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ORIGINAL ARTICLE

Comparative efficiency of microbial enzyme preparations versus pancreatin for in vitro alimentary protein digestion

Mireille Andriamihaja · Alain Guillot · Allan Svendsen · Joerg Hagedorn · Sahondra Rakotondratohanina · Daniel Tomé · François Blachier

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Abstract Utilisation of microbial enzymes may represent an alternative strategy to the use of conventional pancreatin obtained from pig pancreas for the treatment of severe pancreatic insufficiency. In this study, we focused on the capacity of two microbial preparations for their capacity to digest alimentary proteins (caseins and soya proteins) in comparison with pancreatin. These microbial enzymatic preparations were found to be able to generate small, medium-size and larger polypeptides from caseins and soya proteins but were inactivated at pH 3.0. As determined by Liquid Chromatography–Mass Spectrometry analysis, microbial enzymes generated very different peptides from caseins when compared with peptides generated through pancreatin action. These microbial preparations were characterised by relatively low trypsin- and low carboxypeptidase-like activities but high chymotrypsin-like activities and strong capacity for cleavage of caseins at the methionine sites. Although the efficiency of these microbial preparations to increase the rate of absorption of nitrogen-containing compounds in severe pancreatic insufficiency remains to be tested in vivo, our in vitro data indicate proteolytic capacities of such preparations for alimentary protein digestion.

Keywords Proteins · Digestion · Pancreatin · Microbial enzymes

Introduction

Severe exocrine pancreatic insufficiency may result in nutrient malabsorption (Layer and Keller 1999; Meier and Beglinger 2006). The normal human pancreas secretes more than 20 enzymes in the form of zymogens which are, after activation, responsible for the digestion of luminal proteins, lipids and carbohydrates (Whitcomb and Lowe 2007). The clinical signs of exocrine pancreatic insufficiency are evident only when 90 % or more of the exocrine pancreas function are lost (Lankisch et al. 1986). In that case, decreased digestion of fat precedes decreased digestion of proteins and carbohydrates (Layer and Groger 1993; Sikkens et al. 2010). As a consequence, steatorrhea is generally more severe and occurs several years before clinical signs of protein and carbohydrate decreased digestion (Layer et al. 2001). The pancreatic enzyme replacement therapy is based on the use of exogenous pancreatic enzymes which are primarily extracted from porcine sources. These preparations, including pancreatin, contain a mixture of proteases, lipases and amylases (Bruno et al. 1998; Dominguez-Munoz et al. 1997; Halm

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M. Andriamihaja · S. Rakotondratohanina · D. Tomé · F. Blachier (✉)
INRA, CNRH-IdF, AgroParisTech, UMR 914 Nutrition Physiology and Ingestive Behaviour,
16 Rue Claude Bernard, 75005 Paris, France
e-mail: francois.blachier@agroparistech.fr

A. Guillot
TGU : MICALIS, Plate Forme D'Analyse Protéomique de Paris Sud-Ouest INRA, Batiment 526, Domaine de Vilvert,
78352 Jouy en Josas cedex, France

A. Svendsen
Department of Protein Design, Novozymes A/S,
Krogshoejvej 36, 2880 Bagsvaerd, Denmark

J. Hagedorn
Abbott laboratories GmbH, Hannover, Germany

et al. 1999; Lohr et al. 2009; Savoie et al. 2005). Treatment with pancreatin was found to be effective regarding the rate of absorption of dietary fat and also to improve the rate of absorption of N-containing compounds as well as to improve the nitrogen balance (DiMagno et al. 1973; Van Hoozen et al. 1997). Although pancreatin treatment for up to 2 years has been considered as safe (Littlewood et al. 2011), an alternative strategy for exocrine pancreatic insufficiency would be the use of enzymes originating from bacterial and/or fungal origin instead of enzymes from animal source (Layer and Keller 2003; Sikkens et al. 2010). In this study, we focused exclusively on the effects of two microbial enzyme preparations for their capacity to degrade alimentary proteins in comparison with pancreatin. Indeed, in severe pancreatitis, high mortality and morbidity rates are related to the failure of establishing a positive nitrogen balance (Ioannidis et al. 2008). Because the time-course of protein hydrolysis is known to be partly related to the specificity of enzymes for target amino acids (Valette et al. 1993); we have used mass spectrometry to determine the overall amino acid cleavage sites of microbial enzymes using whole casein as a substrate, and we have made comparison with the cleavage sites of proteases in pancreatin.

Materials and methods

Material

Protein substrates (whole caseins and soya proteins) and pancreatin were obtained from Solvay Pharmaceuticals (Germany). NS4 and NS5 were obtained from Novozymes (Denmark). The protease activities in pancreatin was equal to 4275 Pharmaceutical European (PE) units per g solid. All other chemicals were purchased from Sigma Aldrich. Filters used for separation of proteins and peptides were obtained from Millipore (Amicon Ultra) and gel electrophoresis system was from Biorad.

Preparation of the microbial enzyme solutions NS47024 (NS4) and NS47025 (NS5)

NS 4 proteases were prepared from *Nocardiopsis prasina* and NS 5 proteases from *Bacillus subtilis* (Nedkov et al. 1983). NS 4 proteases were dissolved in a 50-mM boric acid solution containing 10 mM acetic acid, 1 mM $CaCl_2$ and 250 mM NaCl (pH 4.5). NS 5 proteases were dissolved in a 50 mM boric acid solution containing 5 mM dimethyl glutaric acid, 1 mM $CaCl_2$, 100 mM NaCl (pH 6.0). Protease activities in NS4 and NS5 solutions were 212 and 512 PE units/g liquid, respectively.

In vitro protein digestion

All studies were performed with a fixed amount of substrate (i.e. 18 mg casein or soya protein) incubated at 37 °C in 1.5 mL medium containing 43 mM NaCl, 7.3 mM disodium tetraborate, 171 mM boric acid and 1 mM $CaCl_2$ (pH 7.4) in the absence or presence of an increasing amount of the different enzyme sources. Digestion of protein substrates was performed during various periods of time up to 2 h. At the end of the incubation, proteolytic digestion was halted by mixing with 500 µL ice-cold trichloroacetic acid (TCA, final concentration 25 %). This TCA concentration is suitable to precipitate proteins and polypeptides. The mixture was left for 1 h at 4 °C to allow complete protein/polypeptide precipitation. Then, the extracts were centrifuged 5 min at 10,000*g*. In order to avoid any acid-induced protein hydrolysis during storage (Zellner et al. 2005), the precipitates were rapidly washed in acetone to remove TCA. Then supernatants were discarded and the pellets containing proteins and peptides were washed two times with ice-cold acetone containing dithiothreitol (DTT, 80 mM). The pellets were recovered by centrifugation and dried at room temperature. The pellets were finally resuspended in NaOH solution (0.25 M) and protein quantity was determined using the Lowry method (Lowry et al. 1951) with BSA used as a standard. In order to study the effects of the pH on protease activities, protein hydrolysis were monitored in incubation medium with pH equal to 3 and 7.4 and also in incubation medium shifted from 3 to 7.4.

Electrophoresis

Protein samples were solubilised in denaturing Laemmli buffer (Tris 0.25 M, SDS 4 %, DTT 4 % and bromophenol blue 0.002 %) before loading an aliquote (50 µg protein) on ready XT precast gel 18 % (Biorad) for migration in running buffer (Tris/glycine/SDS). Molecular weight (MW) markers (Fermentas) were run in parallel to determine the MW of substrates and products in the protein mixture (range between 170 and 11 kDa).

Filtration

The range of MW of digestion products was determined using Amicon filters (Millipore). These latter are able to retain proteins and peptides above a specified MW. For these experiments, protein hydrolysis was stopped by addition of formic acid 0.7 M at 4 °C. Formic acid was used because of its compatibility with the columns used for Liquid Chromatography–Mass Spectrometry (LC/MS)/MS analysis. All the incubation media containing digestion products were first filtered on Amicon filters 10 kDa. These filters retain proteins with MW up to 10 kDa in the retentate

phase. Ultrafiltrate 10 was then filtered on filter 3 kDa. The resulting retentate and ultrafiltrate fractions were recovered and protein quantity was assayed using the Lowry procedure.

LC/MS/MS analysis

All digest ultrafiltrates at 3 kDa threshold were diluted a hundred times before the analysis by mass spectrometry. LC–MS/MS analysis was performed on an Ultimate 3000 nano-HPLC system (Dionex, Voisins le Bretonneux, France) connected by nanospray ion-source to LTQ-Orbitrap Discovery mass spectrometer (Thermo Fisher). Peptides were separated in 50 min at 300 nL/min using a nano-column Pepmap C18 (0.075 × 15 cm, 100 Å, 3 µm, Dionex) with an acetonitrile gradient (1.6–29 %) connected to the mass spectrometer by a liquid junction with a spray voltage of 1.2 kV applied to a non-coated capillary probe (PicoTip emitter 10 µm i.d.; New Objective) and analysed online by the mass spectrometer. Peptide ions were automatically analysed by the data-dependent method: full MS scan (m/z 300 to 1600) on Orbitrap analyser and MS/MS on the four most abundant precursors on the LTQ linear ion trap. In the present study, only the mono-, doubly- and triply charged peptides were subjected to MS/MS experiments with an exclusion window of 1.5 min, with classical peptide fragmentation parameters as follows: Qz = 0.22, activation time = 50 ms and 35 % collision energy. The peptide masses were measured in the Orbitrap mass analyser with activation of lock mass option that enhanced the system accuracy. Protein identification was performed by mass matching approach using X!tandem (version 2011.12.01.1). In a first analysis, the proteins present in the casein sample were identified and validated in a large *Bos Taurus* protein database (33889 proteins). The analysis of proteolysis was made using a short database corresponding to the proteins that were identified in the first analysis (26 proteins). The X!tandem search parameters included no cleavage specificity, variable oxidation of methionine and phosphorylation of serine and threonine. The mass tolerance was fixed to 10 ppm for precursor ions and 0.5 Da for the ion daughter. We validated the search result using a filter applied at the peptide level to the value calculated by X!tandem, i.e. less than 0.03. We thus have estimated the False Positive Rate (FPR) to 0.15 % by including to the analysis a reverse of the casein database. We used a home-made perl script (written by Benoît Valot at INRA/PAPPSO in Gif sur Yvette, France) to calculate the frequencies of cleavage after each amino acid. We normalised the frequencies of cleavages after each amino acid for all caseins according to the following formula: Amino acid × (%) = $\sum$ amino acids × (cleavage in all peptides) × 100/$\sum$ amino acids × in whole casein; × representing a single amino acid.

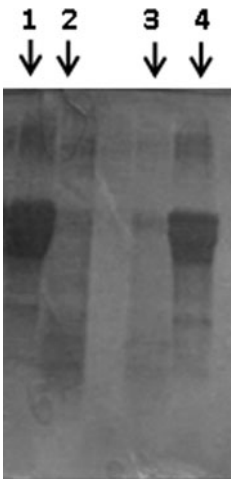


Fig. 1 Gel electrophoresis of casein incubated for 60 min at 37 °C in the absence of enzyme (control, *line 1*) or with 40 µg pancreatin (*line 2*), 400 µg pancreatin (*line 3*) or 0.4 µg pancreatin (*line 4*)

Data analysis

The results are expressed as the mean value ± SEM and represent between 3 and 12 independent experiments. The statistical difference between mean amounts of proteins and polypeptides measured in the TCA-precipitable material and in polypeptides produced from caseins and soya proteins through the catalytic action of pancreatin, NS4 or NS5 was assessed by Student's *t* test. The differences were considered significant when $p < 0.05$.

Results

Effect of pancreatin on the digestion of casein and soya

As indicated on Fig. 1, after 1 h of incubation at 37 °C of 18 mg casein in the presence of 400 µg pancreatin, the casein bands completely disappeared on the electrophoresis gel. The digestion was also almost complete in the presence of 40 µg pancreatin; but with 0.4 µg pancreatin, the digestion of casein was not clearly detectable. Even after 2 h of incubation in the presence of 0.4 µg pancreatin, the digestion of casein could not be evidenced (data not shown). Therefore, we choose for next time-course experiments the 40 µg pancreatin dose (which correspond to 0.171 PE unit). In such condition, the casein digestion appears partial after 30 min with the apparition of bands with smaller molecular weights (data not shown).

Comparison of pancreatin and microbial enzyme preparations for the digestion of casein: effects of pH

As indicated on Fig. 2, the two microbial enzyme preparations NS4 and NS5 (0.171 PE unit each) were both efficient for casein digestion at pH 7.4 as assessed using electrophoresis gel. When the pH of the incubation medium

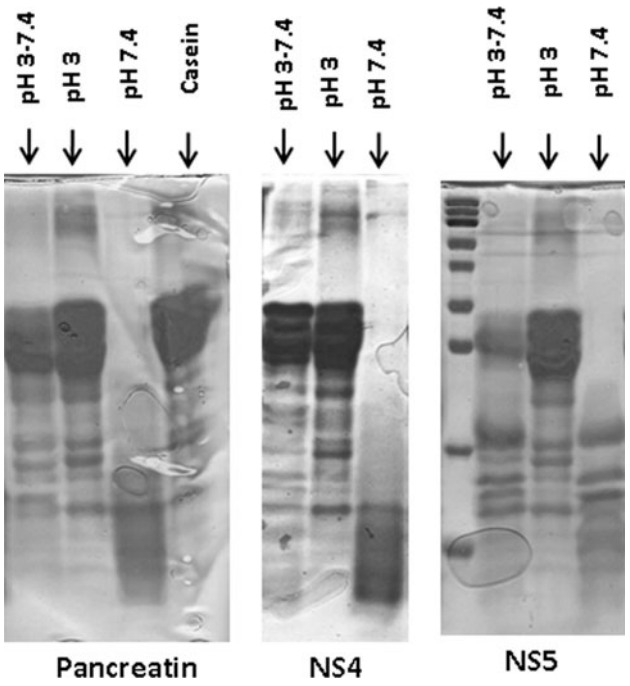


Fig. 2 Gel electrophoresis of caseins incubated at 37 °C for 60 min in the absence of enzyme (casein), or in the presence of 0.171 PE unit (40 µg) pancreatin, or 0.171 PE unit NS4, or 0.171 PE unit NS5 at pH 3.0 or pH 7.4 or at pH 3.0 and then at pH 7.4 (pH 3–7.4)

was equal to 3.0, there was no apparent casein digestion. However, when the pH was switched from 3.0 to 7.4, there was a very slight degradation of the caseins for pancreatin and both NS enzyme preparations.

Comparison of pancreatin and microbial enzyme preparations for the digestion of casein and soya protein

Using the measurement of the remaining TCA-precipitable products after incubation for various periods of time with either pancreatin or NS4 and NS5 (0.171 PE unit each) together with 18 mg caseins, we found that the time-course of proteolytic activities of the three enzyme preparations was not vastly different when comparing NSs and pancreatin (Fig. 3a). When using whole soya proteins (18 mg also), we found that although there was a modest proteolytic activity of three enzyme preparations after 30 min of incubation, NS5 and NS4 were more efficient than pancreatin for soya protein digestion after 60 and 120 min of incubation (Fig. 3b).

Pancreatin was more efficient than NS4 and NS5 for generating peptides with molecular weight less than 3 kDa from 18 mg caseins (Fig. 4a). When polypeptides with molecular weight between 3 and 10 kDa were measured after incubation of caseins with the three enzyme preparations, the amounts of these polypeptides were not different according to the preparation used (Fig. 4b). When the same experiments were performed with 18 mg of soya proteins, the release of peptides with molecular weight less than 3 kDa was again not vastly different between pancreatin and the 2 NSs (Fig. 4c). The release of polypeptides with molecular weight between 3 and 10 kDa from soya proteins was similar after 30 and 60 min of incubation whatever the enzyme preparations used (Fig. 4d). After 120 min of incubation, the amounts of such peptides were higher with NS4 and NS5 than with pancreatin.

Liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS) analysis of peptides generated through pancreatin and NS4 and NS5 treatments

As indicated on Fig. 5a, b, c, after incubation of 18 mg whole caseins in the presence of 0.171 PE unit of pancreatin or NS4 or NS5 at 37 °C, an important number of peptides were found to be generated in all three cases. The complete list of the peptides identified, together with mass spectrometric data are given in Online resource 1. These peptides were produced in all cases in a time-dependent manner (Fig. 5a–c). With regard to the ionic current measurement, the pattern of peptides generated from caseins was very reproducible since they were almost superimposable when obtained after 60 min of incubation from three independent experiments (data not shown). From these experiments, it was determined that the total number of peptides generated from the various caseins (alphaS1, alphaS2, beta and kappa) were ranging from 20 to more than 80 according to the enzymatic preparation used (Fig. 6). Regarding the total number of distinct identified peptides, pancreatin appeared to be more efficient than NS5 and NS4 for casein digestion in our experimental conditions. As indicated on Fig. 5a, b, c, the casein ionic profile distribution was very different after hydrolysis in the presence of pancreatin or NS4 or NS5 indicating heterogeneity of the peptides produced from the three enzyme preparations.

As shown on Fig. 7, the cleavage sites on whole caseins of pancreatin or NS4 or NS5 were rather different. As expected, pancreatin displayed tryptic cleavage sites (Arg/R and Lys/K sites), chymotrypsic cleavage sites (Phe/F, Trp/W, Tyr/Y, Leu/L…) and elastase cleavage site (Ala/A…). NS4 and NS5 were characterised by lower tryptic activities than pancreatin but by relatively high chymotryptic activity. Elastase-like activity was much lower in NS4 and NS5 than in pancreatin (Fig. 7). A weak activity of pancreatin, NS4 and NS5 enzymatic preparations on the amino acids Pro/P, Asp/D and Glu/E was observed. When comparing cleavage sites of NS4 and NS5, NS5 appears to be more efficient than NS4 for cleavage at His/H and at Tyr/Y sites. As a matter of comparison, the composition of amino acids in whole casein is given in Table 1.

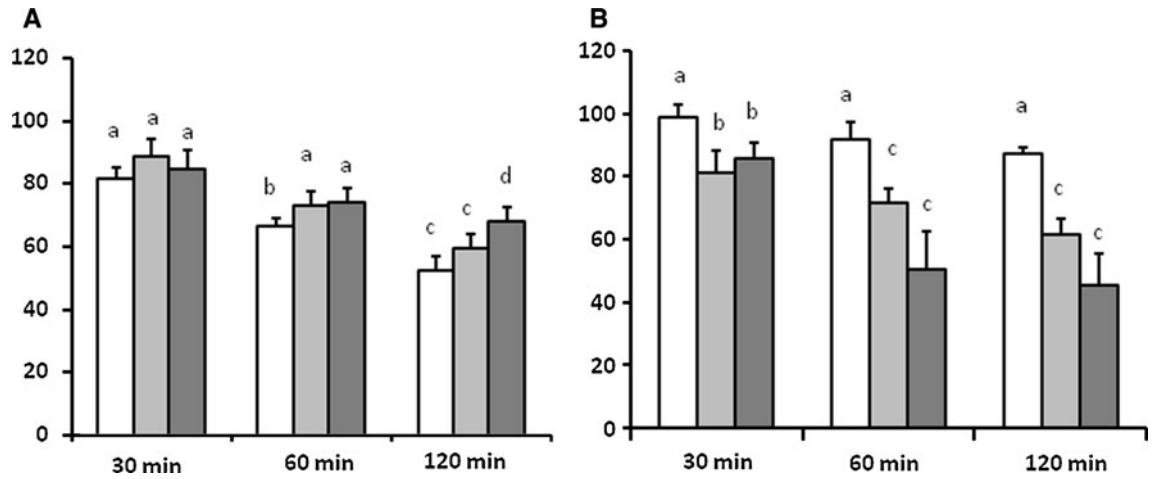


Fig. 3 **a** Trichloroacetic acid-precipitable material originating from casein incubated with pancreatin (*white bars*), NS4 (*light grey bars*) or NS5 (*dark grey bars*). Caseins were incubated at 37 °C for different periods of time in the presence of enzymatic preparations (0.171 PU) and proteins/polypolypeptides precipitated by TCA was measured. **b** TCA-precipitable material originating from soya proteins incubated with 0.171 PE unit of pancreatin, NS4 or NS5. Values (mean ± SEM) represent at least four independent experiments. Histogram bars with different letters are significantly different ($p < 0.05$)

Discussion

Pancreatin is a complex mixture of numerous enzymatic degrading activities including amylase, lipase, RNAse, DNase, phosphatase and various protease activities. These protease activities include trypsin-like activities which cleave protein at the cationic amino acid Arg and Lys sites, chymotryptic like activities which cleaves at aromatic and branched-chain amino acid sites (Tyr, Phe, Trp, Leu), carboxypeptidase A which cleaves at all amino acid sites (except Asp, Glu, Arg, Lys) and elastase which cleaves at Ala site. Our LC/MS/MS analysis indicates clearly that pancreatin is characterised by major proteolytic activities of trypsic, chymotryptic and elastase types as described in the literature (Lauwers and Ruyssen 1968). Although caseins are rich in the acidic amino acid glutamate and also in proline (Table 1), our analysis following incubation with pancreatin, NS4 and NS5 has detected very little amounts of peptides which would have been produced through cleavage of caseins at these amino acid sites. NS4 and NS5 display lower tryptic activity than pancreatin and NS4 has lower activity for cleavage at the Tyr, Trp and His sites than pancreatin and NS5. NS5, and to a lower extent NS4, display major chymoptryptic activities. NS4 and NS5 display a much weaker cleavage activity than pancreatin for the amino acids glycine, isoleucine, valine and alanine. In contrast, NS4 and NS5 were characterised by a stronger activity than pancreatin for cleavage at the methionine sites. Last, carboxypeptidase activities appeared likely weaker in NS4 and NS5 than in pancreatin, even if this latter point is difficult to quantify due to the broader enzymatic specificity of these enzymatic activities.

The peptide diversity was overall more important on pancreatin hydrolysate than on NS4 and NS5 hydrolysates. When taking into account the total number of peptides generated from caseins, pancreatin appears more efficient that NS5 and NS4 (in that order) for digestion. However, the efficiency of NS5 for casein digestion remains not vastly different than the pancreatin efficiency for alphaS1, alphaS2, beta and kappa casein digestion. NS5 enzymatic preparation appears markedly less efficient than NS4 for alphaS2 and kappa casein digestion.

Another important conclusion which can be made from digest profiling is that NS4 and NS5 are generating peptides of much different chemical natures, likely because they do not share common mechanisms of action for protein digestion. Indeed, the peptidic patterns obtained after incubation with pancreatin, NS4 or NS5 were all much different.

All enzymatic preparations (i.e. pancreatin, NS4 and NS5) were all unable to digest casein at pH3. We can thus presume from these in vitro data that these enzyme mixtures would not digest alimentary and endogenous proteins in the stomach lumen. Furthermore, only very partial restoration of the capacity of enzymatic preparations to digest caseins was recovered when the pH was increased from 3 to neutrality. This indicates that NS4 and NS5 are very likely markedly inactivated at acidic pH, raising the view that they should be tested in vivo in a gastric-resistant form.

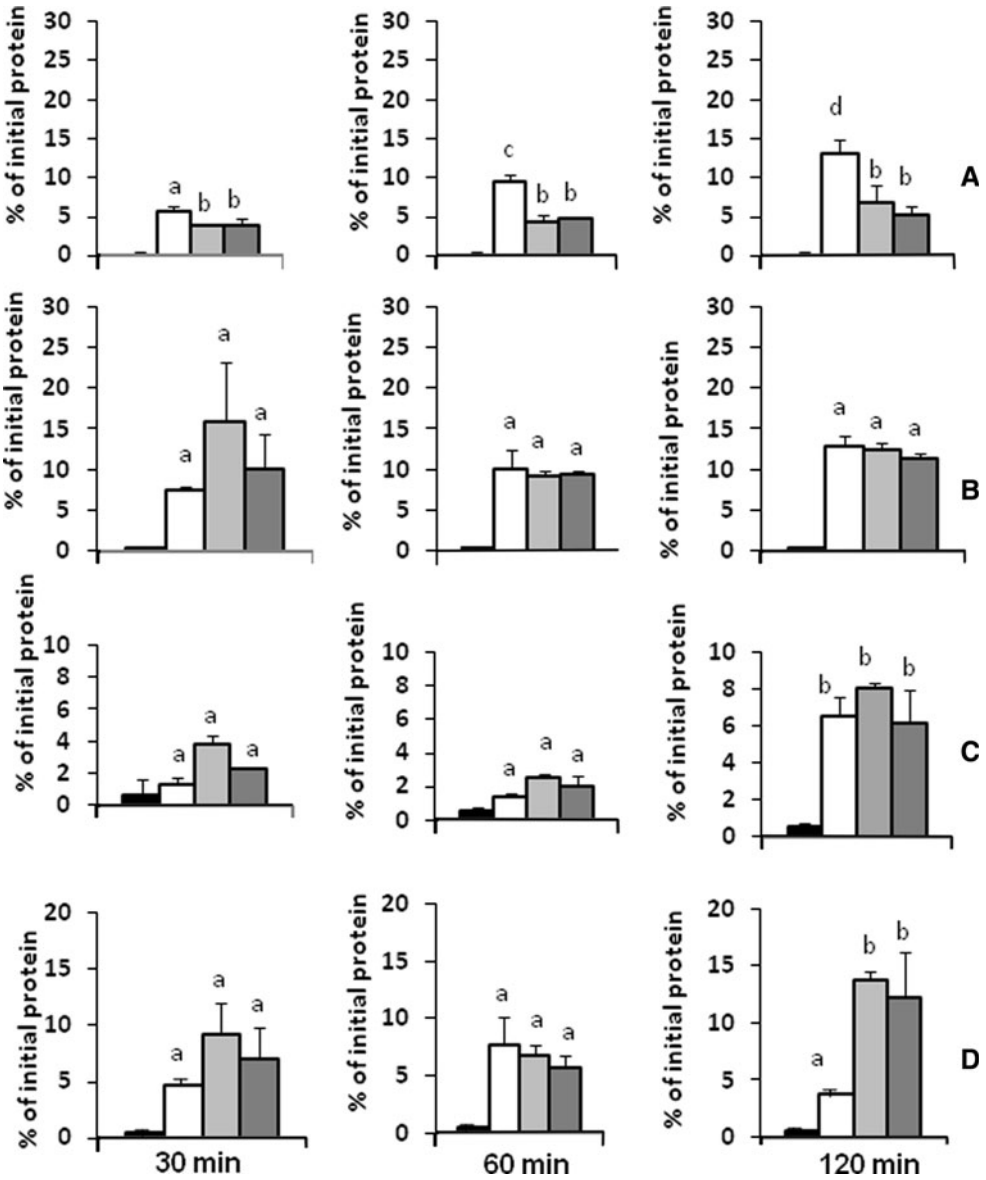


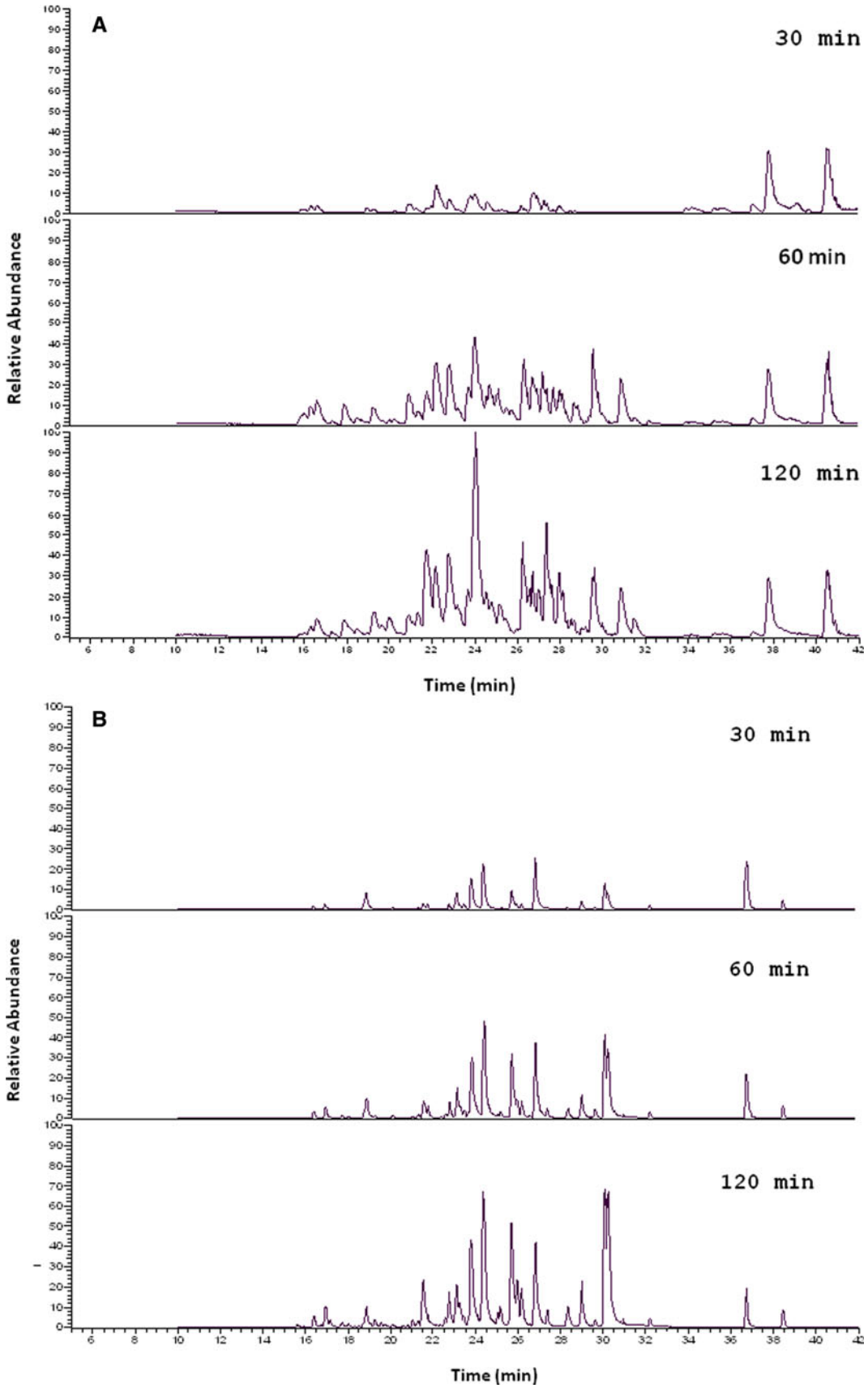
Fig. 4 **a** Measurement of the amounts of peptides with molecular weight less than 3 kDa generated after incubation of caseins without (*black bars*) or with pancreatin (*white bars*), NS4 (*light grey bars*) or NS5 (*dark grey bars*). Caseins were incubated at 37 °C for different periods of times with 0.171 PE unit of pancreatin, NS4 or NS5, and peptides were recovered through centrifugation on filters. **b** Measurement of polypeptides with MW between 3 and 10 kDa after incubation of caseins with 0.171 PE unit of pancreatin, NS4 or NS5. **c** Measurement of peptides with MW less than 3 kDa generated after incubation of soya proteins with 0.171 PE unit of pancreatin, NS4 or NS5. **d** Measurement of polypeptides with MW between 3 and 10 KDa after incubation of soya proteins with 0.171 PE unit of pancreatin, NS4 or NS5. Values (mean ± SEM) represent at least four independent experiments. Histogram bars with different letters in a line are significantly different ($p < 0.05$)

NS4 and NS5 enzymatic preparations were found able to digest soya proteins in a time-dependent manner. NS5 was found to be more efficient than NS4 for such a process, and both of them were more efficient than pancreatin, a result which is in contrast with the data obtained from casein digestion.

Both NS4 and NS5 preparations were able to produce small peptides (less than 3 kDa) and polypeptides with higher molecular weights (between 3 and 10 kDa) from caseins and soya proteins. However, pancreatin appears more efficient than NS4 and NS5 for small peptide release from caseins, a result which is in accordance with the LS/MS/MS data. In contrast, NS4 and NS5 preparations were as efficient as pancreatin for the release of small peptides from soya proteins, and, after 2 h of incubation, more efficient than pancreatin for the release of polypeptides with molecular weight between 3 and 10 kDa, reinforcing the view that NS4 and NS5 efficiency towards alimentary proteins, when compared with pancreatin efficiency, is different according to the chemical nature of the substrates.

Taken as a whole, our in vitro experiments indicate that NS5, and to a lesser extent NS4, represent microbial enzymatic preparation with relatively high chymotryptic activities but lower tryptic activities when compared with

Fig. 5 **a** LC–MS mass signal detection after incubation of caseins with pancreatin. Caseins were incubated at 37 °C for different periods of time with 0.171 PE unit of pancreatin. **b** LC–MS mass signal detection after incubation of caseins with NS4. Caseins were incubated at 37 °C for different period of times with 0.171 PE unit of NS4. **c** LC–MS mass signal detection after incubation of caseins with NS5. Caseins were incubated at 37 °C for different period of times with 0.171 PE unit of NS5. These mass signal patterns are representative of three independent experiments ▶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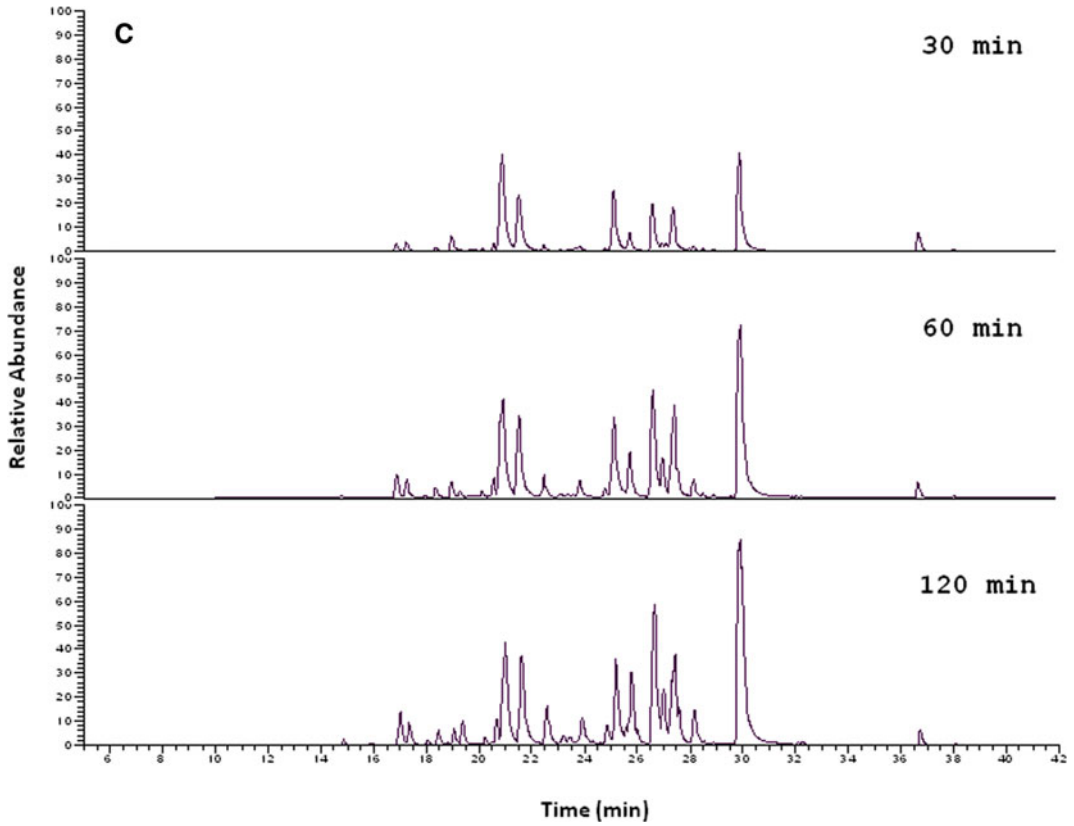

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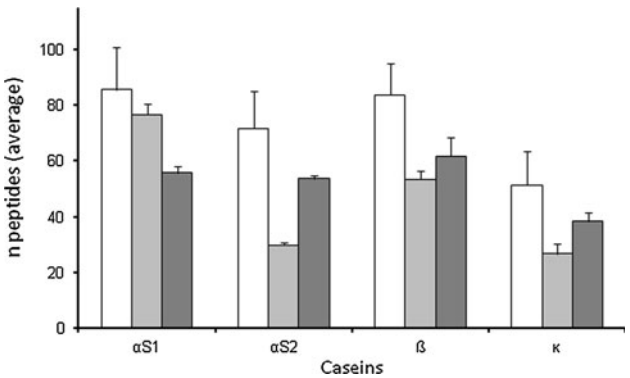

Fig. 6 Number of peptides identified after incubation of caseins with pancreatin (*white bars*), NS4 (*light grey bars*) or NS5 (*dark grey bars*). Caseins were incubated for 60 min at 37 °C and following LC–MS analysis, the numbers of peptides generated from the various forms of caseins were calculated. Values (mean ± SEM) represents three independent experiments

pancreatin. It is worth noting that in the present study, we compared mixtures of protease activities. Then, the results obtained should be considered as representing the overall action of the different proteases in the different preparations. With this reservation in mind, it appears clearly from our results that both enzymatic activities and proteolytic

Table 1 Amino acid composition of whole caseins

Amino-acids		Composition (g/100 g)
Ala	A	2.8
Cys	C	0.4
Asp	D	3.9
Glu	E	9.4
Phe	F	4.9
Gly	G	1.9
His	H	2.8
Ile	I	4.9
Lys	K	7.5
Leu	L	8.8
Met	M	2.6
Asn	N	2.6
Pro	P	10.8
Gln	Q	11.2
Arg	R	3.4
Ser	S	5.1
Thr	T	4.1
Val	V	6.0
Trp	W	1.2
Tyr	Y	5.1

These data are from Li et al. (2011)

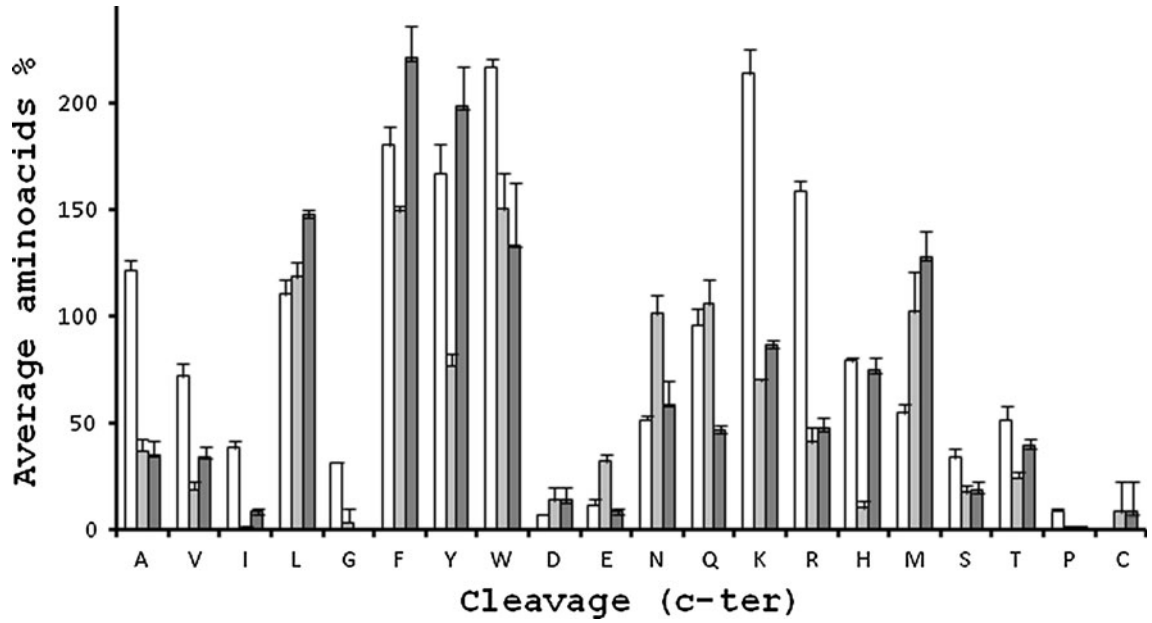

Fig. 7 Cleavage sites in the whole caseins according to pancreatin (*white bars*), NS4 (*light grey bars*) or NS5 (*dark grey bars*) proteolytic enzymatic activities

specificity are much different between NS4/NS5 and pancreatin. Interestingly, NS4 and NS5 are able to digest very different alimentary proteins like caseins and soya proteins.

These microbial preparations may thus represent an alternative way to increase the rate of absorption of nitrogen-containing compounds in severe pancreatic insufficiency. According to the Codex alimentarius from the Food and Agriculture Organisation and to the Food and Drug Administration, respectively, *Nocardiopsis prasina* and *Bacillus subtilis* are both considered as GRAS (Generally Recognised As Safe). New experiments are obviously needed to test in vivo if these enzymatic preparations (in a gastro-resistant form) are efficient in terms of protein, lipid and carbohydrate digestion in animal models of severe pancreatic insufficiency. The safety of such preparations needs also to be evaluated notably in terms of gastrointestinal physiology and of possible activation of the intestinal mucosa-associated immune system.

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