml from the milk solution and 0.5 ml of culture supernatant were pre-incubated for 10 min at 35 ˚C and mixed. The curd formation was observed at 35 ˚C while manually rotating the test tube from time to time. The end point was recorded when discrete particles were discernible. One milk clotting unit was defined as the amount of enzyme which clots 1 ml of milk within 1 min.

2.3.4 Peptidolytic activity (PA)

PA was measured according to (Iversen & Jorgensen, 1995). 25 µL of the culture supernatant, 200 µL 2.5% azo-casein in potassium phosphate 100 mM pH 6 and 275 µL of

potassium phosphate 100 mM pH 6 were mixed gently and incubated at 35 ˚C for 20 min, the reaction stopped with 1 ml of 3% trichloroacetic acid (TCA), the tubes were kept at 0 ˚C for 10 min and centrifuged on 12,000 x g for 10 min, 1 ml of the supernatant was taken and the absorbance read at 366 nm. A blank was prepared in the same way, but the enzyme was added after the addition of TCA. One PA unit was defined as the amount of enzyme which increased the absorbance at 366 nm by 1 in one min. The ratio of MCA to PA was calculated. The highest MCA/PA ratio for each species during cultivation was chosen.

2.3.5 Screening of MCA

All of the tested basidiomycetes were chosen on the basis of that they exhibited PA in preliminary work (data are not shown). A first round of screening was performed by cultivating five basidiomycetes, *Flammulina velutipes* (*Fve*), *Fomes fomentarius* (*Ffo*), *Marasmius scorodonius* (*Msc*), *Trametes versicolor* (*Tve*) and *Wolfiporia cocos* (*Wco*) in skim milk and gluten media. MCA and PA were checked daily in the culture supernatant. A second screening was performed by cultivation 28 basidiomycetes in SNL and gluten media, MCA and PA were checked daily in the culture supernatant.

2.3.6 Cheese making

2.3.6.1 White soft cheese

Fresh Feta-type cheeses were prepared from pasteurized cow's milk (3.8% fat) according to (Abd El-Salam & Alichanidis, 2004). The milk was pre-ripened using 0.5% of mesophilic homofermentative culture R-704 on 35 ˚C for 30 min. Culture supernatant of *Tve, Wco* as well as calf rennet, with clotting activity adjusted to coagulate the milk in 40 min, were used as coagulants, and the resultant cheeses were stored in a brine 6% sodium chloride for two weeks. The cheeses were assessed organoleptically by a group of 15 panelists. The assessed parameters were flavour, colour & appearance and body & texture using a 10-point scale, in which one referred to the worst and ten to the best quality.

2.3.6.2 Semi-hard cheese (Gouda)

Wco culture supernatant concentrated with Vivaspin-20 concentrator 3 kDa cut off (Sartorius, Göttingen, Germany) was added to the pre-ripened milk and left for 40 min at 30 ˚C until the gel formed. The gel was cut into 8 to 15 mm cubes and slowly stirred for 15 min. About 45% of the whey was removed and the curd gently stirred for 15 min. About 30% (of the starting volume) of hot water at 60 ˚C was added and stirred for 30 min. Most of the whey was drained off, and the curd was lightly pressed for 15 to 30 min. The curd was cut into suitable size blocks, put into the moulds and pressed for 2 to 3 hours. The cheese loaf was submerged into the cooled brine (17%) for 3 days. Then the cheese was stored at 13 ˚C and 90% RH for two months. Sensory analysis of four common flavour attributes (milky, bitter, salty and fruity), four texture attributes (firmness, rubbery, grainy and pasty) and four appearance attributes (chalky, colour, mouldy and openness) for the resultant cheese were carried out and compared to a mild commercial Gouda cheese from the local supermarket.

2.3.7 Gelatin zymography

Electrophoresis was performed in a Mini-Protean tetra cell (Bio-Rad Laboratories, USA). Culture supernatant was mixed with sample loading buffer (1:1) containing 150 mM Tris-HCl buffer, pH 6.8, 4% SDS, 20% glycerol and 25 mg/l bromophenol blue. A volume of 20 µL of the sample was loaded onto SDS polyacrylamide gels (SDS-PAGE) containing 0.1% (w/v) gelatin. Electrophoresis was performed using a 12% polyacrylamide single gel in a running buffer containing 25 mM Tris base, 1.44% (w/v) glycine and 0.1 % SDS at 4 ˚C and 10 mA/gel (Laemmli, 1970). After electrophoretic migration, the gel was washed two times with 2.5% (v/v) Triton X-100 for 20 min. The activity reaction then proceeded inside the gel during incubation at 35 ˚C for 4 h in a bath of 50 mM sodium acetate buffer, pH 5.5. The active enzymes were detected as translucent bands after incubation of the gel, first in a mixture of 25% (v/v) ethanol, 10% (v/v) acetic acid, and 0.05% (w/v) R-250 Coomassie Brilliant Blue for 2 hours, and second in a destaining solution containing 25% (w/v) ethanol and 8% (v/v) acetic acid with several washings.

2.3.8 *β*-Casein degradation

β-Casein degradation was carried out according to (Kobayashi & Kim, 2003): A solution of 0.1% (w/v) of *β*-casein in 50 mM potassium phosphate buffer, pH 6 was prepared. 30 µL of *Wco* SNL, *Pso* gluten culture supernatants and *Mucor* rennin (1.3 U/ml) were mixed with 1.5 ml of *β*-casein solution and incubated at 35 ˚C. During incubation period, aliquots (0.2 ml) were taken at 0, 30, 60 and 120 min. Samples were prepared by mixing 20 µL of each aliquot with 20 µL of loading buffer with a final concentration of 0.1 M Tris-HCl (pH 6.8), 0.2 M DTT, 4% SDS, 20% glycerol, 0.2% bromophenol blue, boiled for 10 min and applied to SDS-PAGE 18% acrylamide separating gel and 3% acrylamide stacking gel containing 0.1% SDS. After electrophoresis at 12 mA/gel at room temperature, the gels were stained with Coomassie Brilliant Blue. For the determination of molecular mass, ultra low molecular marker proteins from 26.6 to 1.06 kDa (BioRad, Germany) were used.

Statistical analysis

Experiments were performed in triplicate and the data reported were expressed as mean values with standard deviation using Microsoft Office Excel 2007.

2.4 Results and discussion

2.4.1 PA of basidiomycetes

The results of the first orientating experiments with basidiomycetes cultivated in nutrient media supplemented with skim milk or gluten as inducers of PA are shown in Table 1. In the presence of skim milk three out of five species were tested positive for MCA, while all were positive in the gluten medium. Surprisingly, the MCA was higher in the presence of gluten, except for *Fomes fomentarius*. The gluten substrate was autoclaved before use and was thus excluded as a source of peptidase activity. The MCA/PA ratio was adopted as an indicator for selecting the most suitable basidiomycetes. On that basis, *Wco* gluten culture supernatant was chosen because of its high MCA/PA ratio, whereas *Tve* gluten culture supernatant was chosen as a negative reference because of its high PA.

Table 1: Gluten and skim milk as inducers of MCA in basidiomycetes; values are means ± SD, n = 3.

Basidiomycete	MCA (Uml^{-1})		PA ($U\ ml^{-1}$) λ 366 nm		MCA/PA	
	Skim milk	Gluten	Skim milk	Gluten	Skim milk	Gluten
Flammulina velutipes	0.40 ± 0.01	0.67 ± 0.02	1.01 ± 0.10	1.68 ± 0.18	0.40 ± 0.02	0.40 ± 0.05
Marasmius scorodonius	ND	0.32 ± 0.02	NT	0.35 ± 0.01	---	0.91 ± 0.09
Fomes fomentarius	1.54 ± 0.14	1.50 ± 0.11	1.73 ± 0.12	1.78 ± 0.14	0.89 ± 0.04	0.84 ± 0.10
Trametes versicolor	1.04 ± 0.04	1.91 ± 0.26	1.89 ± 0.15	2.12 ± 0.18	0.55 ± 0.04	0.90 ± 0.11
Wolfiporia cocos	ND	1.71 ± 0.17	NT	0.172 ± 0.01	---	9.94 ± 0.66

ND: not detected, NT: not tested, ---: not calculated, n: number of replicates, SD: standard deviation.

Both culture supernatants and commercial calf rennet served to produce white soft cheeses under identical conditions. The results of the organoleptic assessment of these cheeses are summarized in Table 2. The flavour, body and texture attributes of *Tve* cheese were not preferred by most of the panelists. It showed a creamy texture, rather pronounced bitterness and strong taste. Some of the flavour attributes reminded of a ripened cheese. By contrast, *Wco* derived cheese had no off-flavour, bitterness or too soft texture and was hard to distinguish from the conventional calf rennet cheese.

Table 2: Sensory assessment of white fresh cheese produced by basidiomycetous enzymes; values are means ± SD (average of 15 panelists' scores).

Enzyme source	Flavour	Color & Appearance	Body & Texture
Calf rennet	7.9 ± 1.07	9.1 ± 0.43	8.7 ± 0.71
Tve gluten	2.9 ± 0.87	5.8 ± 0.82	3.3 ± 0.65
Wco gluten	7.8 ± 0.86	8.9 ± 0.43	8.5 ± 0.80

2.4.2 Extensive screening using gluten and SNL media

According to the first milk clotting screening results, gluten was considered as a suitable inducer of milk clotting activity. This plant protein fraction is distinguished by a high content

of L-prolin. Peptide bonds flanked by a prolin or prolyl-moiety bend the protein chain and are particularly difficult substrates for most peptidases which could explain its efficiency as an inducer of potent peptidases. Another argument may be derived from the fact that basidiomycetes tend to grow on insoluble or suspended materials, and gluten added to nutrient basal medium yielded suspensions. The selected basidiomycetes (28 strains) were cultivated in gluten as well as in SNL reference medium (Table 3).

Table 3: Screening of basidiomycetes induced by gluten and skim milk proteins for milk clotting properties; values are mean ± SD, n = 3.

Basidiomycete	**MCA (U ml^{-1})**		**PA (U ml^{-1}) $\lambda_{366\ nm}$**		**MCA/PA**	
	SNL	**gluten**	**SNL**	**gluten**	**SNL**	**Gluten**
Agrocybe aegerita	ND	0.26 ± 0.04	--	0.54 ± 0.06	--	0.48 ± 0.03
Bjerkandera adusta	0.33 ± 0.03	1.80 ± 0.19	0.13 ± 0.01	1.43 ± 0.11	2.60 ± 0.14	1.26 ± 0.13
Flammulina velutipes	0.34 ± 0.03	0.67 ± 0.02	0.035 ± 0.005	1.68 ± 0.18	9.71 ± 0.54	0.40 ± 0.05
Fomes fomentarius	0.45 ± 0.05	1.50 ± 0.11	0.136 ± 0.01	1.78 ± 0.14	3.31 ± 0.32	0.84 ± 0.10
Fomitopsis pinicola	1.43 ± 0.13	1.43 ± 0.14	0.11 ± 0.005	0.172 ± 0.01	13.0 ± 0.95	8.31 ± 0.69
Grifola frondosa	0.83 ± 0.09	0.40 ± 0.05	0.212 ± 0.01	0.25 ± 0.02	3.92 ± 0.65	1.60 ± 0.18
Lentinula edodes	ND	0.33 ± 0.02	--	0.42 ± 0.03	--	0.79 ± 0.10
Marasmius scorodonius	ND	0.32 ± 0.02	--	0.35 ± 0.01	--	0.91 ± 0.09
Piptoporus soloniensis	0.56 ± 0.05	1.25 ± 0.11	0.020 ± 0.002	0.076 ± 0.005	28.0 ± 1.65	16.4 ± 1.14
Pleurotus euosmus	0.17 ± 0.01	ND	0.20 ± 0.01	NT	0.85 ± 0.09	--
Polyporus betulinus	0.83 ± 0.10	0.17 ± 0.02	0.072 ± 0.004	0.053 ± 0.005	11.5 ± 1.20	3.21 ± 0.56
Pycnoporus cinnabarinus	ND	0.20 ± 0.03	NT	0.19 ± 0.03	--	1.1 ± 0.15
Pycnoporus sanguineus	ND	3.23 ± 0.40	--	3.03 ± 0.25	--	1.1 ± 0.11
Serpula lacrymans	0.19 ± 0.01	ND	0.10 ± 0.005	NT	1.9 ± 0.16	--
Trametes versicolor	ND	1.91 ± 0.26	--	2.12 ± 0.18	--	0.90 ± 0.11
Wolfiporia cocos	2.86 ± 0.27	1.71 ± 0.17	0.188 ± 0.01	0.172 ± 0.01	15.20 ± 1.27	9.94 ± 0.66

ND: not detected, NT: not tested, ---: not calculated, n: number of replicates, SD: standard deviation.

Among the tested basidiomycetes more than half (16 basidiomycetes) showed MCA. When basidiomycetes were cultivated in SNL, six of them did not exhibit MCA, and the others were low except *Wolfiporia cocos*, *Fomitopsis pinicola*, *Grifola frondosa*, *Polyporus betulinus* and *Piptoporus soloniensis*. The presence of gluten resulted in significant MCA, except for *Pleurotus euosmus* and *Serpula lacrymans*. However, gluten not only induced the MCA, but

promoted PA, and in some of the species even stronger. As a result, the MCA/PA ratio was generally more favorable for cheese making in culture supernatants obtained from SNL cultures. *Flammulina velutipes*, *Fomitopsis pinicola*, *Polyporus betulinus*, *Piptoporus soloniensis* and *Wolfiporia cocos* cultivated in SNL showed the highest MCA/PA ratio, and the best ones, *Wco* and *Pso* recorded 15.2 and 28, respectively. The MCA/PA ratio of *Mucor* rennet was 78 under the same conditions. It should be noted that this comparison resulted from clotting experiments in which pH and temperature conditions were set optimal for rennin.

2.4.3 Production of a semi-hard Gouda cheese

The sensory evaluation of a semi-hard cheese produced using *Wco* culture supernatant compared to commercial semi-hard Gouda cheese is comprised by Figure 1. All of the flavour attributes were nearly identical for both cheeses. For the appearance attributes chalky, colour, mouldy and shiny the *Wco* cheese recorded almost the same scale points as the commercial one, while the openness was slightly different. The texture attribute assessments showed no clear differences between both cheeses.

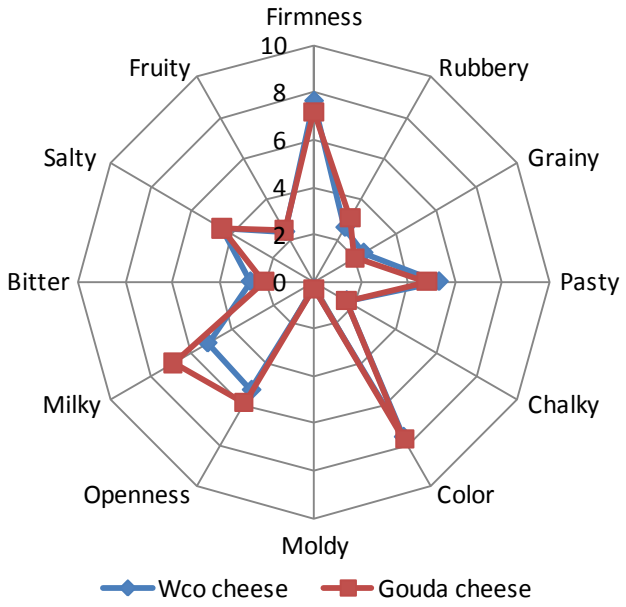

Fig. 1 Spider web diagram of average sensorial scores for a Gouda-type cheese after two months of ripening and a commercial mild Gouda cheese (av. of 15 panelists' scores).

2.4.4 Gelatin zymography

The PA of *Wco* culture supernatant was monitored through the course of an entire cultivation cycle using the activity staining of a semi native SDS gel (Figure 2).

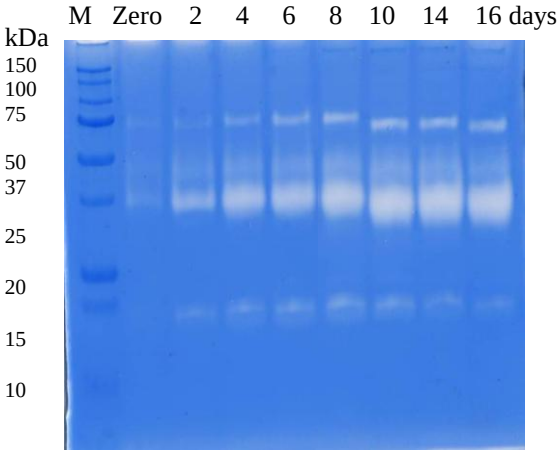

Fig. 2 Peptidolytic activity of *Wco* during cultivation period.

Wco generated a whole set of peptidases of different molecular mass (~ 18, 35, 48 and 75 kDa).The activity progressively increased during the cultivation period. It remains unclear at this point whether a single potent enzyme was responsible for the observed MCA, or if synergistic actions accounted for the effect.

2.4.5 Degradation of *β*-casein

β-Casein is considered as the major source of bitter peptides formed by the peptidolytic action of clotting preparations (Kobayashi & Kim, 2003; Singh et al., 2005). The effect of *Wco* SNL, *Pso* gluten and *Mucor* rennin on *β*-casein was investigated using SDS-PAGE of partial hydrolysates (Figure 3). *Mucor* rennin produced one major band with a molecular weight of ~ 24 kDa while *Wco* produced two major bands, one with the same molecular weight as the one of *Mucor*, and another one in the range of ~ 20 kDa. The gel also reflects the higher PA of the culture supernatant of *Pso* grown on gluten medium. All of the peptides formed by the action of *Pso* gluten and *Wco* SNL and visualized on the gel show a molecular weight higher than that of typical bitter peptides (0.5 to 3 kDa, Lee et al., 1996).

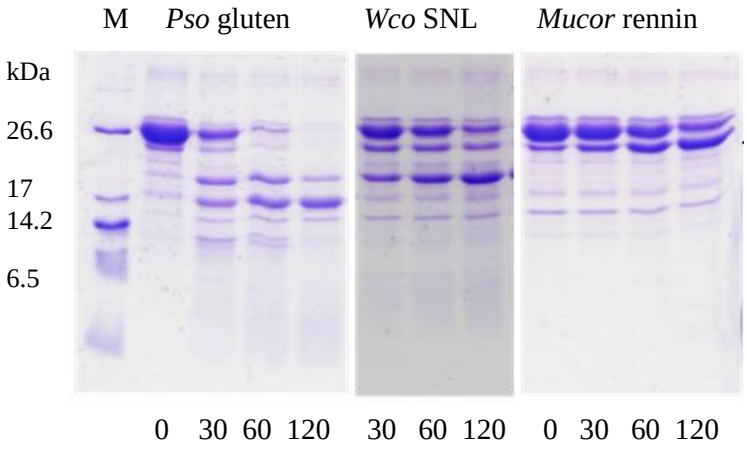

Fig.3 SDS-PAGE degradation of *β*-casein by *Pso* gluten, *Wco* SNL and *Mucor* rennin

Minor peptides not visible on the gel may still impart bitter taste to the curd; however, the electrophoretic data fit the sensory evaluation data reported above.

2.5 Conclusion

Cheese sensory analysis, MCA/PA ratio measured, and *β*-casein degradation visualized indicate that the peptidase mixtures from *Wolfiporia cocos* and *Piptoporus soloniensis* are likewise suited as rennet substitutes. Major advantages are the food character of the enzyme sources and the metabolic flexibility of the producer species as indicated by their different response towards different inducer proteins. Purification and characterization of individual milk clotting enzymes, quantification of cheese yield, investigation of effects on aging and of the stability of the enzyme preparation during storage are underway.

2.6 Acknowledgements

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3 The extracellular aspartic peptidase of basidiomycete *Wolfiporia cocos* is a highly efficient milk clotting enzyme

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3.1 Abstract

An extracellular milk clotting peptidase from submerged culture of the basidiomycete *Wolfiporia cocos* was purified 22-fold to an electrophoretical homogeneity with a recovery of 47% using preparative native-PAGE as the single purification step. The enzyme showed a molecular mass of 51 kDa with 17 kDa subunits indicating a homotrimer, an isoelectric point of 3.2, and an optimum clotting activity at 45 ˚C and 0.04 M $CaCl_2$. The enzyme was most stable in the range of pH 6 and at temperatures below 40 ˚C. The specific milk clotting activity (MCA) was 1.9 times higher than of commercial *Mucor* rennet. The enzyme was identified as an aspartic peptidase A1 by its complete inhibition by 0.02 mM pepstatin A and homology analysis of peptide sequences (ESI-Tandem MS). The peptides formed after hydrolysis of *β*-casein had a molecular mass of around 18 kDa, much larger than those of bitter peptides typically generated using rennet substitutes.

Eine extrazelluläre, Milch dicklegende Peptidase aus der Submerskultur des Basidiomyceten *Wolfiporia cocos* wurde einstufig durch präparative native PAGE 22-fach mit 47% Ausbeute elektrophoretisch rein erhalten. Das Enzym wurde als Homotrimer mit 17 kDa Untereinheiten und einer nativen Molmasse von 51 kDa, einem isoelektrischen Punkt von pH 3,2 und einem Optimum der Koagulations-Aktivität bei 45 ˚C und 0,04 M $CaCl_2$ charakterisiert. Das Enzym war um pH 6 und bei Temperaturen unter 40 ˚C am stabilsten. Die spezifische dicklegende Aktivität übertraf ein Handelspräparat von Lab aus *Mucor* um das 1,9

fache. Das Enzym wurde aufgrund der vollständigen Hemmung durch 0,02 mM Pepstatin A und des Homologievergleichs von Peptidsequenzen (ESI-Tandem MS) als Aspartat-peptidase A1 identifiziert. Bei der Hydrolyse von *β*-Casein wurden 18 kDa Fragmente gefunden, die damit viel größer als die durch Lab-Ersatz häufig generierten Bitterpeptide waren.

Wolfiporia cocos, preparative native-PAGE, peptidase, clotting, *β*-casein

3.2 Introduction

Calf rennet mainly catalyses the hydrolysis of the Phe_{105}–Met_{106} bond of bovine *κ*-casein, thereby causing destabilization of the casein micelles and milk coagulation to form the cheese curd (Green, 1973). The motifs stimulating the search for rennet substitutes are manifold: the increase of both world cheese production and calf rennet's price, the increase of vegetarian life-styles, ethical considerations, laws restricting the slaughter of young animals, and a strong antipathy of consumers against genetically engineered enzymes (Bruno et al., 2010). Intensive research has been carried out in order to find milk clotting enzymes of animal or plant origins (Bruno et al., 2010; Law, 2010; Llorente et al., 2004). With the exception of some *Cynara* extracts, however, most plant sources produce bitter cheeses.

Several attempts were reported to obtain milk clotting enzymes (MCEs) from microorganisms (Arima et al., 1970; Law, 2010; Vishwanatha et al., 2010). Most of these are not able to produce high-quality cheese with a good flavour, and some of them produce bitter peptides as well. Some peptidases from basidiomycetes were studied (Kobayashi & Kim, 2003; Kobayashi et al., 1994; Kobayashi et al., 1983; Nerud et al., 1989; Okamura-Matsui et al., 2001), but only two of them were suggested for cheese making (Kobayashi & Kim, 2003; Kobayashi et al., 1985a). In a previous work, 28 basidiomycetes were screened for milk clotting activity (MCA) (Abd El-Baky et al., 2011a). A Gouda-type cheese was produced using *Wolfiporia cocos* (Wco) culture supernatant, and after two months of ripening the organoleptic characteristics of the product were comparable to those of a commercial standard Gouda cheese without any bitter taste. The objective of the present study was to purify and biochemically characterize this milk clotting peptidase from Wco.

3.3 Materials and methods

3.3.1 Culture conditions

Wco (CBS, Nr. 279.55) was purchased from the Centraalbureau voor Schimmelcultures (CBS), Netherlands. Media composition and preparation of pre- and main-cultures were reported previously (Abd El-Baky et al., 2011a).

3.3.2 Purification of Wco milk clotting enzyme

Wco submerged culture was harvested on the 7th day with 2.8 U/ml MCA, and the supernatant was concentrated using Vivaspin-20 concentrator (MWCO 3 kDa, Sartorius, Germany). One ml of the concentrated sample was mixed with 1 ml of sample buffer containing 40% glycerol in 3 mM Histidine-MES and applied to the preparative native-PAGE system (Model 491 Prep Cell, Biorad, USA) using a 16% polyacrylamide gel (9 cm gel length) prepared with Histidine-MES buffer (30 mM each, pH 6.1). The electrophoresis was done at 5 watts constant power, Na-acetate (50 mM, pH 5.7) was used as elution buffer with a flow rate of 0.75 ml/min. The eluted proteins were collected in 2.5 ml fractions at 4 ˚C. All fractions were checked for MCA.

3.3.3 Enzyme assays and protein determination

MCA was determined according to (Arima et al., 1970). One milk clotting unit was defined as the amount of enzyme which clotted 1 ml of milk within 1 min. PA was measured as described by (Iversen & Jorgensen, 1995). One PA unit was defined as the amount of enzyme which increased the absorbance at 366 nm by 1 within 1 min. Protein content was measured according to (Lowry et al., 1951) using bovine serum albumin as a standard.

3.3.4 Biochemical characterization

Native molecular mass of the enzyme was estimated on a Superdex 75 column (1 x 3 cm, GE Healthcare, USA) equilibrated with sodium phosphate buffer (50 mM pH 6.2) using calibration standards (conalbumin, ovalbumin, carbonic anhydrase, ribonuclease A, aprotinin). SDS–PAGE was done according to (Laemmli, 1970) using marker proteins from 250 to 10 kDa

(BioRad, Germany). Isoelectric focusing with an immobilized pH gradient (pH 3 to 10) was performed according to (Eisele et al., 2011).

Effects of pH and temperature on stability and activity: pH stability of the enzyme was determined in the range of pH 3 to 10 with glycine-HCl, sodium acetate, sodium phosphate and glycine-NaOH buffer (0.1 M each). After incubation 1:1 (v/v) in buffer at 25 ˚C for 22 hours residual MCA was measured. To determine temperature stability, the enzyme was incubated at 30 to 80 ˚C for 30 min and residual MCA was determined. The effect of temperature and pH on MCA was studied by performing MCA on temperature range 25-75 ˚C and pH range 5.5 to 7.

For inhibition studies, the purified enzyme was incubated at 30 ˚C for 15 min with different inhibitors (antipain, EDTA, pepstatin A and PMSF) and residual MCA was estimated. Effect of $CaCl_2$ on MCA was studied by subsequently increasing the salt concentration in the milk from 0 to 0.5 M.

3.3.5 Protein concentration of the whey

1.7 units/ml of Wco peptidase and *Mucor* rennet, respectively, were used to coagulate skim milk (10%) at 35 ˚C. The whey was centrifuged at 8000 x g for 15 min and protein content was determined in the clear supernatant as described in **3.3.3**

3.3.6 *β*-Casein degradation

The procedure was carried out as described previously (Abd El-Baky et al., 2011a).

3.3.7 ESI-Tandem MS analysis of tryptic peptides

Sequencing of tryptic peptides for Wco peptidase was done according to (Eisele et al., 2011)

3.3.8 Statistical analysis

Experiments were performed in triplicate and the data presented were expressed as mean values with standard deviation using Microsoft Excel 2007.

3.4 Results and discussion

3.4.1 Purification of milk clotting enzyme

After preparative native-PAGE milk clotting-active fractions were analyzed by SDS-PAGE, and the enzyme was electrophoretically homogeneous and ready for sequencing (Figure 1). These active fractions were pooled and concentrated up to a specific MCA of 22.2 units/mg with a recovery of 47% and a purification factor of 22 (Table 1). Most of the purification procedures of clotting enzymes were comprised of several purification steps and, thus, complicated and time consuming with low recovery of activity. The one-step purification by preparative native-PAGE was superior in terms of speed and recovery.

Table 1: Purification of milk clotting peptidase from *Wolfiporia cocos* by preparative native-PAGE, mean±SD (n=3).

Purification step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Purification fold
Crude extract	234 ± 9.76	236.6 ± 7.38	1.01 ± 0.02	100	
After PAGE	4.98 ± 0.13	110.72 ± 5.43	22.23 ± 1.38	46.80 ± 2.35	22.1 ± 1.78

3.4.2 Milk clotting activity

Table 2 summarizes milk clotting and PA of the Wco enzyme compared to *Mucor* rennet. The clotting and the peptidolytic activities of the basidiomycetous enzyme were 6.67 and 0.165 U/ml, respectively, and those of *Mucor* rennet were 3.51 and 0.045 U/ml.

Table 2: Comparison of MCA of Wco milk clotting peptidase with *Mucor* rennet, both preparations were adjusted to a protein content of 0.3 mg/ml, mean ± SD (n = 3).

Enzyme	MCA units/ml	PA units/ml	MCA/PA
Wolfiporia cocos	6.67 ± 0.20	0.165 ± 0.01	40.40 ± 2.19
Mucor rennet	3.51 ± 0.10	0.045 ± 0.002	78.00 ± 2.91

When both enzyme solutions were adjusted to the same protein content (0.30 mg/ml), the specific MCA of the Wco enzyme was 1.9 times higher than that of *Mucor* rennet indicating the high potential clotting activity of the Wco counterpart. The calculated MCA/PA ratio of the purified enzyme was half of that of *Mucor* rennet.

3.4.3 Biochemical characterization of Wco peptidase

The purified enzyme was a homotrimeric peptidase composed of 17 kDa subunits (Figure 1).

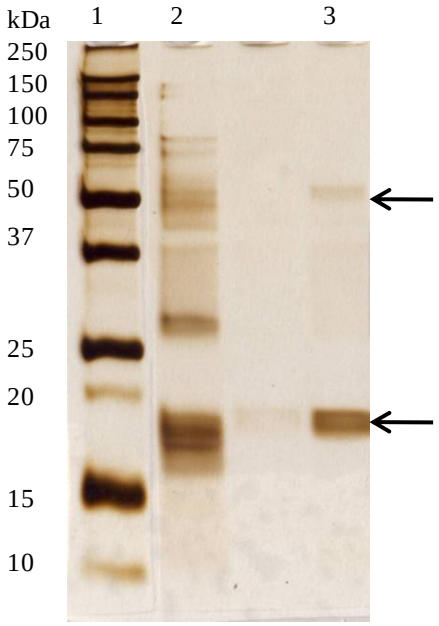

Fig. 1: SDS-PAGE (12 %) of the purification procedure of milk clotting peptidase from Wco. Lane 1: molecular mass marker, lane 2: crude extract and lane 3: purified enzyme after preparative native-PAGE.

Similar findings were reported by (Bleukx et al., 1998), who purified a heterodimeric aspartic peptidase with two subunits of 11 and 29 kDa, and (Llorente et al., 2004), who isolated a heterodimeric proteinase consisting of 30 and 15 kDa subunits from globe artichoke. The native molecular mass of the Wco peptidase was estimated via gel filtration to be 52 kDa, while most milk clotting aspartic peptidases showed a mass of 32 to 66 kDa (Ahmed et al., 2010; Chitpinityol & Crabbe, 1998; Kobayashi & Kim, 2003; Park et al., 2000). The isoelectric point of the Wco enzyme was 3.2 and comparable to milk clotting peptidases from *Laetiporus*

sulphureus (Kobayashi & Kim, 2003), whereas Chymosin showed a pI of 4.6 (Foltmann, 1970).

The MCA of the peptidase of Wco increased by decreasing the pH as was observed for other milk clotting enzymes previously (Crappe, 2004) (Figure 2a). The Wco peptidase was unstable from pH 3 to 5, possibly due to auto-degradation (Mickelson & Ernstrom, 1967), but it maintained its activity between pH 5.5 and 6.5 for the most part and rapidly denatured above pH 6.5. Maximum stability of Chymosin was recorded between pH 5.3 and 6.3 (Foltmann, 1959).

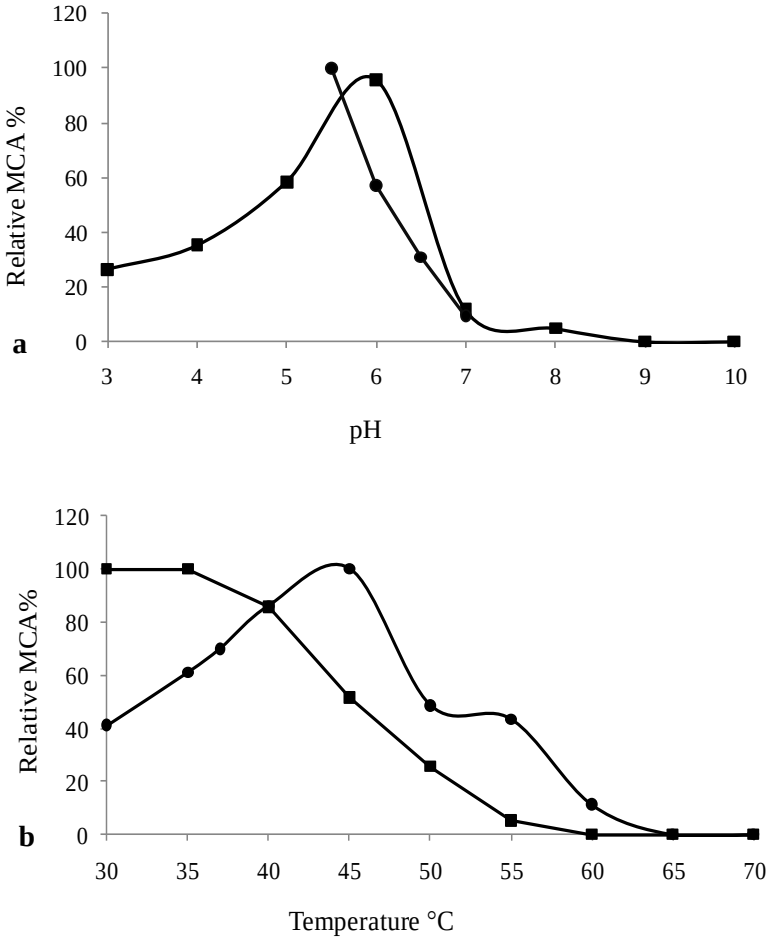

Fig. 2: Effect of pH (a) and temperature (b) on activity (●) and stability (■) of Wco milk clotting peptidase, SD values are less than 5%, n=3.

The purified enzyme had an optimum temperature of 45 ˚C (Figure 2b). This result differs from, e. g., milk clotting enzymes of *Rhizomucor miehei* (65 ˚C) (Walsh & LI, 2000) and

Solunum dubium Fresen seeds (70 ˚C) (Ahmed et al., 2010). The basidiomycetous enzyme was stable at 35 ˚C for 30 min, but incubating at 45 ˚C for 30 min resulted in a loss of 50% of activity, while it was completely inactivated at 55 ˚C for 30 min. A similar behavior was observed for a clotting peptidase of *Laetiporus sulphureus* (Kobayashi & Kim, 2003). By contrast, Chymosin was relatively stable below 50 ˚C (Kawaguchi et al., 1987). The vast majority of rennet substitutes showed a high thermal stability. Thus, they remain active in the curd and produce off-flavours during continuing peptidolysis.

The relative thermal and pH instability of the peptidase of Wco turns into a technological advantage, because it allowed producing a ripened, semi hard Gouda-type cheese without bitter taste (Abd El-Baky et al., 2011a).

Calcium ions play an important role in milk coagulation by creating isoelectric conditions and acting as a bridge between casein micelles (Merheb-Dini et al., 2010). For the Wco peptidase an optimum concentration of 0.04 M $CaCl_2$ was recorded. At higher salt concentrations the activity decreased. Similar results were reported by (Merheb-Dini et al., 2010).

3.4.4 Determination of enzyme class

Peptidase inhibitors were used to classify the Wco peptidase (Table 3). The enzyme exhibited 94% of its activity in the presence of antipain, an inhibitor of serine and cysteine peptidases. Likewise, EDTA, a metallo peptidase inhibitor, did not affect the enzyme activity, while pepstatin A, an aspartic peptidase inhibitor, completely inhibited the Wco enzyme at 0.02 mM. These results indicate that the peptidase of Wco, like Chymosin and other milk clotting enzymes, belonged to the group of aspartic peptidases. This was confirmed by N-terminal sequences. By means of ESI-tandem MS, four tryptic peptides, ALNTQLGGESGK, NLGSESGQGLDFLNGFAWLQR, VANGELTFGGTDSTR and LYLVASNLGSESGQGLDFLNGFAWLQR were identified from the denatured protein band excised from the SDS-Gel. Homology searches against public databases identified the enzyme as a member of aspartic peptidase A1 family, similar to an aspartic peptidase of Laccaria bicolor (Martin et al., 2008).

Table 3: Effect of peptidase inhibitors on MCA of Wco milk clotting peptidase, mean ± SD (n = 3)

Inhibitor	Concentration mM	Residual activity %
Control	0	100
Antipain	0.01	93.15 ± 2.65
EDTA	1	97.10 ± 2.44
Pepstatin A	0.02	0
PMSF	0.1	25.20 ± 1.15

3.4.5 Protein concentration of whey

Extensive hydrolysis of milk proteins by less specific microbial enzymes resulted in loss of protein and yield, off-flavors and bitter peptides in cheese (Vishwanatha et al., 2009). The protein concentration of the whey after coagulation using the Wco peptidase was compared to *Mucor* rennet. The protein concentration in the whey recorded about the same value, 4.50 ± 0.03 and 4.44 ± 0.04 mg/ml for the Wco peptidase and for *Mucor* rennet, respectively, indicating the almost equal potential of the basidiomycetous enzyme. Looking at the worse MCA/PA ratio of the peptidase of Wco this was an unexpected result. It appears that this widely used measure has less predictive power for the clotting efficiency when it comes to assessing new peptidases.

3.4.6 *β*-Casein degradation

Bitterness is one of the most common off-flavors in cheese, and it has been associated with bitter peptides rich in hydrophobic amino acid moieties. Accordingly, the majority of potentially bitter peptides identified in cheeses were found to originate from α_{s1}- and *β*-casein (Kobayashi & Kim, 2003; Singh et al., 2003). The effect of calf rennet versus the Wco peptidase on *β*-casein after four hours of incubation is shown in Figure 3. Calf rennet produced one major smaller band indicating that *β*-casein contained few dipeptidyl sites suitable as a substrate for calf rennet. The peptidase of Wco produced two fragments, one major band with a mass of 18 kDa and another band at 24 kDa. Similar results have been stated by

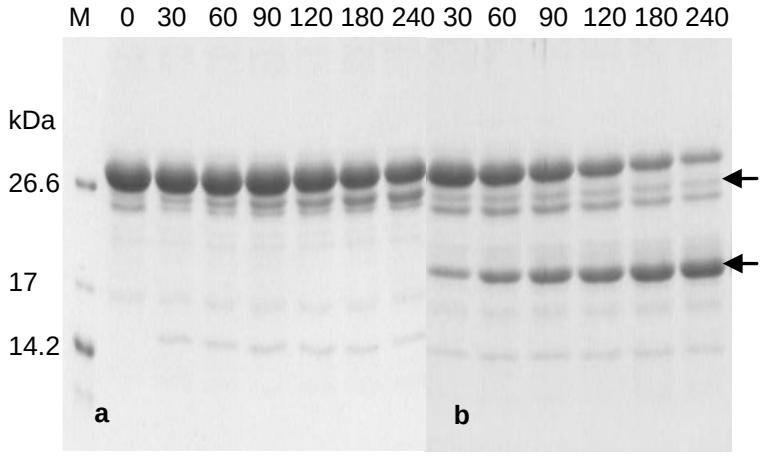

Fig. 3 SDS-PAGE (18%) of the time-dependent (min) degradation of *β*-casein by calf rennet (a) and Wco milk clotting peptidase (b). Arrows indicating pre-dominant fragments produced.

Similar results have been stated by (Kobayashi & Kim, 2003) who reported one major band with Chymosin and three bands when using the enzyme of *L. sulphureus*. These results agree well with the experimental observation that the semi hard Gouda-type cheese produced using the Wco peptidase was without any perceivable bitter taste (Abd El-Baky et al., 2011a).

3.5 Conclusion

Purified aspartic peptidase of *Wolfiporia cocos* showed a MCA comparable to *Mucor* rennet, the reference. The protein concentration of the whey after coagulation was about the same as with *Mucor* rennet. The peptidase of Wco lost 50% of its activity by incubating at 45 ˚C for 30 min and is thus supposed to become more rapidly inactivated in the curd than other clotting enzymes. The peptidase did not find many cleavage sites in *β*-casein and yielded only a few larger fragments. In summary, the peptidase from *Wolfiporia cocos* showed a number of favorable properties not found in other non-animal rennet substitutes. Its biotechnological production could supply the dairy industry with an inexhaustible source of an efficient clotting activity.

3.6 Acknowledgment

Financial support by the Egyptian Ministry of Higher Education and the DAAD, Bonn, is gratefully acknowledged

4 PsoP1, an aspartic peptidase from the brown-rot fungus *Piptoporus soloniensis*

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(Accepted)

4.1 Abstract

A peptidase was isolated from submerged cultures of the basidiomycete *Piptoporus soloniensis* (PsoP1) and purified by fractional precipitation and preparative native-PAGE to an electrophoretical homogeneity. The native molecular mass of PsoP1 was 38 kDa with an isoelectric point of 3.9. Similar to chymosin from calves, PsoP1 showed a maximum activity at 35 to 40 ˚C and was most stable at pH 6 and below 40 ˚C. The complete inhibition by pepstatin A identified this enzyme as an aspartic peptidase. ESI-tandem MS showed an amino acid partial sequence which was more homologous to mammalian milk clotting peptidases than to the chymosin substitute from *Mucor miehei*, a Zygomycete. According to SDS-PAGE patterns, the peptidase cleaved *κ*-casein in a way similar to chymosin, and only slowly hydrolyzed *β*-casein, as it would be expected from an efficient chymosin substitute.

Keywords

Piptoporus soloniensis, milk clotting, *β*- and *κ*-casein, aspartic peptidase, Preparative PAGE

4.2 Introduction

Among the vast number of peptidases with applications in the food industry, aspartic peptidases, such as chymosin (EC 3.4.23.4) used in cheese-making, are particularly well investigated (Egito et al., 2007). Milk clotting by chymosin occurs essentially by cleaving the Phe_{105}–Met_{106} bond of *κ*-casein, resulting in a release of a short hydrophilic glycopeptide (106–169 residues), which dissolves in the whey. Para-*κ*-casein becomes positively charged at neutral pH and causes a decrease of the repulsive forces between casein micelles, thereby causing aggregation (Vishwanatha et al., 2009).

The scarcity of traditional rennet, the increase of the world cheese production, ethical considerations associated with the slaughtering of milk calves, the rejection of consumers of genetically modified enzymes and the increase of vegetarian life-styles have promoted the research for alternative coagulants (Bruno et al., 2010).

Plant sources, with few exceptions, showed a broader peptidolytic activity, causing extensive digestion of curd, off-flavour, impaired taste and a pasty texture (Kumar et al., 2010). An extensive cleavage of casein, owing to a lesser specificity and high thermal stability of microbial enzymes, resulted in a loss of protein and yield, off-flavour and bitter peptide formation (Vishwanatha et al., 2010). Different trials were made to isolate peptidases from basidiomycetes (Kawai & Mukai, 1970; Kobayashi & Kim, 2003; Kobayashi et al., 1994; Kobayashi et al., 1983; Nerud et al., 1989; Rudenskaia et al., 1980; Saboti et al., 2007). Milk clotting enzymes from *Irpex lacteus* and *Laetiporus sulphureus* were suggested as calf rennet substitutes (Kobayashi & Kim, 2003; Kobayashi et al., 1985a). In a previous paper (Abd El-Baky et al., 2011a), peptidolytic activities of 28 basidiomycetes were screened using different nutrient media. Among the tested basidiomycetes more than half (16 species) showed milk clotting activity (MCA).

Piptoporus soloniensis attracted particular attention, because it combined acceptable growth in submerged culture with high specific clotting and low peptidolytic activities. This brown-rot fungus, a close relative of *Piptoporus betulinus*, the edible birch polypore, is preferably found on oak trees. Apart from the formation of γ-decalactone, a fruity flavour compound, no particular metabolic traits of *P. soloniensis* were reported (Okamoto et al., 2002). The focus of

the present study was on the purification and physicochemical characterization of its milk clotting peptidase, (PsoP1).

4.3 Materials and Methods

4.3.1 Culture and cultivation conditions

Piptoporus soloniensis (CBS Nr. 492.76 Centraalbureau voor Schimmelcultures CBS, Netherlands) was inoculated on standard agar plates, SNL, (media composition was described by (Wu et al., 2007)). Pre-cultures were prepared by homogenisation of a 10x10 mm agar plug with mycelium of *Piptoporus soloniensis* (Pso) in 100 ml of sterile standard nutrition solution using an Ultra turrax. Cultures were maintained at 24 ˚C and 150 rpm for eight days and 20 ml pre-culture were transferred into 250 ml SNL main culture. After 14 days of cultivation at 24 ˚C and 150 rpm, the culture was harvested with 0.56 U/ml MCA.

4.3.2 Assay of MCA

MCA was measured according to (Arima et al., 1970), which is based on the visual evaluation of the appearance of the first clotting aggregates. 10% skim milk solution containing 10 mM $CaCl_2$ was prepared and the pH was adjusted to pH 6. 0.2 ml of enzyme and 2 ml of the skim milk were preincubated at 35 ˚C for 10 min. The mixture was mixed well in a test-tube. The coagulation point was determined periodical at 35 ˚C by manual rotation of the test tube. One milk clotting unit was defined as the amount of the enzyme which clotted the milk solution in 1 min.

4.3.3 Assay of peptidolytic activity (PA)

PA was determined according to (Iversen & Jorgensen, 1995), with slight modifications. The reaction mixture was made up with 0.2 ml of azocasein 2.5% (w/v) in 0.1 M potassium phosphate buffer pH 6 and 0.275 ml of 0.1 M potassium phosphate buffer pH 6, to which 0.025 ml of the enzyme solution was added. The reaction was carried out at 35 ˚C for 20 min and stopped with 1 ml of 10% trichloroacetic acid (TCA). The tubes were kept at zero ˚C for 10 min and centrifuged at 12,000 x g for 10 min. The absorbance of the supernatant was read at 366 nm. A blank was prepared by pipetting the enzyme after the addition of the TCA. One

peptidolytic activity unit was defined as the amount of enzyme which increased the absorbance at 366 by 1 in 1 min.

4.3.4 Determination of the protein content

The protein content of the fractions obtained after ammonium sulphate precipitation and preparative native-PAGE was estimated by measuring the absorbance at 750 nm according to (Lowry et al., 1951) using bovine serum albumin as standard.

4.3.5 Purification of the enzyme

Culture broth of Pso was centrifuged at 5000 x g for 40 min and 4 ˚C to pellet the mycelia. Culture supernatant was first gradually saturated with ammonium sulphate between 60-80% and centrifuged. The precipitate was dissolved in Histidine-MES buffer, pH 6.1, washed three times and concentrated using Vivaspin-15 concentrator (MWCO 3 kDa, Sartorius).

The second purification step was performed using preparative native-PAGE (Model 491 Prep Cell Biorad). 1 ml of the concentrated sample was mixed with 1 ml of sample buffer containing 40% glycerol in 3 mM Histidine-MES buffer, pH 6.1 and separated on a 14% SDS gel (8 cm gel length, 8.2 cm^2 gel surface area, at 5 W constant power and 4 ˚C with a flow rate of 0.75 ml/min of 50 mM potassium phosphate, pH 6 as elution buffer. 2.5 ml-fractions were collected and checked for MCA.

4.3.6 Characterization of PsoP1

4.3.6.1 Temperature optimum and thermostability

In order to determine the optimum temperature for PsoP1, MCA was assayed between 30 and 70 ˚C. Thermal stability of the purified enzyme was determined by incubating the enzyme at 30 to 75 ˚C for 30 min. After incubation, residual MCA was determined.

4.3.6.2 Effect of pH on the activity and stability

The effect of pH on MCA was tested by assaying the clotting activity in the range of pH 5.5 to 7.5 by adjusting the pH of skim milk with 0.2 M NaOH or 0.2 M HCl. For pH stability, the enzyme was diluted 1:1 in the following 0.1 M buffer solutions: glycine-HCl (pH 3), sodium

acetate (pH 4-5), sodium phosphate (pH 6-8) and glycine-NaOH (pH 9-10) and maintained at 25 ˚C for 22 hours afterwards, residual MCA was determined.

4.3.6.3 SDS-PAGE and preparative isoelectric focusing

Active samples eluting from the preparative native-PAGE were analyzed by SDS-PAGE using 12% acrylamide in a Mini-Protean tetra cell (Bio-Rad), according to (Laemmli, 1970). After electrophoresis, gels were stained with silver. To determine the isoelectric point of the enzyme, preparative isoelectric focusing was performed using Rotofor® cell system (Bio-Rad). The conditions were: 0.5% Servalyt 40%, pH 2-4 (Serva) and 12 W constant at 4 ˚C for 4 hours. The pH of the fractions was measured using Qph 70 pH-meter (Merck).

4.3.6.4 Determination of molecular mass

The native molecular mass of the enzyme was estimated on a Superdex 75 column (10 x 30 mm, GE Healthcare) equilibrated with 50 mM sodium phosphate buffer, pH 6.2 containing 150 mM NaCl with a flow rate of 0.5 ml/min. Eluted fractions (0.5 ml) were checked for MCA. Estimation of molecular mass was done by means of a calibration with standard proteins.

4.3.6.5 Inhibition study

To classify PsoP1, the purified enzyme was incubated for 15 min at 30 ˚C with inhibitors: antipain (serine and cysteine peptidases inhibitor), ethylenediamine tetraacetic (EDTA) (metallo peptidase inhibitor), pepstatin A (aspartic peptidase inhibitor) and phenylmethysulphonylfluoride (PMSF) (serine peptidase inhibitor). Residual MCA was determined and the control enzyme activity without inhibitor was taken as 100%.

4.3.6.6 Effect of $CaCl_2$ on MCA

The effect of $CaCl_2$ ions at different concentrations (0 to 0.5 M) on MCA was determined. The substrate (10% skim milk) was equilibrated at different concentrations of $CaCl_2$ for five minutes at room temperature, afterwards the enzyme was added and MCA was determined as described above.

4.3.6.7 Hydrolysis of *κ*-casein

Hydrolysis of *κ*-casein was carried out by dissolving bovine *κ*-casein in 100 mM sodium phosphate buffer, pH 6.5, to a final concentration of 3 mg/ml. PsoP1 as well as chymosin (1.7 U/ml) were added to the *κ*-casein substrates at a ratio of 0.5:10 ml (v/v), and the reaction was allowed to proceed at 35 ˚C. Aliquots were taken periodically, and the reaction was stopped by heating at 95 ˚C for 10 min. Samples were applied to SDS-PAGE according to (Laemmli, 1970) using 160 g acrylamide/l separating gel and 30 g acrylamide/l stacking gel containing 1 g SDS/l. Ultra low molecular marker proteins from 26.6 to 1.06 kDa (Bio-Rad) were used. The gel was stained with Coomassie Brilliant Blue.

4.3.6.8 *β*-Casein degradation

Degradation of *β*-casein was monitored as follows: a solution of 0.1% (w/v) of *β*-casein in 20 mM potassium phosphate buffer, pH 6 was prepared. 40 µl of PsoP1 or chymosin (1.3 U/ml) were mixed with 2 ml of *β*-casein solution and incubated at 35 ˚C. During incubation period, aliquots (0.2 ml) were taken at 0, 30, 60, 90, 120, 180, 240 min, and 24 hours. Samples were prepared by mixing 20 µl of each aliquot with 20 µl of loading buffer with a final concentration of 0.1 M Tris-HCl (pH 6.8), 0.2 M DTT, 4% SDS, 20% glycerol, 0.2% bromophenol blue, boiled for 10 min and applied on an SDS gel consisting of a 18% acrylamide separating gel and 3% acrylamide stacking gel containing 0.1% SDS. After electrophoresis at 12 mA/gel at room temperature, the gels were stained with Coomassie Brilliant Blue. For the determination of molecular mass, ultra low molecular marker proteins were used.

4.3.6.9 Electrospray ionization (ESI)-tandem mass spectrometer (MS)

Coomassie stained protein bands were excised from SDS-PAGE gels, dried, and digested with trypsin. The resulting peptides were extracted and purified according to standard protocols. A Qtof II MS (Micromass, Manchester, UK) equipped with a nanospray ion source and gold-coated capillaries (Protana, Odense, Denmark) were used for ESI-MS of peptides. For collision induced experiments, multiple charged parent ions were selectively transmitted from the quadrupole mass analyzer into the collision cell (25 to 30 eV). The resulting daughter ions were separated by an orthogonal time-of-flight mass analyzer. The acquired MS-MS spectra

were enhanced (Max. Ent. 3, Micromass) and used for the *ab initio* sequencing of tryptic peptides.

4.4 Results and discussion

4.4.1 Purification of PsoP1

After removing the fungal pellets by centrifugation the protein in the crude supernatant was precipitated with ammonium sulphate. The fractions from 60 to 80% were dissolved in Histidine-MES buffer. 70% of MCA were recovered with a specific MCA of 2.33 U/mg and a purification factor of 23 (Table1). Histidine-MES buffer, 30 mM pH 6.1, was chosen to keep the enzyme active during the run. Using the preparative native PAGE, PsoP1 was purified to electrophoretic homogeneity, showing a single band on a silver stained SDS gel (Figure 1).

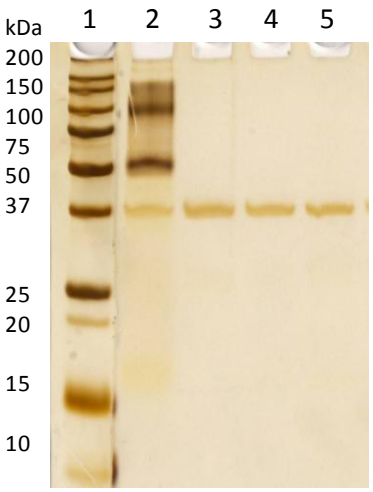

Fig. 1 SDS-PAGE of PsoP1 purification steps. Molecular mass marker (1), precipitate after ammonium sulphate treatment (2) and purified PsoP1 after preparative native-PAGE (3-5).

Active pooled fractions exhibited a specific MCA of 8.50 U/mg with a recovery of 26% and a purification factor of 83 (Table 1).

Table 1: Purification of milk clotting peptidase from Piptoporus soloniensis by ammonium sulphate precipitation and preparative native-PAGE, mean ± SD (n = 3).

Step	Total protein (mg)	Total activity (units)	Specific activity (U/mg)	Yield %	Purification fold
Crude extract	888.25 ± 12.51	90.25 ± 2.38	0.102 ± 0.001	100	0
Amm. sulphate	27.06 ± 0.90	63.14 ± 1.75	2.33 ± 0.10	69.96 ± 2.74	22.84 ± 1.29
P-native-PAGE	2.75 ± 0.13	23.37 ± 1.07	8.50 ± 0.37	25.89 ± 1.10	83.33 3.86

4.4.2 Milk clotting activity

Table 2 compiles the clotting and peptidolytic activities of PsoP1 compared to the reference enzyme from *Mucor miehei*. Both activities were similar for both sources, but PsoP1 showed a somewhat lower MCA/PA ratio, a measure generally regarded as an index of the clotting efficacy. Commercial *Mucor* rennin is supposed to be chemically or genetically improved to obtain a maximum MCA/PA ratio (Chitpinityol & Crabbe, 1998). However, the absolute values of peptidolytic activities were very low for both enzymes, and thus small deviations will result in large changes of the MCA/PA ratio. Moreover, data on peptidolytic activity inevitably depend on the substrate used, and different substrates are known to yield different absolute activities. Nonetheless, the results clearly indicated high clotting activity and low peptidolytic activity of PsoP1.

Table 2 MCA of Piptoporus soloniensis milk clotting peptidase and Mucor rennet. Both preparations were adjusted to a protein content of 0.3 mg/ml, mean ± SD (n = 3).

Enzyme	MCA (U/ml)	PA (U/ml)	MCA/PA
PsoP1	2.55 ± 0.12	0.05 ± 0.003	51.0 ± 2.21
Mucor miehei	3.51 ± 0.10	0.045 ± 0.002	78.0 ± 2.71

4.4.3 Temperature optimum and thermostability

PsoP1 exhibited its maximum clotting activity between 35 to 40 ˚C (Figure 2). This is in agreement with (Berridge, 1952), who determined the optimum temperature for purified

chymosin to be between 30 and 40 ˚C. On the other hand, this result differed from other microbial clotting enzymes: The peptidase from *Penicillium oxalicum*, for example, showed a maximum activity at 65 ˚C (Hashem, 2000), while the peptidase from *Aspergillus oryzae* was most active at 55 ˚C (Vishwanatha et al., 2010). The temperature of cheese processing is between 30 to 35 ˚C. At this temperature, PsoP1 retained its maximum activity. PsoP1 remained stable until 40 ˚C for 30 min, while it lost 83% of its activity after incubation at 55 ˚C for 30 min. A complete inhibition was observed at 60 ˚C (Figure 2).

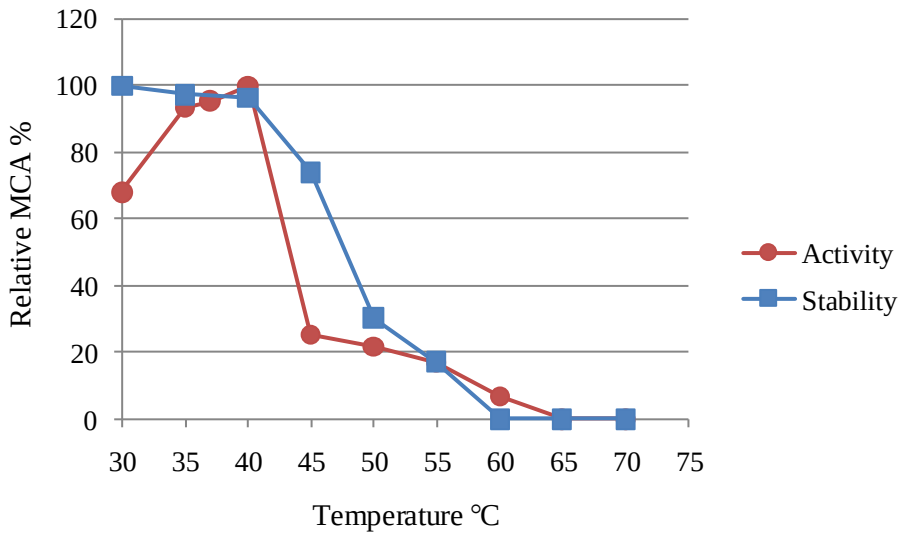

Fig. 2 Effect of temperature on MCA (●) and stability (■) of PsoP1 milk clotting peptidase, SD values were less than 5 %, n=3.

Hence, PsoP1 was also more sensitive to thermal treatment than the commercial clotting enzyme from *Rhizomucor miehei* which exhibited high stability at 60 ˚C (Walsh & LI, 2000). As a result, the temperature inactivation characteristics of PsoP1 rather resemble the chymosin reference than most of the microbial sources.

4.4.4 Effect of pH on the activity and stability of PsoP1

Highest MCA was observed at pH 5.5 followed by a decrease to pH 7.5 (Figure 3). Other milk clotting enzymes exhibited the same trend (Kobayashi & Kim, 2003; Merheb-Dini et al., 2010). PsoP1 was stable from pH 3 to -5 and retained about 70 % of its activity with a maximum stability at pH 6 (Figure 3). At higher pH values the enzyme completely lost its

activity (Mezina et al., 2001) reported that chymosin was stable at acidic pH values and denaturated above pH 6.

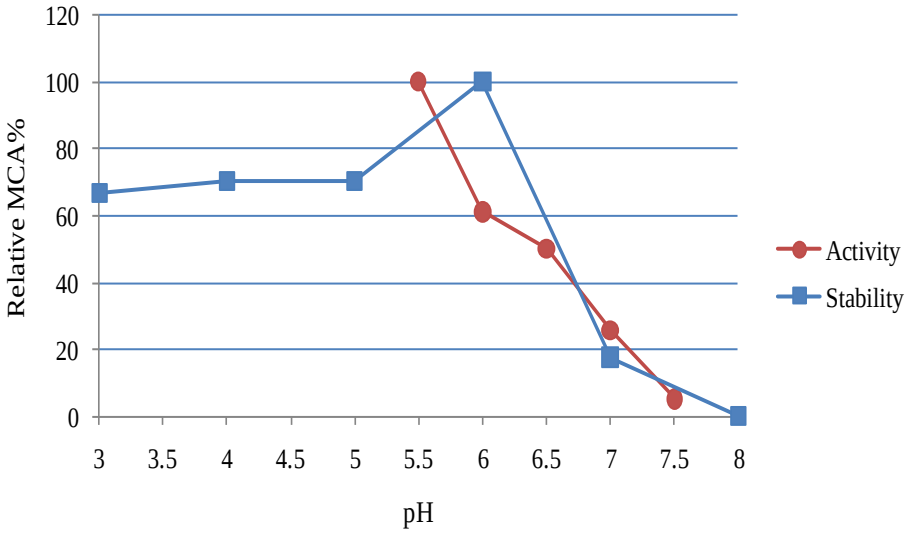

Fig. 3 Effect of pH on MCA (●) and stability (■) of PsoP1, SD values were less than 5 %, n=3.

4.4.5 Molecular mass and isoelectric point (p*I*) of PsoP1

The native molecular mass of the PsoP1 was estimated by gel filtration (Superdex 75 column) to be 38 kDa. This result was similar to the findings for other commercial milk clotting enzymes, for example from *Mucor miehei* (39 kDa, (Lagrange et al., 1980) and calf chymosin (36.3 kDa (Kleinert et al., 1988). The p*I* of the Pso enzyme was 3.9 which was exactly the same as the p*I* of milk clotting enzyme from *Rhizomucor pusillus* (Etoh et al., 1979) and comparable to that of an enzyme from *Laetiporus sulphureus* (p*I* 3.5, (Kobayashi & Kim, 2003). On the other hand the p*I* of chymosin was 4.5 (Kleinert et al., 1988).

4.4.6 Inhibition studies

To classify PsoP1, peptidase inhibitors were employed to identify functional groups at the active site of this enzyme. PsoP1 retained 94.5% of its activity when treated with antipain, an inhibitor of serine and cysteine peptidases (Table 3). EDTA did not alter the activity of the enzyme showing that the enzyme did not belong to metallopeptidases group.

Table 3 Effect of peptidase inhibitors on MCA of PsoP1, mean ± SD (n = 3).

Inhibitors	Concentration mM	Relative MCA (%)
Control	0	100
Antipain	0.01	94.5 ± 2.00
EDTA	1	98.5 ± 1.51
Pepstatin A	0.02	0
PMSF	0.1	51.1 ± 1.1

In the presence of PMSF, a serine peptidase inhibitor, Pso enzyme lost ~ 50% of its activity. A complete inhibition was observed in the presence of pepstatin A. It was reported that aspartic peptidases are inhibited by pepstatin A, a hexapeptide from *Streptomyces* that contains two statin residues, an unusual amino acid (Yegin et al., 2011).

4.4.7 Effect of $CaCl_2$ on the MCA of PsoP1

Calcium has been considered to be stimulator for coagulation by creating isoelectric conditions and by acting as an ion bridge between the phosphate moieties of casein micelles (Merheb-Dini et al., 2010). As shown in Figure 4, MCA of PsoP1 was increased 2.5 fold by the addition of 0.04 M $CaCl_2$.

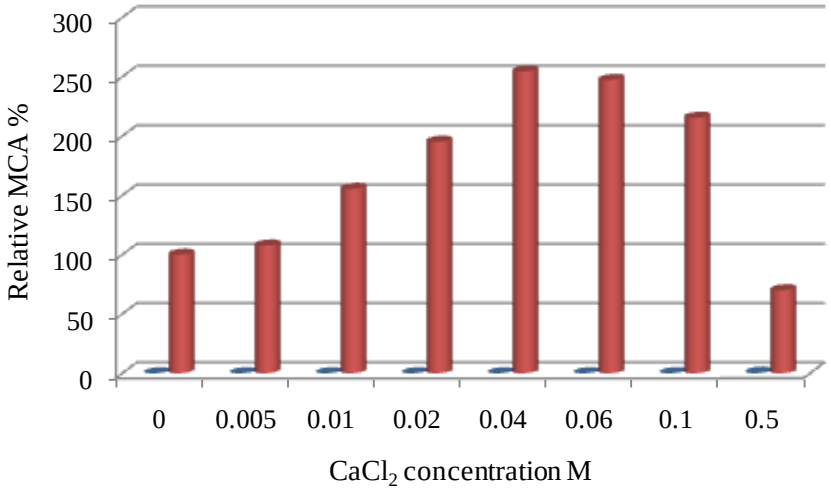

Fig. 4 Effect of $CaCl_2$ on MCA of PsoP1, SD values were less than 5%, n=3.

Afterwards, MCA decreased at concentrations higher than 0.04 M, probably due to the saturation of negative residues of the micelles in the enzyme substrate mixture (Vairo-Cavalli et al., 2005). This was in the range of the genuine calcium concentration of cow´s milk (around 0.03 M). The distinct response of PsoP1 treated milk to the addition of $CaCl_2$ is in line with the high MCA of this enzyme and is expected to support the gel strength, aggregation rate and curd firmness in cheese production (Solorza & Bell, 1998).

4.4.8 Hydrolysis of κ-casein

Hydrolysis of *κ*-casein by PsoP1 as well as chymosin was monitored by SDS-PAGE (Figure 5). Both enzymes rapidly produced one major band after 5 min with a molecular mass of about 16 kDa which is assumed to be para-*κ*-casein. The intensity of the para-*κ*-casein band increased with the incubation time. After 30 min *κ*-casein was almost completely degraded by chymosin while it was more stable towards the action of PsoP1. Only one thin band with a molecular mass of 18 kDa was visible even after 24 hours. The desired limited hydrolysis of *β*-casein by chymosin is well-known (Kobayashi & Kim, 2003). On the other hand *Mucor miehei*, *Irpex lacteus* and *Endothia parasitica* milk clotting peptidases exhibited high peptidolytic activity towards *β*-casein (Kobayashi et al., 1985b).

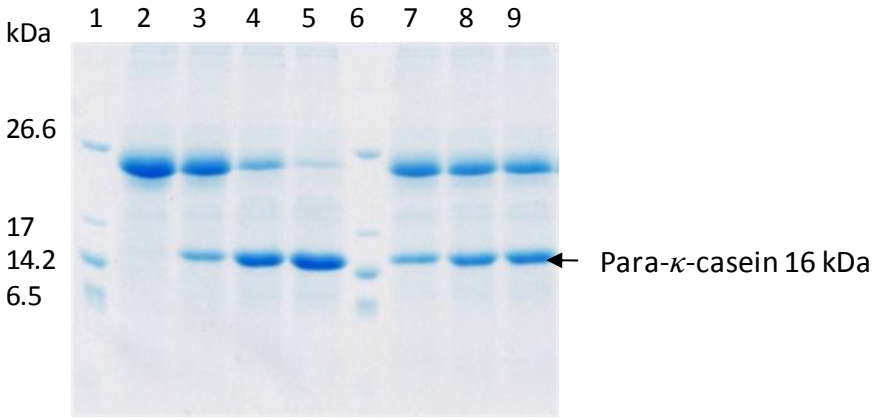

Fig. 5 SDS-PAGE pattern of *κ*-casein hydrolyzed by chymosin and PsoP1. Molecular mass marker (1, 6), non-hydrolyzed *κ*-casein (2), *κ*-casein hydrolyzed by chymosin for 5, 15 and 30 min (2-5), and *κ*-casein hydrolyzed by PsoP1 for 5, 15 and 30 min (7-9), the generated peptide indicated by arrow.

The slower proteolysis of *κ*- and para-*κ*-casein is supposed to increase the yield of the curd. In contrast, commercial rennet substitutes of *Mucor miehei and Endothia parasitica* caused an extensive non-specific hydrolysis of both *κ*-casein and para-*κ*-casein (Shammet et al., 1992).

4.4.9 *β*-Casein degradation

The differences in cheese properties during ripening and storage are mainly caused by the combined effects of the ongoing degradation of *α*- and *β*-casein. Unwanted bitter peptides may arise from their hydrophobic sequences in the course of random hydrolysis (Vishwanatha et al., 2010). Figure 6 shows the degradation of *β*-casein by PsoP1 compared to chymosin. *β*-Casein was slightly hydrolyzed by both chymosin and PsoP1 after 30 min. The intensity of the generated bands increased slightly during four hours of incubation.

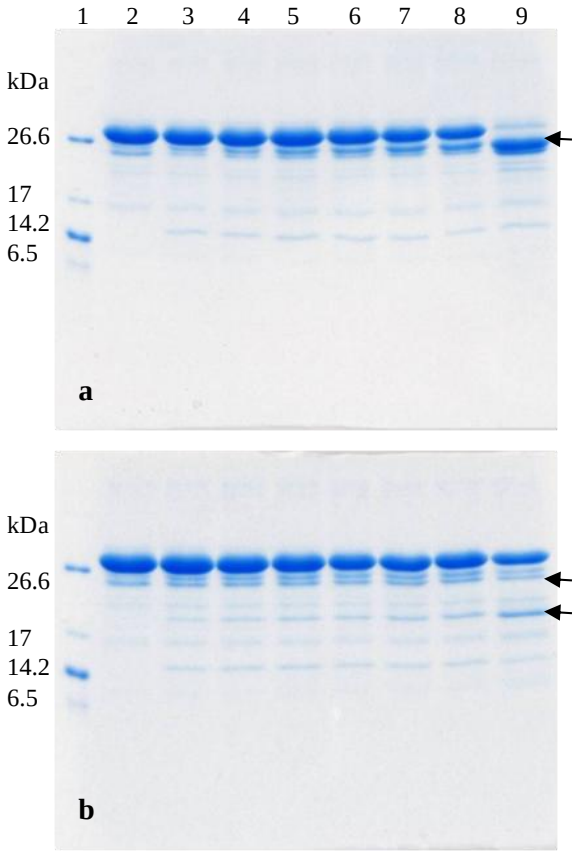

Fig. 6 SDS-PAGE pattern of *β*-casein hydrolyzed by chymosin (a) and PsoP1 (b). Molecular mass marker (1), non-hydrolyzed *β*-casein (2), and *β*-casein hydrolyzed for 30, 60, 90, 120, 180, 240 min and 24 hours (3-9), the generated peptides indicated by arrows.

After 24 hours, *β*-casein was completely degraded by the action of chymosin to a major band with a molecular mass of 24 kDa while it was hardly hydrolyzed by PsoP1. The limited peptidolytic activity of PsoP1 is considered as a unique characteristic among the various microbial origins, and suggests its applicability in cheese production.

4.4.10 Partial peptide sequences

Three tryptic peptides were identified from the protein band excised from the SDS-Gel by means of ESI-tandem MS. Homology searches against public databases identified the enzyme as a member of aspartic peptidase A1 family, similar to aspartic peptidases of *Laccaria bicolor*, *Schizophyllum commune* and *Irpex lacteus*. The N-terminal sequence of the tryptic peptides of PsoP1 showed 50 to 91% identity to the aspartic peptidases from *L. bicolor* and *S. commune* and 42 to 82 % identity to the one from *Irpex lacteus* (Figure 7 a). *Irpex lacteus* and *Piptoporus soloniensis* are taxonomically related, both of them belonging to the order *Polyporales*, while *Laccaria* and *Schizophyllum* are *Agaricales*.

```
PsoP peptide 1      ----------------TLNTTLGGEEGK-
L. bicolor          GVTYTLTANAQLFPRSLNTLIGGTAGKI 358
S. commune          DTTFEFTPNAQIWPRKLNSLLGGDDNKI 281
I. lacteus          GQTFELTANAQIWPRNLNTAIGGSASSV 300

PsoP peptide 2&3    ---FYSVFDTTNSRVGVATTPYTDATTN
L. bicolor          LERFYSVFDTTNKRVGFATTAYTTATTN 409
S. commune          LQRFYSVYDTTNSRVGLAKTEYTNATTN 332
I. lacteus          FERFYSVYDTTNKRLGLATTSFTTATSN 340
```

Fig. 7 a Amino acid sequence alignment of the peptides of the PsoP1 compared to cDNA-derived amino acid sequence of *Laccaria bicolor* (Martin et al., 2008), *Schizophyllum commune* (Ohm et al., 2010) and *Irpex lacteus* (Fujimoto et al., 2004) peptidases.

This agrees with the above mentioned observation of milk clotting activities in basidiomycete species from different orders during the initial screening. Alignment of the three tryptic peptides of PsoP1 with MCEs of calf, camel, dog, sheep and *Mucor miehei* revealed that two of them did not show a good homology with any other source. The third peptide

(FYSVFDTTNSR) was surprisingly highly homologous to all mammalian sources (Figure 7 b) but less to *Mucor miehei*.

```
PsoP peptide 3        ---FYSVFDTTNS---------
Calf                  IREYYSVFDRANN-LVGLAKAI------ 324
Camel                 IREYYSVFDRANN-RVGLAKAI------ 323
Dog                   IRKFYSVFDRGNN-RVGLALAVP----- 428
Sheep                 IREYYSVFDRANN-LVGLAKAI------ 381
M.miehei              LRFFVNVYDFGNN-RIGFAPLASAYENE 430
```

Fig. 7 b Amino acid sequence alignment of single peptide of the PsoP1compared to cDNA-derived amino acid sequence of calf prochymosin (Hidaka et al., 1986), camel chymosin (Kappeler et al., 2006), dog cathepsin E (NCBI-XP- 545694.2), sheep preprochymosin (Pungercar et al., 1990) and *Mucor miehei* (Yang et al., 1997). Identical amino acids are shaded.

Moreover, this agrees with the physicochemical features of PsoP1 which were closer to calf chymosin than to microbial rennets. Speculations on sequence-property relationships, however, must remain premature, until the full sequence of PsoP1 has become available.

4.5 Conclusion

A milk clotting aspartic peptidase from *Piptoporus soloniensis* was purified using a simple two-step purification procedure. The enzyme showed a unique combination of properties: High MCA with a low general peptidolytic activity, moderate temperature instability, maximum activity at a weakly acidic pH, and a very slow peptidolysis of *β*-casein. As a result, the biotechnology of basidiomycetes represents an inexhaustible source of milk clotting activity. The transfer of peptidase genes into heterologous hosts, such as *E. coli*, was successful and could offer an alternative route, if the superiority of genetically engineered food enzymes will find wider appreciation by consumers.

4.6 Acknowledgements

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