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(54) Titre : NOUVELLES PROTEASES CAPABLES D'HYDROLYSER DES PEPTIDES ET PROTEINES DU GLUTEN A
UN PH ACIDE PROVENANT DE L'ACTINOMYCETE ACTINOALLOMURUS
(54) Title: NEW PROTEASES ABLE TO HYDROLYZE GLUTEN PEPTIDES AND PROTEINS AT ACIDIC PH, FROM
THE ACTINOMYCETE ACTINOALLOMURUS

(57) **Abrégé/Abstract:**

The invention relates to a new family of proteolytic enzymes having the ability to hydrolyze at a pH between 3 and 8 gluten oligopeptides which are resistant to cleavage by gastric and pancreatic enzymes and whose presence in the intestinal lumen results in toxic effects. The enzymes have been identified as endopeptidases of the S8/S53 family and are produced by an Actinoallomurus strain. The object of the invention includes also methods for producing enzymes composition comprising the endopeptidases by cultivation of native Actinoallomurus strains, mutants thereof, or recombinant host cells comprising nucleic acids codifying for the endopeptidases. Said nucleic acids constitute a further object of the invention. The enzyme compositions comprising at least one endopeptidase of the invention are useful for the treatment and/or prevention of celiac sprue, dermatitis herpetiformis and any other disorder associated with gluten intolerance as ingredients of pharmaceutical formulations or as additives of foods and drinks.



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(54) Title: NEW PROTEASES ABLE TO HYDROLYZE GLUTEN PEPTIDES AND PROTEINS AT ACIDIC PH, FROM THE ACTINOMYCETE ACTINOALLOMURUS

(57) Abstract: The invention relates to a new family of proteolytic enxymes having the ability to hydrolize at a p H between 3 and 8 gluten olygopeptides which are resistant to cleavage by gastric and pancreatic enzymes and whose presence in the intestinal lumen results in toxic effects. The enzymes have been identified as endopeptidases of the S8/S53 family and are produced by an Actinoal-lomurus strain. The object of the invention includes also methods for producing enzymes composition comprising the endopepti-dases by cultivation of native Actinoallomurus strains, mutants thereof, or recombinant host cells comprising nucleic acids codifying for the endopeptidases. Said nucleic acids constitute a further object of the invention. The enzyme compositions comprising at least one endopeptidase of the invention are useful for the treatment and/or prevention of celiac sprue, dermatitis herpetiformis and any other disorder associated with gluten intolerance as ingredients of pharmaceutical formulations or as additives of foods and drinks.



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NEW PROTEASES ABLE TO HYDROLYZE GLUTEN PEPTIDES AND PROTEINS AT ACIDIC PH, FROM THE ACTINOMYCETE *ACTINOALLOMURUS*.

5 Field of the Invention

The present invention relates to a novel endopeptidase family having unique catalytic properties that render it able to degrade large polypeptides, including those rich in proline. The present invention further relates to methods for producing the enzyme composition as well as pharmaceutical composition and a food supplement containing the enzyme
10 composition and its use in the degradation of polypeptides.

Background of the Invention

Celiac Disease (CD) is a chronic gastrointestinal tract disorder in which ingestion of gluten, present in food products made from wheat, rye, barley and their cross-related varieties, leads to damage of the small intestinal mucosa by an autoimmune mechanism in genetically
15 susceptible individuals (Green P.H.R., Cellier C. "Celiac Disease" N. Engl. J. Med., 2007, 357,1731-1743; Kagnoff M.F. "Celiac disease: pathogenesis of a model immunogenetic disease" J. Clin. Invest., 2007, 117, 41-9). Mechanisms through which gluten induces its pathogenic effects have been explained in recent years. Both innate and adaptive immunity mechanisms are involved and are responsible for the ultimate mucosal damage.

20 Gluten consists of gliadins and glutenins, the water/salt insoluble fractions of storage proteins present in cereal grains. A gluten network is created by interaction between the two proteins when flour and water are mixed in the preparation of dough.

Once ingested, gluten goes towards a partial digestion by gastric-pancreatic and brush-border proteolytic enzymes which results in many peptides of different length (few to more
25 than 30 aminoacids) which are resistant to further digestion due to the high content of proline residues as many proteases are unable to cleave peptide bonds located at N- or C-termini of proline (Hausch F., Shan L., Santiago N.A., Gray G.M., Khosla C. "Intestinal digestive resistance of immunodominant gliadin peptides". Am. J. Physiol. Gastrointest. Liver Physiol., 2002, 283, 996-1003; Shan L., Molberg O., Parrot I., Hausch F., Filiz F.,
30 Gray G.M., Sollid L.M., Khosla C. "Structural basis for gluten intolerance in celiac sprue" Science, 2002, 297, 2275-2279). The lack of proline-specific cleaving enzymes is not a specific enzyme deficiency in celiac subjects, as suggested in the past, but is proper of the mammalian digestive apparatus which has not evolved to consume proteins with so high proline content in its diet.

Undigested gluten peptides can pass the epithelial barrier through mechanisms not yet clearly explained, although a zonulin-mediated paracellular passage and a transcellular way, *via* transcytosis and retrotranscytosis, have been recently proposed (Drago S., El Asmar R., Di Pierro M., Grazia Clemente M., Tripathi A., Sapone A., Thakar M., Iacono G., Carroccio A., D'Agate C., Not T., Zampini L., Catassi C., Fasano A. “Gliadin, zonulin and gut permeability: Effects on celiac and non-celiac intestinal mucosa and intestinal cell lines” Scand. J. Gastroenterol., 2006, 41, 408-19; Schumann M., Richter J.F., Wedell I., Moos V., Zimmermann-Kordmann M., Schneider T., Daum S., Zeitz M., Fromm M., Schulzke J.D. “Mechanisms of epithelial translocation of the alpha(2)-gliadin-33mer in coeliac sprue” Gut, 10 2008, 57, 747-754; Matysiak-Budnik T., Moura I.C., Arcos-Fajardo M., Lebreton C., Ménard S., Candalh C., Ben-Khalifa K., Dugave C., Tamouza H., van Niel G., Bouhnik Y., Lamarque D., Chaussade S., Malamut G., Cellier C., Cerf-Bensussan N., Monteiro R.C., Heyman M. “Secretory IgA mediates retrotranscytosis of intact gliadin peptides via the transferrin receptor in celiac disease” J. Exp. Med., 2008, 205, 143-154).

15 Once in the *lamina propria* (LM), deamidation of glutamine to glutamate residues by tissue transglutaminase (tTG, the autoantigen in CD) reinforces their presentation to DQ2 or DQ8 CD4+ T cells (Molberg O., McAdam S., Lundin K.E., Kristiansen C., Arentz-Hansen H., Kett K., Sollid L.M. “T cells from celiac disease lesions recognize gliadin epitopes deamidated in situ by endogenous tissue transglutaminase” Eur. J. Immunol., 2001, 31, 20 1317-23) producing a pro-inflammatory response with interferon-gamma (IFN- γ) as main cytokine effector. Several gluten peptides have also been shown to cause mucosal damage independently from a specific recognition by CD4+ T-lymphocytes, but inducing an innate immune response by up-regulating the expression of IL-15, cyclo-oxygenase-2 and the activation markers CD25 and CD83 in LM mononuclear cells: among them, the best 25 characterized are peptides p31-43/49 of the α 1-gliadins (PGQQQPFPPQQPY/PQPQPF) (Ciccocioppo R., Di Sabatino A., Corazza G.R. “The immune recognition of gluten in coeliac disease” Clin. Exp. Immunol., 2005, 140, 408-16).

CD is estimated to affect about 1% of both European and North American population, with a study from Finland showing increasing rates (1:47) in elder people (Vilppula A., Kaukinen 30 K., Luostarinen L., Krekelä I., Patrikainen H., Valve R., Mäki M., Collin P. “Increasing prevalence and high incidence of celiac disease in elderly people: a population-based study” BMC Gastroenterol., 2009, 29, 9-49). However, many studies indicate that CD is diffused all over the world with similar prevalence values (Barada K., Bitar A., Mokadem M.A., Hashash J.G., Green P. “Celiac disease in Middle Eastern and North African countries: a

new burden?” World J. Gastroenterol., 2010, 16, 1449-57; Dalgic B., Sari S., Basturk B., Ensari A., Egritas O., Bukulmez A., Baris Z., Turkish Celiac Study Group “Prevalence of celiac disease in healthy Turkish school children” Am. J. Gastroenterol., 2011, 106, 1512-7; Makharia G.K., Verma A.K., Amarchand R., Bhatnagar S., Das P., Goswami A., Bhatia V., Ahuja V., Datta Gupta S., Anand K. “Prevalence of celiac disease in the northern part of India: a community based study” J. Gastroenterol. Hepatol., 2011, 26, 894-900; Wang X.Q., Liu W., Xu C.D., Mei H., Gao Y., Peng H.M., Yuan L., Xu J.J. “Celiac disease in children with diarrhea in 4 cities in China” J. Pediatr. Gastroenterol. Nutr., 2011, 53, 368-70).

No therapies are available at this time and the only remediation to disease is a strict, lifelong gluten-free diet necessary to prevent not only CD specific mucosal damage and consequent malabsorption-related disorders (like iron-deficient anemia or osteoporosis) but also other autoimmune diseases which have been associated with CD, like type 1 diabetes and autoimmune thyroiditis, or heavier complications like enteropathy-associated T-cell lymphomas.

Total avoidance of gluten (safe gluten intake threshold is generally indicated in 50 mg/day, although 10 mg/day is considered more safe) maintains CD in remission in all but a small percentage of patients (2-5%) which suffer of a non-responsive form. Such a diet is, however, strongly demanding for patients, which are restricted in their common activities and suffer from social isolation. The use of gluten as additive in food processes is widespread and is the main cause of unaware ingestion of gluten, making this diet really difficult to maintain.

For these reasons it would be strongly welcome by CD patients any alternative allowing them to assume in their daily diet at least minimal amounts of gluten.

The use of exogenous proteolytic enzymes for gluten detoxification is one of the most promising strategies for CD treatment. Different ways of application can exploit these enzymes potential: treatment of gluten containing flours, before or during dough fermentation, thus going towards the production of “novel food”, as well as concomitant consumption of gluten and suitable proteolytic enzymes, thus going as “food supplement”, similarly to the use of lactase for lactose intolerance. Necessarily, different enzyme properties are requested to meet the different objectives.

The enzymatic approach for CD treatment is based on the demonstration by Shan *et al.* (Science, 2002) of microbial enzymes’ ability to cleave gluten peptides on specific residues and remove toxic /immunotoxic specific peptide sequences. In particular, they showed that an exogenous prolyl-specific endoprotease derived from *Flavobacterium meningosepticum*

(FM-PEP) resulted helpful in the digestion of gliadin peptides. The addition of a PEP either in vitro in the presence of brush border membrane (BBM) extracts or during in vivo perfusion of rat small intestine caused a rapid degradation of the immunodominant 33-mer peptide ("33-mer") and a loss of its capacity to stimulate gliadin-specific T-cells (Hausch *et al.*, 2002).

Other enzymes of the same family (EC. 3.4.21.26) from other bacterial strains (*i.e.* *Sphingomonas capsulata* and *Myxococcus xanthus*) have been evaluated for this aim by the same authors (Shan L., Marti T., Sollid L.M., Gray G.M., Khosla C. "Comparative biochemical analysis of three bacterial prolyl endopeptidases: implications for coeliac sprue" *Biochem. J.*, 2004, 383, 311-318). Globally, these studies showed substantial differences among the three enzymes with respect to chain-length and subsite specificity and confirmed the potential of oral enzyme therapy, although raised concerns regarding their possible efficacy *in-vivo*, due to restrictions on substrates specificity, pH of activity (optimal activity at almost neutral pH instead of acidic pH as needed to act in the stomach), long time necessary to complete the digestion of toxic peptides and resistance to degradation by pepsin. A combination of two enzymes with gastric activity and complementary substrate specificity was then suggested (Gass J., Bethune M.T., Siegel M., Spencer A., Khosla C. "Combination enzyme therapy for gastric digestion of dietary gluten in patients with coeliac sprue" *Gastroenterology*, 2007, 133, 472-480): PEP from *S. capsulata* associated to EP-B2, the glutamine-specific endoprotease B isoform 2 from *Hordeum vulgare*, a cysteine-protease derived from germinating barley seeds that is activated at acidic pH and by pepsin (Bethune M.T., Strop P., Tang Y., Sollid L.M., Khosla C. "Heterologous expression, purification, refolding, and structural-functional characterization of EP-B2, a self-activating barley cysteine endoprotease" *Chem. Biol.*, 2006, 13, 637-47), showed to be a potentially more potent therapeutic tool. Another study reports that a PEP derived from *Aspergillus niger*, deploying its main activity under acid conditions in the stomach, can start to degrade gliadin before it reached the intestinal lumen (Stepniak D., Spaenij-Dekking L., Mitea C., Moester M., de Ru A., Baak-Pablo R. van Veelen P., Edens L., Koning F. "Highly efficient gluten degradation with a newly identified prolyl endoprotease: implications for coeliac disease" *Am. J. Physiol. Gastrointest. Liver Physiol.*, 2006, 291, G621-9).

These findings have been dealt by several patent documents. In WO2003/068170 (EP572127), inventors claim that administering an effective dose of glutenase to a coeliac or dermatitis herpetiformis patient reduces levels of toxic gluten oligopeptides, thereby attenuating or eliminating the damaging effects of gluten. Further support to this approach is

given in WO2005/107786 (EP1740197), where pharmaceutical formulations of glutenase enzymes for use in the treatment of celiac or dermatitis herpetiformis patients are disclosed. WO2005/027953 (EP16663298) describes a treatment with a new prolyl-specific endoprotease from *Aspergillus niger* (AN-PEP) which resulted helpful in digestion of toxic
5 gluten peptides. WO2005/019251 provides leucine aminopeptidase (LAP) of two different fungal species, *Trichophyton rubrum* and *Aspergillus fumigatus* in combination with dipeptidyl peptidase IV (DppIV). These enzymes have been evaluated for cleavage of the 33-mer under neutral pH conditions since the optimal activity of LAPs was estimated around 7.0 with a range of activity between pH 6 and 8, thus precluding or limiting a
10 possible breakdown of gliadin in the gastric fluid.

It has also been shown that *A. fumigatus* tripeptidyl peptidases can degrade proteins at acidic pH (Reichard U., Lechenne B., Asif A.R., Streit F., Grouzmann E., Jousson O., Monod M. "Sedolisins, a new class of secreted proteases from *Aspergillus fumigatus* with endoprotease or tripeptidyl-peptidase activity at acidic pHs" Appl. Environ. Microb., 2006,
15 72, 1739-48). In WO2011/077359 it is provided a kit composed by a prolyl-protease (AfuS28) and at least one tripeptidyl protease belonging to the family of sedolisin to be used as food supplement useful also for CD treatment.

It is clear that the therapeutic value of an enzyme or enzyme composition for CD treatment is related to the enzymes a) being resistant to degradation by other gastrointestinal enzymes,
20 b) being efficient in the environment where 33-mer and the others toxic peptides are produced, and c) exhibiting rapid and high proteolytic activity toward gluten peptides. These enzymes should be active at acidic pH and should be able to access a complex composition of gluten hindered by other components of normal foodstuffs baked or cooked.

25 Summary of the invention

The Applicants in the present invention have identified a new family of enzymes and provide an enzyme or a composition of this enzyme family having unique catalytic properties.

The Applicants have identified an *Actinoallomurus* sp. as producer of this enzyme family.

30 The endopeptidases of the invention are produced by cultivation of *Actinoallomurus* in an appropriate culture medium, for example containing soya. The strain, originally identified with the Applicant code A8, was isolated from an Italian soil and deposited on June 24, 2011, with the DSMZ,-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, (now Leibniz-Institut DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen

GmbH), Inhoffenstarsse 7b, D-38124 Braunschweig, Germany, under the provision of the Budapest Treaty. The strain was accorded accession number DSM 24988.

The endopeptidases of the present invention are able to hydrolyze certain gluten oligopeptides, which are resistant to cleavage by gastric and pancreatic enzymes and whose presence in the intestinal lumen results in toxic effects. The enzymatic treatment can remove such peptides and their toxic effects; it can degrade gliadin and so detoxify gluten without any PEP addition. The peptidases of the invention have a wide range of pH activities and are able to exert proteolytic activity from pH3 to pH8. Such activity will be defined as glutenase activity.

These glutenase enzymes provide methods for preventing the symptoms of Celiac Sprue and/or dermatitis herpetiformis by decreasing the levels of toxic gluten oligopeptides in foodstuffs, either prior to or after ingestion by a patient. These enzymes can be used together with other proteolytic enzymes such as proteinases and aminopeptidases to effectively produce protein hydrolyzates used for foods and drinks, and medicines.

These enzymes are secreted by different *Actinollomurus* strains by cultivating them in suitable media and their activity can be evaluated by using both chromogenic substrates and zymographic analysis.

Furthermore, the enzymes of the invention can be produced into host cells by introducing a nucleic acids encoding for these enzymes, cultivating the cells in a culture medium under conditions suitable for producing and recovering the enzymes.

Description of the drawings

Figure 1: Phylogenetic tree inferred from the sequences of glutenases of the invention and the sedolisin or kumamolisin proteases.

Protein sequences were aligned with CLUSTALW program and the maximum likelihood tree with bootstrap values was calculated using MEGA5 software. *Actinoallomurus* sp. endopeptidases are in bold. SedE was used as outgroup. The tree was then visualized with MEGA5 software. Bootstrap values equal or higher than 50% are indicated at nodes. The scale bar indicates the number of amino acid substitutions per site.

SedE = sedolisin E from *A. fumigatus* Af293 (EAL86850); SedB = sedolisin B from *Aspergillus fumigatus* Af293 (CAE17674); TPPa = tripeptidyl peptidase A from *A. oryzae* RIB40 (BAC56232); SedD = sedolisin D from *A. fumigatus* Af293 (CAE17675); SedC = sedolisin C from *A. fumigatus* Af293 (CAE46473);

SedA = sedolisin A from *A. fumigatus* Af293 (CAE51075); KumaA = Kumamolisin-As from *Alicyclobacillus sendaiensis* (Q8GB88); KumaB = Kumamolisin from *Bacillus* sp. MN-32 (Q8RR56); sedolisinA = sedolisin from *Pseudomonas* sp. 101 (P42790); sedolisinB = sedolisin-B from *Xanthomonas* sp. T-22 (Q60106); Endopep- are the glutenases of the invention. Protein sequence accession numbers are reported in brackets.

Figure 2: Activity profile of endopep-140 (>100 kDa) and endopep-40 (<100 kDa) measured on the fluorescent substrate Succinyl-Ala-Ala-Pro-Phe-AMC under various pH conditions. Fluorescence measured after 2 h at 37°C. Specific pH values are given on X-axis, percentage of relative activity on Y-axis.

Figure 3: Endopeptidase activity of endopep-140 (>100 kDa) and endopep-40 (<100 kDa) measured on the fluorescent substrate Succinyl-Ala-Ala-Pro-Phe-AMC at pH3 and pH5. Fluorescence measured at various time intervals at 37°C. Incubation times are given on X-axis, arbitrary fluorescence units (rfu) on Y-axis.

Figure 4: 8% SDS-PAGE stained with Coomassie blue of recombinant glutenases. Protein expression was induced with IPTG 0.2 μ M at 22°C overnight. S1 indicates the *E. coli* strain transformed with pET28b-endopep-140; S3 indicates the *E. coli* strain transformed with pET28b-endopep-40. Molecular weights of the marker used are indicated on the left.

From the left: lane 1 = molecular weight marker; lane 2 = S1 not induced; lane 3 = S1 induced, 3h harvest; lane 4 = S1 induced, overnight (o.n.) harvest; lane 5 = blank; lane 6 = S3 not induced; lane 7 = S3 induced, 3h harvest; lane 8 = S3 induced, o.n. harvest.

Figure 5: Breakdown of the 33-mer gliadin peptide by endopep-140.

The time course reaction products were separated by reversed phase HPLC. Figure 5A: MS traces, ion counts $m/z=300-2000$; figure 5B: UV-profile 230 nm, μ AU are absorbance micro units. The profile obtained at time 0, 30min, 2h are compared. Only one signal corresponding to the intact 33-mer peptide ([M-2H]=1957) was present at time 0. This signal decreased effectively with time. Several peaks appeared after 30 min incubation and their amount increased at time 2h. All the molecular ions observed are reported in Table 2. The size of the most characteristic peak are highlighted in the figure.

Figure 6: Breakdown of the 33-mer gliadin peptide by endopep-140 in the presence of pepsin (1mg/ml).

The time course reaction products were separated by reversed phase HPLC. Figure 6A: MS traces, ion counts $m/z=300-2000$; figure 6B: UV-profile 230 nm, μAU are absorbance micro units. The profile obtained at time 0, 30min, 2h are compared. While only one signal corresponding to the intact 33-mer peptide ($[M-2H]=1957$) was present at time 0, several peaks appeared after 30 min incubation and their amount increased at time 2h. All the molecular ions observed are reported in Table 3. The size of the most characteristic peaks are highlighted in the figure. The signal at ($[M-H]=1068$) corresponds to a peptide of nine aminoacids present in the 33-mer sequence. The signal 689.7 is due to pepsin.

Figure 7: HPLC-MS analysis. 33-mer gliadin peptide was incubated with pepsin. Figure7A: MS traces; figure7B: UV-profile 230nm, μAU are absorbance micro units. The profile obtained at time 0 and 2h are compared. Almost no degradation of 33-mer was observed.

Figure 8: Proteolysis of gliadin SDS-PAGE stained with Coomassie blue.

Gliadin digestion by the two different glutenases in presence or absence of pepsin. Gliadin was incubated 2h at 37°C .

From the left: lane 1 = Molecular weight marker; lane 2 = gliadin; lane 3 = gliadin + endopep-40 (<100 kDa); lane 4 = gliadin + endopep-40 (<100 kDa) + pepsin; lane 5 = reaction 3 stopped after 10 min incubation; lane 6 = gliadin + endopep-140 (>100 kDa); lane 7 = gliadin + endopep-140 (>100 kDa) + pepsin.

Brief description of tables

Table 1. Putative proteins purified from *Actinoallomurus* secretome.

Table 2. Peptides released by 33-mer digestion with endopep-140.

Table 3. Peptides released by 33-mer digestion with endopep-40.

Detailed description of the Invention

Actinomycetes are filamentous gram-positive bacteria, mainly known for their ability to produce secondary bioactive metabolites. Actinomycetes have been used as source of hydrolases, although only few, particularly the alkaliphilic ones, have so far been explored for their enzymatic potential (Endo A., Murakawa S., Shimizu H., Shiraishi Y. "Purification and properties of collagenase from a *Streptomyces* species" J. Biochem., 1987, 102, 163-70; Sakurai Y., Inoue H., Nishii W., Takahashi T., Iino Y., Yamamoto M., Takahashi K.

“Purification and Characterization of a Major Collagenase from *Streptomyces parvulus*” Biosci. Biotechnol. Biochem., 2009, 73, 21-28; Mehta V.J., Thumar J.T., Singh S.P., “Production of alkaline protease from an alkaliphilic actinomycete” Bioresource Technology, 2006, 97, 1650-1654).

- 5 Moreover, the complete genome sequence of *Streptomyces coelicolor* A3(2) predicted the presence of 60 secreted putative proteases and peptidases among the 819 potential secreted proteins (Bentley S.D., Chater K.F., Cerdeño-Tárraga A.M., Challis G.L., Thomson N.R., James K.D., Harris D.E., Quail M.A., Kieser H., Harper D., Bateman A., Brown S., Chandra G., Chen C.W., Collins M., Cronin A., Fraser A., Goble A., Hidalgo J., Hornsby T.,
- 10 Howarth S., Huang C.H., Kieser T., Larke L., Murphy L., Oliver K., O'Neil S., Rabinowitsch E., Rajandream M.A., Rutherford K., Rutter S., Seeger K., Saunders D., Sharp S., Squares R., Squares S., Taylor K., Warren T., Wietzorrek A., Woodward J., Barrell B.G., Parkhill J., Hopwood D.A. “Complete genome sequence of the model actinomycete *Streptomyces coelicolor* A3(2)” Nature, 2002, 417, 141-7) giving
- 15 actinomycetes further value as potential source for new hydrolytic enzymes. Among the actinomycetes, acidophilic strains offer higher chances to produce enzymes with properties best fitting the requirements for being effective in CD, namely activity at acidic pH. Several new genera of acidophilic actinomycetes (i.e. *Catenulispora*, *Actinospica*, *Rugosimonospora*, *Streptacidiphilus*) as well as filamentous bacteria with acidophilic
- 20 properties belonging to a previously unknown bacterial lineage (i.e. *Ktedonobacter*) have been described for the first time in recent years (Busti E., Cavaletti L., Monciardini P., Schumann P., Rohde M., Sosio M., Donadio S. “*Catenulispora acidiphila* gen. nov., sp. nov., a novel, mycelium-forming actinomycete, and proposal of *Catenulisporaceae* fam. nov.” Int. J. Syst. Evol. Bacteriol., 2006, 56, 1741–1746; Cavaletti L., Monciardini P.,
- 25 Bamonte R., Schumann P., Rohde M., Sosio M., Donadio S. “New lineage of filamentous, spore-forming, gram-positive bacteria from soil” Appl. Env. Microbiol., 2006, 72, 4360-4369; Cavaletti L., Monciardini P., Schumann P., Rohde M., Bamonte R., Busti E., Sosio M., Donadio S. “*Actinospica acidiphila* gen. nov., sp. nov. and *Actinospica robiniae* gen. nov., sp. nov.; proposal for *Actinospicaceae* fam. nov. and *Catenulisporinae* subordo. nov.
- 30 in the order *Actinomycetales*” Int. J. Syst. Evol. Bacteriol., 2006, 56, 1747-1753; Monciardini P., Cavaletti L., Ranghetti A., Schumann P., Rohde M., Bamonte R., Sosio M., Mezzelani A., Donadio S. “Novel members of the family *Micromonosporaceae*, *Rugosimonospora acidiphila* gen. nov., sp. nov. and *Rugosimonospora africana* sp. nov” Int. J. Syst. Evol. Bacteriol., 2009, 59, 2752–2758; Kim S.B., Lonsdale J., Seong C.M.,

Goodfellow M. “*Streptacidiphilus* gen. nov., acidophilic actinomycetes with wall chemotype I and emendation of the family *Streptomycetaceae* (Waksman and Henrici (1943)^{AL}) emend. Rainey et al. 1997)” *Antonie van Leeuwenhoek*, 2003, 83, 107-116).

Actinoallomurus, like many other actinomycetes, can grow in a medium containing protein as the sole nitrogen and carbon source. This ability to grow in a protein medium depends on the synergic action of secreted endo- and exoproteases since only aminoacids and short peptides can be assimilated via membrane transporters. *Actinoallomurus* strains have optimal growth at slightly acidic pH, thus suggesting that proteolytic enzymes may be expressed at this pH and may be able to digest complex proteins in acidic conditions. At Applicants’ knowledge no other report describes the production of proteases acting at acidic pH from actinomycetes.

Actinoallomurus sp. DSM 24988 was isolated by Applicants from an Italian soil, stored in the Applicants’ strain collection, and deposited on June 24, 2011 with the DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Inhoffenstr.7b, D-38124 Braunschweig, Germany (now Leibniz-Institut DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH), under the provision of the Budapest Treaty. The strain was accorded accession number DSM 24988. *Actinoallomurus* sp. DSM 24988 grows on various standard solid media (Shirling E.B., Gottlieb D. “Methods of characterization of *Streptomyces* species” *Int. J. Syst. Bacteriol.*, 1966, 16, 313-340) acidified at pH 5.5 with HCl. After 15 days of incubation at 28°C, the substrate mycelium is convolute, its colour is cream becoming violet with ageing and no aerial mycelium is produced on ISP2 agar. The colour of the aerial mycelium is white when formed on HSA5 (Busti *et al.*, 2006). Abundant growth and production of convolute cream/brown vegetative mycelium was observed on ISP3 agar after 15 days of incubation. Slight production of brownish soluble pigments is present after 20 days of incubation. The strain can be grown at temperature between 21°C and 35°C, optimal temperature being 28°C on ISP2 and HSA5. *Actinoallomurus* sp. DSM 24988 is able to grow in the presence of NaCl up to 2.5% (w/v); at concentration of 1% (w/v) and higher the strain does not differentiate violet vegetative mycelium but only convolute cream substrate mycelium. The strain, plated on ISP2 agar adjusted to the desired pH values with HCl or NaOH, grows well at pH between 4.0 and 7.0, with an optimum pH5.5. Abundant convolute cream vegetative mycelium is observed when grown onto acidic ISP9 added with gluten. No aerial mycelium and soluble pigments are produced.

Proteomic investigation of the secreted *Actinoallomurus* proteins confirms that different

sets of proteases active at neutral, basic or acidic pH are secreted and, in particular, several members of the S8 / S53 peptidase family.

The glutenases of the invention belong to the MEROPS peptidase S8 [subfamilies S8A (subtilisin) and S8B (kexin)] and S53 (sedolisin) family (Rawlings N.D., Barrett A.J.,
5 Bateman A. "MEROPS: the peptidase database" Nucleic Acids Res., 2010, 38, D227-D233).

Gluten is a proteic component of wheat, barley, rye and related species, unique in its ability to provide elasticity and other desired characteristics to dough and many other food products. Gluten proteins are rich in glutamine (35%) and proline (15%) residues, a feature
10 that is especially notable among gluten epitopes that are recognized by disease-specific T cells. The principal toxic components of wheat gluten are gliadins, a family of proline- and glutamine- rich proteins that contain several T-cell stimulatory epitopes. Their partial degradation in the gastrointestinal tract by pepsin, trypsin, chymotrypsin leads to the formation of several toxic peptides out of which peptides p31-49 and its derived p31-43 of
15 the α 1-gliadin fraction, and p56-88 (33-mer) of the α 2-fraction are the best characterized. The 33-mer is a peptide fragment of 33 residues obtained also by *in-vitro* mimicking the physiological gastrointestinal enzymatic digestion (Shan *et al.*, 2002), whose aminoacid sequence is LQLQPFQPQLPYPQPQLPYPQPQLPYPQPQPF (SEQ ID NO: 6). It is the strongest immunostimulator peptide as it carries multiple copies of three epitopes that are
20 immunogenic in patients with celiac disease; it is thus responsible for a strong immunotoxic response (Qiao S.W., Bergseng E., Molberg O., Xia J., Fleckenstein B., Khosla C., Sollid M.L. "Antigen Presentation to Celiac Lesion-Derived T Cells of a 33-mer Gliadin Peptide Naturally Formed by Gastrointestinal Digestion" J. Immunol., 2004, 173, 1757-1762) and therefore it is used as a model for gluten detoxification. The 33-mer peptide is an excellent
25 substrate for the enzyme transglutaminase 2 (TG2) that deamidates the gliadin peptides in the *lamina propria*, increasing their affinity to human leucocyte antigen (HLA) DQ2 or DQ8 molecules and thus enhancing the T cell-mediated mucosal immune response leading to the clinical consequences. Intestinal transport of intact 33-mer across the enterocyte layer may be due to an overexpression of transferrin receptor in CD and/or to an enhanced
30 mucosal permeability. Anyway, the peptides can escape degradation by the acidic endosome-lysosomal pathway and can reach the serosal border unchanged. Degradation of the 33-mer gliadin peptide into peptides containing less than nine residues results in no gluten toxicity.

Since the gastrointestinal tract does not possess the enzymatic equipment to efficiently

cleave the gluten-derived proline-rich peptides, driving in patients with celiac disease the abnormal immune intestinal response, the use of orally active proteases to degrade toxic gliadin peptides before they reach the mucosa can be considered as an alternative treatment to the diet.

5 Applicants have identified a new sub-family of proteins which exhibits a proteolytic activity toward peptides, such as proline rich peptides, at acidic pH, which corresponds to the pH of the gastric fluid, and found that this enzyme composition is also able to degrade the 33-mer gliadin peptide. On the basis of structural properties, obtained by bioinformatic analysis, Applicants have shown that this new subfamily belong to peptidases S8 / S53 (subtilisin
10 kexin sedolisin) and can be grouped in a new subgroup different from the already known sedolisin or kumamolisin that belong to the S53 family (Fig.1).

Applicants have developed a particular composition of these endopeptidases. This composition comprises at least one of the endoproteases of the peptidase S8 / S53 (subtilisin kexin sedolisin) family produced by *Actinoallomurus* which digests full-length
15 polypeptides and degrades a fragment of gliadin known to be resistant to protease action. So at least one endoprotease can be used for the treatment of celiac disease or any disease of the digestive process such as malabsorption. Moreover, as the enzyme is resistant to pepsin, a combination of these two proteolytic activities could result in more extensive degradation of polypeptides and proteins such as gliadin. Applicants have discovered that 33-mer is
20 degraded to peptides of six aminoacids (Table 2 and 3). Although the enzymes of the invention are active alone, the addition to them of one or more peptidases that are acting on proline rich peptides such as prolyl-endoproteases and/or x-prolyl-dipeptidyl aminopeptidases and/or prolyl-aminopeptidases may result in a more rapid action.

The S8/S53 protein family of this invention alone or optionally in combination with other
25 proteases can be useful in the food industry, such as, but not limited to, to degrade substrates for bitterness, treatment of meat, soap industry, or to degrade prions, viruses, and toxic or contaminant proteins into short peptides and/or free aminoacids.

Thus, the present invention provides an enzyme composition, comprising at least one S8/S53 endopeptidase active at pH between 3 and 8 (inclusive), selected from the group
30 consisting of:

a) endopep-140 comprising SEQ ID NO: 1, a biologically active fragment thereof, a naturally occurring allelic variant thereof, or a sequence having at least 50%, 60%, 70%, 80%, 90%, or 95% of identity,

b) endopep-40 comprising SEQ ID NO: 2, a biologically active fragment thereof, a naturally occurring allelic variant thereof, or a sequence having at least 50%, 60%, 70%, 80%, 90%, or 95% of identity,

c) endopep-120 comprising SEQ ID NO: 3, a biologically active fragment thereof, a naturally occurring allelic variant thereof, or a sequence having at least 50%, 60%, 70%, 80%, 90%, or 95% of identity,

d) endopep-60 comprising SEQ ID NO: 4, a biologically active fragment thereof, a naturally occurring allelic variant thereof, or a sequence having at least 50%, 60%, 70%, 80%, 90%, or 95% of identity

e) endopep-41 comprising SEQ ID NO: 5, a biologically active fragment thereof, a naturally occurring allelic variant thereof, or a sequence having at least 50%, 60%, 70%, 80%, 90%, or 95% of identity.

The term “peptidase(s) of the invention”, “protease(s) of the invention”, or “endopeptidase(s) of the invention” as used herein, identifies one or more of the endopeptidases defined above.

The present invention also includes an endopeptidase derived from any one of the sequences indicated above where any of the aminoacids may be changed from the corresponding residues shown in SEQ ID NOs: 1, 2, 3, 4 or 5 still maintaining its biological activity and physiological functions, or a biologically active fragment thereof. The object of this invention includes also any peptidase variant containing substitutions, deletions, side-chain modifications and/or insertions at certain positions within the aminoacid sequence of the native aminoacid sequence which preserves the biological activity and physiological function of said native aminoacid sequence. Examples of substitutions are well known to those skilled in the art and indicated, for example, in WO2011/077359.

In another aspect, the present invention is directed to isolated proteases of the invention, and biologically active fragments thereof (or derivatives, portions, analogs or homologs thereof). Biologically active fragment refers to regions of the proteases of the invention, which are necessary for specific protease activities.

In a further embodiment, the protease of the invention is a protease that comprises an aminoacid sequence having at least 50%, preferably at least 60%, more preferably at least 70%, even more preferably 80%, still more preferably 90% and most preferably 95% of identity to the aminoacid sequence comprising SEQ ID NOs: 1, 2, 3, 4 or 5 and retains the activity of the proteases comprising SEQ ID NOs: 1, 2, 3, 4 or 5.

The term "identity" and "homology" when referred to a nucleotide or aminoacid sequence

are used interchangeably herein and refers to the degree to which two polynucleotide or polypeptide sequences are identical or homologous on a residue-by-residue basis over a particular region of comparison. The alignment and the percent identity or homology can be determined using any suitable software program known in the art, for example those
5 described in Current Protocols in Molecular Biology (Ausubel F.M. et al., "Commercially Available Software", Current Protocols in Molecular Biology, 1987, Supplement 30, Section 7.7.18, Table 7.7.1). Preferred programs include the GCG Pileup program, FASTA (Pearson R. and Lipman D. J. "Improved Tools for Biological Sequence Analysis" Proc. Natl., Acad. Sci. USA, 1988, 85, 2444-2448), and BLAST (Altschul S.F., Gish W., Miller
10 W., Myers E.W., Lipman D.J. "Basic local alignment search tool" J. Mol. Biol., 1990, 215, 403-410).

The invention also provides proteases of the invention operatively-fused to another polypeptide which are called chimeric or tagged proteins by those skilled in the art. The polypeptide can be fused to the N-terminus and/or C-terminus of the protease of the
15 invention. Different tagged fusion proteins can be done by those skilled in the art and produced by standard recombinant DNA techniques or conventional techniques including automated DNA synthesizers. Such fusion proteins can facilitate the purification of the recombinant protease of the invention or their expression and secretion in host cells. These techniques are well known to those skilled in the art.

The Applicants have shown that large peptides such the 33-mer gliadin peptide can be degraded at acidic pH by the endopeptidases. The secreted endopep-140 acts by cleaving the polypeptide chain at several points generating different small peptides of six aminoacids (Table 2, Fig. 5). This endopeptidase shows a preference for sites having tyrosine or leucine or phenylalanine in position P1 and proline in position P2 and P1'. Increasing the amount of
25 endopeptidase and/or time of incubation leads to complete degradation of the 33-mer polypeptide. According to the molecular weights detected, it seems that glutamine residues are transformed to glutamic residues thus suggesting the involvement of an amidotransferase activity. The endopeptidases of the enzyme composition of the invention can operate in the presence of mammalian pepsin (Table 2, Fig. 6).

The same analyses were performed with endopep-40 and the results obtained, shown in Table 3, indicate that this glutenase behaves similarly.

The endopep-140 contains a structural domain with high homology to other endopeptidases of the invention. For example, BLAST alignment gives Identities = 223/370 (60%), Positives = 261/370 (71%) between endopep-140 and endopep-40 or Identities = 227/431

(53%), Positives = 273/431 (63%) between endopep-140 and endopep-41. Similar results are obtained by the BLAST alignment between endopep-40 and endopep-41, with Identities = 230/419 (55%), Positives = 278/419 (66%). Positives are aminoacid residues that are identical or very similar to each other in their physico-chemical features as determined with the BLOSUM62 matrix.

The endopeptidases of the enzyme composition of the invention can be produced by cultivation of a naturally occurring *Actinoallomurus* strain or a mutant thereof which is capable of producing at least one S8/S53 endopeptidase above defined or a host cell obtained by recombinant DNA techniques.

The term "recombinant", when used with reference to a cell, indicates that the cell replicates a heterologous nucleic acid, or expresses a peptide or protein encoded by a heterologous nucleic acid. Genes not found within the native (non-recombinant) form of the cell or found in the native form of the cell wherein the genes are modified and re-introduced into the cell by artificial means can be contained in recombinant cells.

The person skilled in the art will recognize that these cells can be unicellular or multicellular transgenic organisms.

The invention also includes methods based on cultivation of cells that contain a nucleic acid endogenous to the cell that has been modified without removing the nucleic acid from the cell; such modifications include those obtained by gene replacement, site-specific mutation, and related techniques, e.g. by treatment with mutagenic agents or with ionizing radiations.

The term "allelic variant" denotes any of two or more alternative forms of a gene occupying the same chromosomal locus. Allelic variation arises naturally through mutation, and may result in phenotypic polymorphism within populations. Gene mutations can be silent (no change in the encoded polypeptide) or may encode polypeptides having altered aminoacid sequence. The term allelic variant refers also to a protein encoded by an allelic variant of a gene.

Thus, the present invention provides a method for producing the enzyme composition of the invention which comprises:

A) cultivating a naturally occurring *Actinoallomurus* strain capable of producing at least one S8/S53 endopeptidase above defined and recovering the endopeptidase(s) from the cultivation batch; or

B) cultivating an *Actinoallomurus* strain derived from a naturally occurring *Actinoallomurus* strain capable of producing at least one S8/S53 endopeptidase above defined by conventional mutation and/or selection techniques, which maintain the capability

of producing at least one of the above defined endopeptidase(s) and recovering the endopeptidase(s) from the cultivation batch; or

C) a recombinant DNA technique comprising the steps of:

1) introducing into a host cell a nucleic acid encoding for at least one endopeptidase of the

5 S8/S53 subtilisin kexin sedolisin class selected from the group consisting of:

a) endopep-140 comprising SEQ ID NO: 1, a biologically active fragment thereof, a naturally occurring allelic variant thereof, or a sequence having at least 50%, 60%, 70%, 80%, 90% or 95% of identity,

10 b) endopep-40 comprising SEQ ID NO: 2, a biologically active fragment thereof, a naturally occurring allelic variant thereof, or a sequence having at least 50%, 60%, 70%, 80%, 90% or 95% of identity,

c) endopep-120 comprising SEQ ID NO: 3, a biologically active fragment thereof, a naturally occurring allelic variant thereof, or a sequence having at least 50%, 60%, 70%, 80%, 90% or 95% of identity,

15 d) endopep-60 comprising SEQ ID NO: 4, a biologically active fragment thereof, a naturally occurring allelic variant thereof, or a sequence having at least 50%, 60%, 70%, 80%, 90% or 95% of identity, and

e) endopep-41 comprising SEQ ID NO: 5, a biologically active fragment thereof, a naturally occurring allelic variant thereof, or a sequence having at least 50%, 60%, 70%, 80%, 90% or 95% of identity,

2) cultivating the cell of step 1) in a culture medium under conditions suitable for producing the endopeptidase(s), and

3) recovering the endopeptidase(s) from the cultivation batch.

One or more endopeptidases of the invention may be produced in performing one method as
25 above defined. When single endopeptidases are separately produced performing the method defined above, this invention includes optionally the step of combining two or more of the obtained endopeptidases to provide an enzyme composition containing a mixture of the said endopeptidases. Said endopeptidases may be obtained as isolated endopeptidases. With the term “isolated endopeptidase”, as used herein, a purified form of the endopeptidase is
30 intended which is substantially free of other proteins or cellular material from the cell from which the endopeptidase is derived.

All DNA/ RNA nucleic acid terms also referred to their related manipulation techniques herein used are well known to those expert in the molecular biology art and so far these terms are used in all their wide and common meaning as reported in Maniatis T., Fritsch

E.F., Sambrook J. "Molecular cloning: a laboratory manual" Cold Spring Harbor, NY, 1982 or Ausubel F.M. "Current Protocols in Molecular Biology" John Wiley & Sons, New York, NY, 1993 or Sambrook et al., "Molecular Cloning: a Laboratory Manual 2nd Ed.", Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989.

5 The nucleic acids encoding the endopeptidases of the enzyme composition of the invention are a further object of the invention which includes the nucleic acids whose sequences are provided herein and defined as SEQ ID NOs: 7, 8, 9, 10 or 11 or fragments thereof. The invention also includes mutant or variant nucleic acids or portion of these nucleic acids.

Accordingly, the nucleic acids of this invention include a polynucleotide sequence shown in
10 SEQ ID NOs: 7, 8, 9, 10 or 11 or a sequence having at least 50%, preferably 60%, more preferably at least 70%, even more preferably 80%, still more preferably 90% and most preferably at least 95% of identity to the nucleic acid sequence comprising SEQ ID NOs: 7, 8, 9, 10 or 11 and is encoding an amino acid sequence which is still maintaining the biological activity and physiological function of said amino acid sequence.

15 Sequences characterized by identities at the nucleotide level are indicated herein also as "homologous nucleic acid sequences" or variations thereof. Homologous nucleotide sequences encode those sequences coding for isoforms of proteases of the invention. Isoforms can be expressed in the same organism as a result of, for example, alternative splicing of RNA. Alternatively, isoforms can be encoded by different genes.

20 Homologous nucleotide sequences also include, but are not limited to, naturally occurring allelic variations and mutations of the nucleotide sequences set forth herein. Homologous nucleic acid sequences include those nucleic acid sequences that encode conservative amino acid substitutions in SEQ ID NOs: 1, 2, 3, 4 or 5.

In the invention, homologous nucleotide sequences can include nucleotide sequences
25 encoding a protease of the invention of other species belonging to *Actinoallomurus* as well as of genera other than *Actinoallomurus*, such as for example *Catenulispora*, *Actinospica*, *Ktedonobacter*, *Streptomyces*, *Streptacidiphilus*, *Micromonospora*, *Rugosimonospora*.

Applicants determined by BLAST analysis that *Catenulispora*, *Ktedonobacter*, *Streptomyces* genomes contain genes that encode for proteins having more than 50%
30 homology with the corresponding protease of the invention; for example, the putative proteins with accession numbers ACU72534 and ACU72320 of *Catenulispora acidiphila* have Identities = 261/400 (65%), Positives = 295/400 (74%) with endopep-40 SEQ ID NO: 2 and Identities = 705/1342 (53%), Positives = 878/1342 (65%) with endopep-140 SEQ ID NO: 1 respectively, or the putative protein with accession number EFH81837 of

Ktedonobacter racemifer has Identities = 244/402 (61%), Positives = 299/402 (74%) with endopep-40 SEQ ID NO: 2, or *Streptomyces* sp. e14 protein sequence with accession number EFF91270 has Identities = 230/371 (62%), Positives = 274/371 (74%) with endopep-40 SEQ ID NO: 2, as well. Homologous endopeptidase encoding genes may be present in strains belonging to genera phylogenetically related to the above mentioned whose genomic sequences are not available by public databases.

A "biologically-active fragment" of the endoprotease of the invention can be prepared by isolating a nucleic acid fragment of SEQ ID NOs: 7, 8, 9, 10 or 11, that encodes an endoprotease with the same biological activity of the endoproteases of the invention, expressing the encoded portion of endoprotease (for example, by recombinant expression *in vitro*) and assessing the activity of the encoded fragment of endoprotease. The invention further encompasses nucleic acid molecules that differ from the nucleic acid sequences shown in SEQ ID NOs: 7, 8, 9, 10 or 11 due to degeneracy of the genetic code and thus encode the same proteases that are encoded by the nucleic acid sequences shown in SEQ ID NOs: 7, 8, 9, 10 or 11.

The techniques for gene manipulation and protein expression are known to those skilled in the art and can be found also in Kriegler M. "Gene Transfer and Expression: A Laboratory Manual" Stockton Press, NY, 1990.

Applicants indicate with the term "biological activity" or "functional activity" the natural or normal function of the proteases of the invention, for example, the ability to degrade other proteins. Aminoacid residues that are conserved among the proteases of the invention are predicted to be particularly non-amenable to alteration. Aminoacids for which conservative substitutions can be made are well known within the art. As all the persons skilled in the art recognize, each codon in a nucleic acid (apart from AUG, which is ordinarily the only codon for methionine) can be modified to yield a functionally identical molecule by standard techniques. Furthermore, individual substitutions, deletions or additions which alter, add or delete a single aminoacid or a small percentage of aminoacids (typically less than 5%, more typically less than 1%) in an encoded sequence are "conservative mutations" where the alterations result in the substitution of an aminoacid with a chemically similar aminoacid.

Another aspect of the invention pertains to nucleic acid molecules encoding the proteases of the invention that contain changes in aminoacid residues that are not essential for activity. Such proteases of the invention differ in aminoacid sequence from SEQ ID NOs: 1, 2, 3, 4 or 5, yet retain biological activity. In one embodiment, the isolated nucleic acid molecule

comprises a nucleotide sequence encoding a protease, wherein the protease comprises an aminoacid sequence with at least about 50% identity to the aminoacid sequences of SEQ ID NOs: 1, 2, 3, 4 or 5.

The term “isolated nucleic acid” or “isolated polynucleotide sequence”, as used herein, identifies a nucleic acid molecule which is separated from other nucleic acid molecules that are present in the cell from which the nucleic acid is derived and is substantially free of other cellular material or culture medium material when said nucleic acid molecule is obtained from a naturally occurring microorganisms, a microorganism derived therefrom, or microorganisms obtained by recombinant techniques.

An isolated nucleic acid molecule encoding an endoprotease of the invention homologous to the protein of SEQ ID NOs: 1, 2, 3, 4 or 5, can be created by introducing one or more nucleotide substitutions, additions or deletions into the nucleic acid sequence of SEQ ID NOs: 7, 8, 9, 10 or 11, such that one or more aminoacid substitutions, additions or deletions are introduced into the encoded protease. Mutations can be introduced into SEQ ID NOs: 7, 8, 9, 10 or 11, by standard techniques, such as site-directed mutagenesis, PCR-mediated mutagenesis and DNA shuffling. Alternatively, mutations can be introduced randomly along all or part of a coding sequence of the protease of the invention, such as by saturation mutagenesis, and the resultant mutants can be screened for biological activity of the protease of the invention to identify mutants that retain activity.

The glutenase enzyme composition according to the present invention may also be used in immobilized form and used, for example, for the treatment of liquid food products. The enzyme composition of the invention can also be used in the fruit and brewing industry for equipment cleaning and maintenance. For example, a gluten containing liquid food product is allowed to flow along a matrix permeable for gluten in which the enzyme composition of the invention is embedded. The gluten is extracted from the food product and digested by the action of the enzymes. The enzyme composition of the invention can also contribute to the available energy of the food. For example a partially or indigestible proline- comprising protein is fully or partially degraded by the enzyme composition of the invention, resulting in availability of more digestible food for the human or animal. So, the growth rate and/or food conversion ratio (i.e. the weight of ingested food relative to weight gain) of the human or animal is improved.

Methods of production of the endopeptidases and their characterization

The present invention also relates to methods for producing the enzymes of the present invention comprising cultivating (a) a strain, which in its wild-type form, or (b) a form derived therefrom by common mutation techniques, e.g., by treatment with mutagenic agents or with ionizing radiations, or (c) a host cell which is capable of producing the polypeptide of the invention, and recovering said polypeptide from the cultivation batch. In the production methods of the present invention, the media for cell cultivation are differently selected among those known in the art for protein production according to cultivation of wild type strains, strains derived therefrom, or recombinant host cells. The cultivation takes place in a suitable nutrient medium comprising carbon and nitrogen sources and inorganic salts, using procedures known in the art. Suitable media are available from commercial suppliers or may be prepared according to published compositions (e.g., in catalogues of the American Type Culture Collection). For example, medium containing soya are more apt to allow production of enzymes of the invention by wild type microorganisms.

The cells may be cultivated by shake flask cultivation, small-scale or large-scale fermentation (including continuous, batch, fed-batch, or solid state fermentations) in laboratory or industrial fermenters performed in a suitable medium and under conditions allowing the polypeptide to be expressed and/or isolated). If the polypeptide is secreted into the nutrient medium, the polypeptide can be recovered directly from the medium. If the polypeptide is not secreted, it can be recovered from cell lysates. Several proteolytic activities are produced and cultivation is suspended when the production of endopeptidase has reached a maximum. After the completion of cultivation, the culture medium is filtered off to separate microbial bodies, and the filtrate is processed in the usual way for the collection of endopeptidase by several procedures in combination, such as ultrafiltration, concentration under reduced pressure, salting out, precipitation by organic solvent, dialysis, gel filtration, adsorption chromatography, ion-exchange chromatography, electro-focusing, and freeze-drying. Adequate procedures should be selected taking into account the desired physical and chemical properties of endopeptidases.

A representative example of production of endopeptidases of this invention by cultivating a wild-type *Actinoallomurus* strain is provided in Example 1 hereinafter. The endopeptidase(s) of the invention may be purified to the desired degree of purity which may depend on the intended use and on the specific activity of endopeptidase(s).

Heterologous expression in host cells

Recombinant cells and microorganisms can be used to produce the endopeptidases of the present invention. The host cell may be any of the host cells familiar to the person skilled in the art, including prokaryotic cells, eukaryotic cells, mammalian cells, insect cells, fungal cells, yeast cells and/or plant cells. The selection of an appropriate host is within the abilities
5 of the person skilled in the art.

Useful microorganisms are bacterial cells such as gram positive bacteria including, but not limited to, a *Bacillus* cell (e.g. *Bacillus subtilis*, *Bacillus cereus*) or a *Streptomyces* cell, or cells of lactic acid bacteria, or gram negative bacteria such as *E. coli* and *Pseudomonas*. Preferred producing cells include those from organisms known to be generally regarded as
10 safe, such as the prokariots *Lactobacillus*, *Pyrococcus*, *Bacillus*, *Streptomyces*, and the eukariot *Aspergillus*. More preferred cells include those that are already used in the preparation of foodstuffs, such as *Lactobacillus* spp. and/or *Aspergillus oryzae*.

Extracellular production of the enzymes may be obtained from microorganisms such as *Aspergillus oryzae*, *Lactobacillus casei*, *Kluyveromyces lactis* or *Streptomyces lividans*.

15 The introduction of a vector into a bacterial host cell may, for instance, be done by protoplast transformation (e.g., Chang S., Cohen S.N. "High frequency transformation of *Bacillus subtilis* protoplasts by plasmid DNA" Mol. Gen. Genet., 1979, 168, 111-115), using competent cells (e.g., Dubnau D., Davidoff-Abelson R. "Fate of transforming DNA following uptake by competent *Bacillus subtilis*. I. Formation and properties of the donor-
20 recipient complex" J. Mol. Biol., 1971, 56, 209-221), electroporation (e.g., Shigekawa, K., Dower W. J. "Electroporation of eukaryotes and prokaryotes: a general approach to the introduction of macromolecules into cells" Biotechniques, 1988, 6, 742-751) or conjugation (e.g., Koehler T.M., Thorne C.B. "*Bacillus subtilis* (natto) plasmid pLS20 mediates interspecies plasmid transfer" J. Bacteriol., 1987, 169, 5771-5278).

25 Useful multicellular organisms to be used as host cells are, for example, plants, plant parts, seeds or plant cells. Preferred producing multicellular organisms are, for example, transgenic food crops, such as grains, or vegetables, such as tomato, that contain the nucleic acids encoding the proteases of the invention.

A representative example of production of endopeptidases of this invention in recombinant
30 host cells is given in Example 2 hereinafter.

Pharmaceutical formulations

The endopeptidase agents of the present invention may be administered alone or incorporated into a variety of formulations. A pharmaceutical composition of the invention

is formulated to be compatible with its intended route of administration, which is preferably the oral administration. For example, since some of the endopeptidases of the enzyme composition of the invention is secreted, a crude preparation obtained from cell culture medium of *Actinoallomurus* or other useful unicellular recombinant microorganisms such as
5 gram positive bacteria including, but not limited to, a *Bacillus* cell, a *Streptomyces* cell, lactic acid bacteria cells, gram negative bacteria such as *E. coli*, can be administered orally. Lactic acid bacteria include, but are not limited to, species of the genera *Lactococcus*, *Lactobacillus*, *Leuconostoc*, *Streptococcus*, *Pediococcus*, and *Enterococcus*. Alternatively, the enzyme composition of the invention can be administered orally after purification.

10 Such formulations can be prepared with the appropriate ingredients to generate a preparation in liquid form, for example in the form of a solution, emulsion, or in solid form, such as tablets, capsules, or semisolid. The formulation of the enzyme composition can be administered in a variety of ways including those particularly suitable for admixing with foodstuff. The enzyme components can be active prior to or during ingestion, and may be
15 treated, for example, by a suitable encapsulation, to control the timing of activity.

To prepare an appropriate pharmaceutical composition of the endopeptidases of the present invention any method for the stabilization of chemical or biological material known in the art, comprising those based on irradiation or temperature modulation or their combinations, can be used.

20 For treating celiac disease, the pharmaceutical compositions employed are preferably formulated so as to release their activity in the gastric fluid. This type of formulations will provide optimum activity in the right place, for example the release of the endoproteases of the invention in the stomach.

Alternatively a microorganism, such as a bacterial or yeast culture, able of producing the
25 active agents can be administered to a patient. Such a culture may be admixed with food preparations or formulated, for example, as an enteric capsule.

The present invention further provides a food supplement comprising the enzyme composition of the present invention. The term "food supplement" in the context of the present invention is interchangeable with the terms food additive, a dietary supplement and
30 nutritional supplement.

The food supplement of the invention can be formulated, prepared, supplied and dispensed as described in other prior documents regarding the field of this invention (WO2011/077359, WO 2003/068170, WO2005107786) that provide methods for treating and/or preventing a syndrome associated with a human disease, said disease being selected

from the group comprising celiac disease, dermatitis herpetiformis, digestive tract bad absorption, an allergic reaction, an enzyme deficiency, a fungal infection, Crohn disease, mycoses and sprue.

As an example, the food supplement of the invention may be a granulated enzyme coated or uncoated product which may readily be mixed with food components, alternatively, food supplements of the invention can form a component of a pre-mix. Alternatively, the food supplements of the invention may be a stabilized liquid, an aqueous or oil-based slurry. The enzyme composition of the invention can be supplied by expressing the enzymes directly in transgenic food crops (as, e.g., transgenic plants, seeds and the like), such as grains, cereals, corn, soy bean, rape seed, lupin.

The pharmaceutical composition or the food supplement of the invention can be provided prior to meals, immediately before meals, with meals or immediately after meals, so that the endoproteases of the enzyme composition of this invention are released or activated in the upper gastrointestinal lumen where the endoproteases can complement gastric and pancreatic enzymes to detoxify ingested gluten and prevent harmful peptides to pass the enterocytes layer.

The enzyme composition of this invention has numerous applications in food processing industry, in particular they can be used in the manufacture of food supplements as described in the above mentioned prior documents.

From this description it results evident that a further object of this invention consists in providing a method for degrading gluten oligopeptides which are resistant to cleavage by gastric and pancreatic enzymes and whose presence in the internal lumen results in toxic effects which comprises contacting said gluten oligopeptides with an enzyme composition or at least one isolated endopeptidase of this invention.

In particular, one aspect of said method consists in the treatment or prevention of celiac sprue, dermatitis herpetiformis and/or any other disorder associated with gluten intolerance which comprises administering to a patient in need thereof an effective amount of an enzyme composition or of at least one isolated endopeptidase of this invention, preferably, incorporated into a pharmaceutical formulation, food supplement, drink or beverage.

Examples

Example 1: *Actinoallomurus* sp. DSM 24988 endopeptidases identification and characterization

1.1 Strain cultivation and protein production

The *Actinoallomurus* strain used according to this invention derives from the Applicants strain collection.

Actinoallomurus sp. DSM 24988 was maintained on ISP2 agar medium (Shirling and Gottlieb, 1966) acidified at pH 5.5 with HCl. The microbial content of one plate was
5 scraped and inoculated into one 50 ml Erlenmeyer flask containing 15 ml of medium AF5 which is composed of: (g/l) dextrose 20, yeast extract 2, soybean meal 8, NaCl 1 and MES 10. Medium was prepared in distilled water and pH adjusted to 5.5 prior to sterilization at 121°C for 20 min. The inoculated flask was grown at 28°C, on a rotary shaker operating at 200 rpm. After 5-6 days incubation, 5% of culture was inoculated into a second series of
10 500 ml Erlenmeyer flasks containing 100 ml of the same fermentation medium. Protein production was performed in flasks incubated for 15 days at 28°C on a rotary shaker operating at 200 rpm. The production of the protein was monitored by bioassay as described below.

Several proteolytic activities are produced and cultivation is suspended when the production
15 of endopeptidases, followed by the assays described below, has reached a maximum. The fermentation was harvested after 15 days and the broth was centrifuged at 4000 rpm for 15 minutes. The culture medium was filtered off to separate microbial bodies, and the filtrate was processed. Adequate procedures were selected taking into account the desired physical and chemical properties of endopeptidases.

1.2 Proteolytic activities determination

Enzyme activities were measured at different pHs in acetate buffer (ammonium acetate - AMAC, 50 mM final concentration, pH 4.0 to 8.0; acetic acid 20mM, pH3.0) at 37°C in a total volume of 0.2 ml.

25 The enzymatic activity of endopeptidase was determined by measuring the hydrolysis of Succinyl-Ala-Ala-Pro-Phe-AMC (Amino-Methyl-Coumarine) as substrate (Bachem AG, Hauptstrasse 144, 4416 Bubendorf, Switzerland).

Substrate stock solutions were prepared at 10 mM concentration and stored at -20°C. The substrate was prepared from 10-20 µl of 60% methanol solution containing 2mM Succinyl-
30 Ala-Ala-Pro-Phe-AMC and 50-150 µl acetate buffer solution. The reaction mixture contained a concentration of 0.2 mM substrate and the enzyme preparation (between 10-40 µl or 1.0 µg per assay) in 200 µl acetate buffer at different pH values.

To the substrate, which had previously been heated to 37 ° C for 10 minutes, 10-40 µl of the enzyme solution were added, and the reaction was performed at 37 ° C up to 2 hours. The

released AMC was measured with a Fusion micro-plate reader (Perkin Elmer Italia SpA, Monza, Italia) at an excitation wavelength of 360nm and an emission wavelength of 460nm. As control, the substrate was incubated without enzyme.

The enzymatic activity to release 1 mmol of AMC in 1 minute is defined as 1 Unit.

5

1.3 Endopeptidase purification

The mycelium was separated from culture medium by paper filtration (Miracloth from Calbiochem, USA). Thereafter, 200 ml of supernatant were centrifuged for 10 minutes at 5000 rpm to remove debris, then the supernatant was further centrifuged at 10000 rpm for 20 min at 4°C to remove the insoluble fraction. Proteins were precipitated from supernatant by ethanol (1:4 v/v) or ammonium sulphate 20-75% saturation. Pellet was resuspended and dialyzed, at occurrence concentrated using a Centricon Plus-70 with a 5 kDa cut-off (Millipore, Bedford, Mass., USA). The presence of proteases able to hydrolyze Succinyl-Ala-Ala-Pro-Phe-AMC was highlighted when tested at pH5. Aliquots of the protein suspension after dialysis were boiled to be submitted to MS-shotgun analysis. The boiling procedure is necessary in order to avoid auto digestion. At least 5 proteins belonging to the S8/S53 family protease were detected.

Resuspended proteins were chromatographed on ionic exchange resin Amberlite IRA900 (Alfa Aesar GmbH, Karlsruhe, 76185 Germany) or DE-52 cellulose (Whatman Inter. Ltd, Maidstone England) or other matrix; the obtained fractions were tested for Succinyl-Ala-Ala-Pro-Phe-AMC hydrolysis and the pooled active fractions subjected to 1D-SDS-PAGE and zymographic analysis.

Proteins were further fractionated by size exclusion filtration on molecular weight cut-off 300 kDa, 100 kDa, 50kDa, 30 kDa, 10 kDa (Nanosep Pall, Michigan.USA, Vivaspin Sartorius GmbH 37070 Goettingen, Germany). Their activity was tested as described. Active fractions with different molecular weight were obtained. Aliquots of these fractions were boiled and analyzed by MS-shotgun.

Proteins were separated by electrophoresis on a homogeneous 10% or disc-gel 4-15% or any-kd polyacrylamide gel (Bio-Rad, Hercules, 9640, CA, USA) followed by staining with Coomassie Brilliant blue R-250 (Bio-Rad) or silver (SIGMA- Aldrich, USA) or by enzyme activity staining (zymography) performed by incubating the gel at 37° C with 50-100 µM fluorescent substrate Succinyl-Ala-Ala-Pro-Phe-AMC at the desired pH.

Two endopeptidases belonging to the S8/S53 family were purified until the electrophoretic analysis showed the presence of a single band as detected both by silver staining and

zymography.

The first endopeptidase was obtained after molecular weight filtration: it was retained on a cut-off of >100 kDa. The MS-shot gun analysis of the corresponding boiled aliquot revealed the presence of endopep-140, SEQ ID NO: 1 (Table 1A), with a molecular weight of 142449.4 and a theoretical pI of 5.18; traces of two other proteins were detected. This protein fraction resulted active in the pH range from 4 to 8 with a pH optimum of 5 (Fig. 2). When tested at pH3 the enzyme shows 95% of its activity at pH 5 after 30 minutes of incubation at 37°C (Fig. 3). This protein fraction resulted more efficient in 33-mer digestion than in the hydrolysis of the Succinyl-Ala-Ala- Pro-Phe-AMC as shown in example 3.

The second glutenase, indicated as endopep-40, SEQ ID NO: 2, was contained in the <100kDa cut-off protein fraction (Table 1B) with a molecular weight of 39908.4 and a theoretical pI of 5.94. This protein resulted active in the pH range from 4 to 6 with a pH optimum of 5. When tested at pH 3 the enzyme shows 85% of its activity at pH 5 after 30 minutes of incubation at 37°C (Fig. 3). The fluorescent band highlighted by zymography shows a molecular weight of about 40kDa. Either active protein eluted from gel band or correspondent molecular size cut-off retentate were characterized for enzymatic activity. The MS-shot gun analysis revealed the presence of other proteins; the signals indicating the presence of endopep-140 sequences may be due to smaller polypeptides (<100 kDa) formed by its degradation.

The other three endopeptidases (indicated as endopep-120, SEQ ID NO: 3, endopep-60, SEQ ID NO: 4, endopep-41, SEQ ID NO: 5) were not purified to homogeneity. Their presence was detected by MS-shotgun in the secretome and in the active fractions of the first purification steps. The MS-shotgun analysis allowed the identification of their aminoacid sequence, here reported. Applicants named the proteins according to their inferred molecular weight. So, endopep-120 has a molecular weight of 112012.8 and a theoretical pI of 6.75, endopep-60 has a molecular weight of 59646.0 and a theoretical pI of 6.02, endopep-41 has a molecular weight of 41646.1 and a theoretical pI of 5.22.

1.4 Protein MS analysis: *shotgun MS experiments*

Today, proteomic methodologies are of primary importance for discovery-driven biomarker or protein characterization studies.

The classic proteomic approach is based on two-dimensional gel electrophoresis (2DG), where the protein spots of interest are isolated and identified by mass-spectrometry (MS).

The 2DG approach has a relatively high resolution, which is limited however by the difficulty in detecting certain classes of proteins. These include membrane proteins due to their low solubility in gel electrophoresis buffer, proteins with an either low (<10 kDa) or high (>200 kDa) molecular weight, as well as those with an extreme isoelectric point (pI <4 or >9). An additional limitation of this approach resides in the difficulty in analysis of less represented proteins and in the fact that it is tedious and time-consuming.

These problems are solved using a new proteomic methodology based on two-dimensional capillary chromatography coupled to tandem mass spectrometry (2DC-MS/MS), also named MudPIT (Multidimensional Protein Identification Technology) (Washburn, M.P., Wolters D., Yates J.R. 3rd "Large-scale analysis of the yeast proteome by multidimensional protein identification technology" Nat. Biotechnol., 2001, 19, 242-7). It involves the generation of peptides from enzymatic digestion of a complex protein mixture, their separation by means of two micro-HPLC columns and direct analysis of eluted peaks by MS/MS. 2DC-MS/MS combines ion exchange with reversed-phase separation of peptide mixtures obtained from direct digestion of total (or pre-fractioned) proteins. In particular, peptide mixtures are first separated by means of ion-exchange chromatography (SCX column, 5 µm, 0.3 ID ×150 mm) using seven steps of increasing ammonium chloride concentration (0 to 1000 mM). Each salt step is directly loaded onto the reversed phase column (C₁₈, 0.180 ID × 100 mm) and separated with an acetonitrile gradient: eluent A, 0.1% formic acid in water; eluent B, 0.1% formic acid in acetonitrile. Peptides eluted from the C₁₈ column are analyzed directly with an ion trap mass spectrometer; the limit of detection is around 10 fmol or less. Spectra are acquired in positive mode (typically in the range of 400-1600 m/z) using dynamic exclusion for MS/MS analysis. Different proteases are used to digest the protein extract from samples (trypsin, pepsin or proteinase K).

The identification of the corresponding proteins is then obtained through an automated database search with appropriate software, such as the SEQUEST algorithm (Eng, J. K., McKormack, A. L., Yates, J. R. "An Approach to Correlate Tandem Mass Spectra Data of Peptides with Amino Aminoacid Sequences in a Protein Database" J. Am. Soc. Mass Spectrom., 1994, 5, 976-984) for data handling of mass spectra. The experimental mass spectra produced are correlated to peptide sequences obtained by comparison with the theoretical mass spectra in the protein database downloaded from the NCBI (www.ncbi.nlm.nih.gov) or other websites.

1.5 Criteria for protein identification

Peptide identifications were accepted if they could be established at greater than 90.0% probability as specified by the Peptide Prophet algorithm (Keller A., Nesvizhskii A.I., Kolker E., Aebersold R. "Empirical statistical model to estimate the accuracy of peptide identifications made by MS/MS and database search" Anal. Chem., 2002, 15, 5383-92).

5 Protein identifications were accepted if they could be established at greater than 95.0% probability and contained at least one identified peptide. Because not more than 50% identity was found with the protein public databases, the genome of *Actinoallomurus* was sequenced and translated in the 6 possible reading frames. Subsequent alignment by BLAST of the detected peptides against the *Actinoallomurus* putative proteins identified the
10 known homologue proteins.

A total of 94 proteins were putatively identified in the secretome of *Actinoallomurus*. Enzymes constituted a significant fraction of them, 17 proteolytic enzymes were detected, and glycosidases, lipases, acid phosphatases and diesterases were also found. No function could be assigned by BLAST analysis to 25 sequences.

15 Among the proteases, five proteins belonging to the S8/S53 peptidase family could be detected, two of which were among the most represented proteins.

The same analyses were performed onto all subsequent purification steps until only few sequences were detected.

The data obtained by the MS analysis of the boiled active fractions showed few protein
20 sequences although a single band was present on silver stained SDS-PAGE gel. As shown in Table 1, endopep-140 (SEQ ID NO: 1) and endopep-40 (SEQ ID NO: 2) were detected as the proteins responsible of the two described activities. The presence of endopep-140 in the sample identified as < 100 kDa is possibly due to partial degradation of the protein in smaller polypeptides.

25 The *in silico* analysis indicated that both endopep-140 and endopep-40 are glycosylated proteins containing a signal peptide leading to secretion suggesting that they may be produced as preproenzymes.

The *in silico* analysis grouped all the five endopeps in the S8/S53 family and indicated that all are glycosylated proteins. High homology was found among endopep-140, endopep-40
30 and endopep-41 (SEQ ID NO: 5) allowing their clustering in a subgroup as shown by the phylogenetic tree of Fig.1. A signal peptide sequence was detected also for endopep-60 (SEQ ID NO: 4), while it was absent in endopep-120 (SEQ ID NO: 3) and endopep-41.

The phylogenetic tree shown in Fig.1 highlighted the novelty of these S8/S53 glutenases, which do not cluster with kumamolisin nor sedolisin nor with the tripeptidyl-peptidase

enzymes (sedA to sedD) disclosed in WO 2011/077359, that belong to the S53 family. The novelty of these enzymes were further supported by substrate specificity, as shown in Example 3. The degradation pattern of 33-mer obtained by digestion with the endopep-140 or endopep-40 showed an endo-proteolytic activity.

Example 2: endopeptidase production in recombinant host cells

2.1 Strains and plasmids.

Actinoallomurus sp. DSM 24988 was used in this study. All plasmid subcloning experiments were performed in *E. coli* DH10B (Invitrogen, Carlsbad, CA) using the plasmid pTZ57R/T (Fermentas, UAB, Lithuania).

Escherichia coli BL21(DE3) Star (Novagen Italia, Podenzano, PC) was used to produce heterologous (recombinant) peptidases.

2.2 Recombinant protease production

Recombinant *Actinoallomurus* proteases were produced and purified from *Escherichia coli* BL21(DE3) Star used as the expression system.

To construct *E. coli* strains producing Endopep-140 and Endopep-40 nucleotide sequences of genes were amplified using the following set of primers:

Fendopep-140 5'-AAAAAGCTTCAGCTACAGGTGTGGTCGG-3' SEQ ID NO: 12

Rendopep-140 5'-AAAAAACATATGCCCGATCTTCCCACCC-3' SEQ ID NO: 13

Fendopep-40 5'-AAAAAGCTTCAGAAGGCTCCGGTGCC-3' SEQ ID NO: 14

Rendopep-40 5'-AAAAAACATATGTCACGACGCGTGACCG-3' SEQ ID NO: 15

The PCR products were cloned into the pTZ57R/T vector and sequenced prior to cloning into the expression vector to verify that no mutation had been introduced during PCR amplifications.

Then endopep- genes were cloned into the *NdeI-HindIII* sites of pET28b plasmid (Novagen) and transformed into the expression host BL21(DE3) Star.

The transformed cells were grown in 50 ml cultures of LB media containing 30μ/ml of kanamycin at 37°C until OD600 0.6-1 was achieved. The expression of glutenases was induced with the addition of 0.2 μM isopropyl β-D-thiogalactoside (Sigma) and the cultures were further incubated at 22°C overnight. As shown in Fig. 4, protein bands corresponding to the desired molecular weight of endopep-140 and endopep-40 were obtained.

2.3 Purification of heterologously produced endopeptidase(s) .

The cells expressing the recombinant endopeptidases were centrifuged at 5000 g for 20 minutes. The pellet was resuspended in 8 ml of buffer solution (20 mM sodium phosphate 500 mM NaCl, pH 7.8), then lysozyme 1 mg/ml was added to lyse the cells completely.

His-tagged proteins were purified from crude cell extracts by immobilized Ni²⁺-affinity chromatography using the Ni-NTA Purification System (Invitrogen) according to the manufacturer's instructions. Five microliters aliquots of eluted fractions were migrated through a SDS-PAGE and stained with blue-coomassie or silver staining to verify the presence and the degree of purity of the expressed endopeptidases. Figure 4 shows the expression levels obtained for endopep-140 and endopep-40 after induction compared to the non-induced control strain.

Enzymatic activity of the total extracts obtained from *E. coli* strains transformed with pET28b empty and pET28b-endopep-140 or pET28b-endopep-40 respectively was tested with Succinyl-Ala-Ala-Pro-Phe-AMC as a substrate. Enzymatic activity was detected in the extracts from pET28b-endopeps strains while no activity was shown by the negative control.

Example 3: endopeptidase biological activity

3.1 Degradation of 33-mer toxic peptide of gliadin at acidic pH

A solution of 33-mer immunotoxic peptide of gliadin (50 μ M) was incubated at 37°C for up to 2 hours in the presence of 4 μ U of endopep-140 or 2 μ U endopep-40 in presence or absence of pepsin 1mg/ml. The reaction was carried out in acetic acid 20mM pH 3, total volume of 300 μ l. The reaction was monitored at different times from 0 to 120 min (t0 , t3, t15, t30, t60 and t120 min) at 37°C. Disappearance of the 33-mer peptide and appearance of degradation products was monitored by HPLC-MS analysis. 50 μ l aliquots were taken and the enzyme activity was stopped with 50 μ l H₂O: CH₃CN 50:50 (+ 0.1% formic acid). The samples were submitted to HPLC-MS, analyzed on LTQ-XL mass spectrometer (Thermo Fisher Scientific, San Jose, California, USA). The HPLC-MS profiles obtained with endopep-140, endopep-140 and pepsin, pepsin alone are reported in figures 5, 6, 7 respectively. The disappearance of the 33-mer peptide was evident after 2h incubation.

All peptides detected after incubation of the 33-mer peptide with 4 μ U of endopep-140 or 2 μ U of endopep-40 are reported in tables 2 and 3, respectively.

After 2 hours incubation, the most intense peak observed has a charge status of 1 and corresponds to [MH]⁺ of 748.4, indicating the presence of small peptides. This signal is also the first to appear. On the basis of the 33-mer sequence, four (1-6, 8-13, 15-20, 22-27) peptides correspond to this molecular mass, all being composed of 6 residues. According to

the MS analysis of the digested 33-mer, glutamic residues instead of glutamine residues are present in the hydrolyzed peptide sequence, thus suggesting a deamidation of the substrate. The analysis showed that after 2 hours of incubation of 33-mer with pepsin almost no hydrolysis was observed (Fig.7 and Tables 2 and 3) in agreement with literature data.

5 Increasing the amount of endopep-140 up to 8 μ U or of endopep-40 to 9 μ U resulted in complete 33-mer disappearance after 2h at pH 3 (not shown).

Even in the presence of pepsin the same pattern of degradation is observed although the amount of intact peptide after 2h is higher than in its absence, suggesting that the endopeptidases are not destroyed by pepsin.

10

3.2 HPLC/MS analysis

HPLC analysis were performed using ion trap mass spectrometer. LTQ-XL or Advantage coupled to Accela or Surveyor pump (Thermofisher Scientific, San Jose, CA, USA) were used in characterising the enzymatic protein hydrolyzates produced by the enzyme mixture.

15 The peptides were separated using a Thermofisher Scientific Hypersil gold or a C18 symmetry column (Waters, Milford, Mass., USA) in combination with a gradient of 0.1% formic acid in Milli Q water (Millipore, Bedford, Mass., USA) (Solution A) and 0.1% formic acid in acetonitrile (Solution B) for elution. The gradient started at 95% of Solution A and increased to 60% of Solution B. Detailed information on the individual peptides was
20 obtained by using the “scan dependent” MS/MS algorithm, which is a characteristic algorithm for an ion trap mass spectrometer. Full scan analysis was followed by zoom scan analysis for the determination of the charge state of the most intense ion in the full scan mass range. 33-mer (M=3910) was used to tune for optimal sensitivity in MS mode and for optimal fragmentation in MS/MS mode, performing constant infusion of 60 μ g/ml, resulting
25 in mainly doubly and triply charged species in MS mode, and an optimal collision energy of about 35% in MS/MS mode.

Example 4: endopeptidase biological activity

4.1 Degradation of gliadin and gluten hydrolyzed mixture

30 Owing to a specific structural feature, prolyl oligopeptidases cannot digest large peptides, usually longer than 30 aminoacids. Also, the tripeptidyl-protease sedolisin cannot hydrolyze gliadin without prolyl protease addition. This limitation is an obvious disadvantage for an enzyme, which is meant to hydrolyze as quickly and as efficiently as possible all potential toxic proline-rich peptides.

Endopep-140 and endopep-40 from *Actinoallomurus* were incubated with gliadins to see if the *Actinoallomurus* endoproteases are able to digest the whole proteins. The hydrolysis products formed were analyzed by SDS-PAGE.

A solution of gliadin was prepared by dissolving gliadin powder (Sigma G3375) in ethanol 70% at 70°C at a concentration of 10 mg/ml (20 µM). The intact gliadin molecules consist of a protein mixture of 40-55 kDa. Gliadin was incubated at 37° C with 10 µl (5µU) of the *Actinollomurus* endoproteases in acetic acid 20mM pH 3 in a final volume of 0.3 ml. The reactions were performed in a Thermomixer (Eppendorf AG, Hamburg, Germany) both in the absence and in the presence of pepsin at 1mg/ml (Fig. 8). The reactions were monitored at different times interval, 30 µl samples were withdrawn from the incubation mixture at time 0 min, 30 min, 1 and 2h and kept at 4° C until SDS-PAGE. All materials used for SDS-PAGE and staining were purchased from Bio-Rad (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Samples were prepared using sample buffer according to manufactures instructions and separated on any kd Bis-Tris gels using SDS buffer system according to manufactures instructions. Staining was performed using Coomassie R250 or silver.

As it can be seen in Figure 8, gliadin is cleaved by the *Actinollomurus* derived endopeptidases into small peptides to almost complete digestion in 2 hours. The decay of the product can be seen by the decrease in intensity of the band on the SDS gel. This experiment also shows that pepsin does not affect the efficacy of the digestion.

Table 1. Putative proteins purified from *Actinoallomurus* secretome.

Actinoallomurus purified fractions were filtered on 100 kDa molecular weight cut-off and submitted to MS-shotgun analysis. Identified peptides were aligned against proteins inferred by *Actinoallomurus* genome translation (Protein_Id: internal code for putative protein sequence) and subsequent *in silico* analysis by BLAST evidenced the homologue known proteins (BLAST accession number: sequence code; BLAST annotation: protein name; n.r.: no result). Numbers of matched spectra give a semiquantitative measure of protein amounts, indicated as frequency (F_average).

A: data obtained from >100 kDa fraction; B: data obtained from <100 kDa fraction

A

Protein_Id	BLAST accession number	BLAST annotation	F_ Average	notes
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Seq_48773	YP_003835151.1	Peptidase S8/S53	101,9	Endopep-140
Seq_100531	dbj BAJ32040.1	putative lipase	20,2	
Seq_293141	n.r.	n.r.	10.14	

B

Protein_Id	BLAST accession number	BLAST annotation	F_ Average	notes
Seq_44766	ZP_06921971.1	secreted protein	40,14	
Seq_72108	YP_003114375.1	Peptidase S8/S53	30,25	Endopep-40
Seq_48773	YP_003835151.1	Peptidase S8/S53	20,15	Endopep-140
Seq_293141	n.r.	n.r.	10,15	

5 **Table 2.** Peptides released by 33-mer digestion with endopep-140.

All molecular weights detected by HPLC-MS after digestion of 33-mer gliadin peptide (50 μ M) with 4 μ U endopep-140 in the presence and absence of pepsin (1 mg/ml). Control is obtained by incubating the 33-mer with pepsin alone. Incubation at 37°C, acetic acid 20mM, pH3.

1	5	10	15	20	25	30																			
L	Q	L	Q	P	F	P	Q	P	O	L	P	Y	P	Q	P	Q	L	P	Y	P	Q	P	Q	P	F

Incubation time	Mass signal	Charge status	Deduced sequence	peptide	Notes
T₀	1306.27; 1956,64	3 [M-3H] ¹⁺ ; 2 [M-2H] ⁺	1-33		100% residual
T₃₀	1088.73	1 [MH] ⁺	25-33		
	1197.45	1 [MH] ⁺	1-10		
	748.45	1 [MH] ⁺	1-6; 8-13; 15-20; 22-27		
	1306.27; 1956,64	3 [M-3H] ¹⁺ ; 2 [M-2H] ⁺	1-33		50% residual
T₃₀ + pepsin	1088.73	1 [MH] ⁺	25-33		
	1197.45	1 [MH] ⁺	1-10		
	748.45	1 [MH] ⁺	1-6; 8-13; 15-20; 22-27		
	1306.27; 1956,64	3 [M-3H] ¹⁺ ; 2 [M-2H] ⁺	1-33		70% residual
T_{2h}	1088.73	1 [MH] ⁺	25-33		
	1197.45	1 [MH] ⁺	1-10		
	748.45	1 [MH] ⁺	1-6; 8-13; 15-20; 22-27		Most intense peak
	1068.24	1 [MH] ⁺	16-24		
	845.27	1 [MH] ⁺	1-7		traces
	1306.27; 1956,64	3 [M-3H] ¹⁺ ; 2 [M-2H] ⁺	1-33		20% residual
T_{2h} + pepsin	1088.8	1 [MH] ⁺	25-33		
	1197.45	1 [MH] ⁺	1-10		
	748.45	1 [MH] ⁺	1-6; 8-13; 15-20; 22-27		
	1068.24	1 [MH] ⁺	16-24		
	845.27	1 [MH] ⁺	1-7		
	1306.27; 1956,64	3 [M-3H] ¹⁺ ; 2 [M-2H] ⁺	1-33		45% residual
Control T_{2h}	1306.27; 1956,64	3 [M-3H] ¹⁺ ; 2 [M-2H] ⁺	1-33		95% residual

Table 3. Peptides released by 33-mer digestion with endopep-40.

All molecular weights detected by HPLC-MS after digestion of 33-mer gliadin peptide (50 μ M) with 2 μ U endopep-40 in the presence and absence of pepsin (1 mg/ml) at different time intervals. Control is obtained by incubating the 33-mer with pepsin alone. Incubation at 37°C, acetic acid 20mM, pH3.

1	5	10	15	20	25	30																			
L	Q	L	Q	P	F	P	Q	P	O	L	P	Y	P	Q	P	Q	L	P	Y	P	Q	P	Q	P	F

Incubation time	Mass signal	Charge status	Deduced sequence	peptide	notes
T₀	1306.27; 1956,64	3 [M-3H] ¹⁺ ; 2 [M-2H] ⁺	1-33		100% residual
T₃₀	1197.45	1 [MH] ⁺	1-10		
	1088.73	1 [MH] ⁺	25-33		
	748.45	1 [MH] ⁺	1-6; 8-13; 15-20; 22-27		
	1306.27; 1956,64	3 [M-3H] ¹⁺ ; 2 [M-2H] ⁺	1-33		50% residual
T₃₀ + pepsin	1088.73	1 [MH] ⁺	25-33		
	1197.45	1 [MH] ⁺	1-10		
	748.45	1 [MH] ⁺	1-6; 8-13; 15-20; 22-27		
	1306.27; 1956,64	3 [M-3H] ¹⁺ ; 2 [M-2H] ⁺	1-33		70% residual
T_{2h}	1088.73	1 [MH] ⁺	25-33		
	1197.45	1 [MH] ⁺	1-10		
	748.45	1 [MH] ⁺	1-6; 8-13; 15-20; 22-27		
	1306.27; 1956,64	3 [M-3H] ¹⁺ ; 2 [M-2H] ⁺	1-33		20% residual
T_{2h} + pepsin	1088.8	1 [MH] ⁺	25-33		
	1197.45	1 [MH] ⁺	1-10		
	748.45	1 [MH] ⁺	1-6; 8-13; 15-20; 22-27		
	1306.27; 1956,64	3 [M-3H] ¹⁺ ; 2 [M-2H] ⁺	1-33		30% residual
Control T_{2h}	1306.27; 1956,64	3 [M-3H] ¹⁺ ; 2 [M-2H] ⁺	1-33		95% residual

CLAIMS

1. An enzyme composition comprising at least one endopeptidase of the S8/S53 family active at pH between 3 and 8 selected from the group consisting of:

a) endopep-140 comprising SEQ ID NO: 1, a biologically active fragment thereof, a naturally occurring allelic variant thereof, or a sequence having at least 50%, 60%, 70%, 80%, 90%, or 95% of identity,

b) endopep-40 comprising SEQ ID NO: 2, a biologically active fragment thereof, a naturally occurring allelic variant thereof, or a sequence having at least 50%, 60%, 70%, 80%, 90%, or 95% of identity,

c) endopep-120 comprising SEQ ID NO: 3, a biologically active fragment thereof, a naturally occurring allelic variant thereof, or a sequence having at least 50%, 60%, 70%, 80%, 90%, or 95% of identity,

d) endopep-60 comprising SEQ ID NO: 4, a biologically active fragment thereof, a naturally occurring allelic variant thereof, or a sequence having at least 50%, 60%, 70%, 80%, 90%, or 95% of identity, and

e) endopep-41 comprising SEQ ID NO: 5, a biologically active fragment thereof, a naturally occurring allelic variant thereof, or a sequence having at least 50%, 60%, 70%, 80%, 90%, or 95% of identity.

2. An enzyme composition of claim 1 wherein the endopeptidase(s) is/are selected from the group consisting of:

a) endopep-140 comprising SEQ ID NO: 1 or a sequence having at least 95% of identity,

b) endopep-40 comprising SEQ ID NO: 2 or a sequence having at least 95% of identity,

c) endopep-120 comprising SEQ ID NO: 3 or a sequence having at least 95% of identity,

d) endopep-60 comprising SEQ ID NO: 4 or a sequence having at least 95% of identity, and

e) endopep-41 comprising SEQ ID NO: 5 or a sequence having at least 95% of identity.

3. An enzyme composition as in any one of claims 1 and 2 wherein the endopeptidase is selected from the group consisting of:

a) endopep-140 comprising SEQ ID NO: 1 or a sequence having at least 95% of identity, and

b) endopep-40 comprising SEQ ID NO: 2 or a sequence having at least 95% of identity.

4. An enzyme composition as in any one of claims 1 to 3 wherein the endopeptidase(s) is/are obtainable from an *Actinoallomurus* strain.

5. An enzyme composition as in claim 4 wherein the endopeptidase(s) is/are obtainable from *Actinoallomurus* sp. DSM24988.

6. An enzyme composition as in any one of claims 1 to 5 which is able to hydrolyze gluten oligopeptides which are resistant to cleavage by gastric and pancreatic enzymes and whose presence in the intestinal lumen results in toxic effects.

7. An enzyme composition as in any one of claims 1 to 6 which comprises one or more
5 other proteolytic enzymes selected from prolyl-endoproteases, x-prolyl-dipeptidyl aminopeptidases and prolyl-aminopeptidases.

8. An enzyme composition as in any one of claims 1 to 7 wherein the endopeptidase(s) is/are operatively fused to another polypeptide to form chimeric or tapped protein(s).

9. An isolated endopeptidase of the S8/S53 family active at pH between 3 and 8
10 selected from:

a) endopep-140 comprising SEQ ID NO: 1 or a sequence having at least 95% of identity,

b) endopep-40 comprising SEQ ID NO: 2 or a sequence having at least 95% of identity,

c) endopep-120 comprising SEQ ID NO: 3 or a sequence having at least 95% of identity,

d) endopep-60 comprising SEQ ID NO: 4 or a sequence having at least 95% of identity, and

15 e) endopep-41 comprising SEQ ID NO: 5 or a sequence having at least 95% of identity.

10. An isolated endopeptidase of the S8/S53 family of claim 9 which is selected from

a) endopep-140 comprising SEQ ID NO: 1 or a sequence having at least 95% of identity, and

b) endopep-40 comprising SEQ ID NO: 2 or a sequence having at least 95% of identity.

20 11. An enzyme composition as in any one of claims 1 to 8 or an isolated endopeptidase of the S8/S53 family as in any one of claims 9 and 10 for use as a medicament for the treatment or prevention of the celiac sprue, dermatitis herpetiformis and/or any other disorder associated with gluten intolerance.

25 12. Use of an enzyme composition as in any one of claims 1 to 8 or at least one isolated endopeptidase of the S8/S53 family as in any one of claims 9 and 10 to produce protein hydrolyzates used for food and drinks.

13. A pharmaceutical formulation which comprises as the active proteolytic ingredient an enzyme composition as in any one of claims 1 to 8 or at least one isolated endopeptidase of the S8/S53 family as in any one of claims 9 and 10.

30 14. A pharmaceutical formulation of claim 13 which is an oral pharmaceutical formulation.

15. A food supplement which comprises as active proteolytic ingredient an enzyme composition as in any one of claims 1 to 8 or at least one isolated endopeptidase of the S8/S53 family as in any one of claims 9 and 10.

16. An isolated nucleic acid encoding for at least one endopeptidase of any one of claims 1 to 10 which comprises at least one polynucleotide sequence selected from SEQ ID NOs: 7, 8, 9, 10 and 11, or at least one polynucleotide sequence having at least 50%, 60%, 70%, 80%, 90% or 95% of identity to any one of SEQ ID NOs: 7, 8, 9, 10 and 11.

5 17. An isolated nucleic acid as in claim 16 which comprises at least one polynucleotide sequence having at least 95% of identity to any one of SEQ ID NOs: 7, 8, 9, 10 and 11.

18. An isolated nucleic acid as in any one of claims 16 and 17 which comprises at least one polynucleotide sequence selected from SEQ ID NOs: 7 and 8.

19. A process for producing an enzyme composition as in claim 1 which comprises:

10 A) cultivating a naturally occurring *Actinoallomurus* strain capable of producing at least one endopeptidase of the S8/S53 family as defined in claim 1 in a culture medium under conditions suitable for producing the enzyme composition and recovering the enzyme composition from the cultivation batch, or

B) cultivating an *Actinoallomurus* strain derived by conventional mutation and/or selection
15 procedures from a naturally occurring strain as defined under A), which maintains the capability of producing at least one endopeptidase of the S8/S53 family, in a culture medium under conditions suitable for producing the enzyme composition and recovering the enzyme composition from the cultivation batch, or

C) introducing into a host cell a nucleic acid encoding for at least one endopeptidase of the
20 S8/S53 family selected from the group consisting of:

a) endopep-140 comprising SEQ ID NO: 1, a biologically active fragment thereof, a naturally occurring allelic variant thereof, or a sequence having at least 50%, 60%, 70%, 80%, 90%, or 95% of identity,

b) endopep-40 comprising SEQ ID NO: 2, a biologically active fragment thereof, a naturally
25 occurring allelic variant thereof, or a sequence having at least 50%, 60%, 70%, 80%, 90%, or 95% of identity,

c) endopep-120 comprising SEQ ID NO: 3, a biologically active fragment thereof, a naturally occurring allelic variant thereof, or a sequence having at least 50%, 60%, 70%, 80%, 90%, or 95% of identity,

30 d) endopep-60 comprising SEQ ID NO: 4, a biologically active fragment thereof, a naturally occurring allelic variant thereof, or a sequence having at least 50%, 60%, 70%, 80%, 90%, or 95% of identity, and

e) endopep-41 comprising SEQ ID NO: 5, a biologically active fragment thereof, a naturally occurring allelic variant thereof, or a sequence having at least 50%, 60%, 70%, 80%, 90%,

or 95% of identity,

cultivating the cell in a culture medium under conditions suitable for producing the endopeptidase(s) and recovering the endopeptidase(s) from the cultivation batch.

20. A process as in claim 19, paragraph A) or B), wherein the *Actinoallomurus* strain is replaced by a strain belonging to the genus *Catenulispora*, *Ktedonobacter* or *Streptomyces* capable of producing at least one endopeptidase as defined in claim 18.

21. A process as in claim 19 wherein the *Actinoallomurus* strain is *Actinoallomurus sp. DSM 24988*.

22. A process as in claim 19, paragraph C), wherein the nucleic acid comprises at least one of the polynucleotide sequences identified in any one of claims 16 and 17.

23. A process as in claim 22, wherein the nucleic acid comprises at least one of the polynucleotide sequences SEQ ID NOs: 7 and 8 or at least one of the polynucleotide sequence having at least 95% identity to any one of SEQ ID NOs: 7 and 8.

24. A process as in any one of claims 19, paragraph C), 22 and 23, wherein the host cell is a microorganism selected from *Bacillus*, *Streptomyces*, *Lactobacillus*, *Pyrococcus*, *Pseudomonas* and *Escherichia coli*.

25. A process as in claim 24 wherein the host cell is *Escherichia coli DH10B*.

26. The strain *Actinoallomurus DSM 24988*.

27. A method of degrading gluten oligopeptides which are resistant to cleavage by gastric and pancreatic enzymes and whose presence in the internal lumen results in toxic effects which comprises contacting said gluten oligopeptides with an enzyme composition as in any one of claims 1 to 8 or at least one isolated endopeptidase as in any one of claims 9 and 10.

28. A method as in claim 27 for the treatment or prevention of celiac sprue, dermatitis herpetiformis and/or any other disorder associated with gluten intolerance which comprises administering to a patient in need thereof an effective amount of an enzyme composition as in any one of claims 1 to 8 or at least one isolated endopeptidase as in any one of claims 9 and 10.

29. A method as in claim 28 wherein the enzyme composition or the isolated endopeptidase is incorporated into a pharmaceutical formulation, a food supplement, a drink or beverage.

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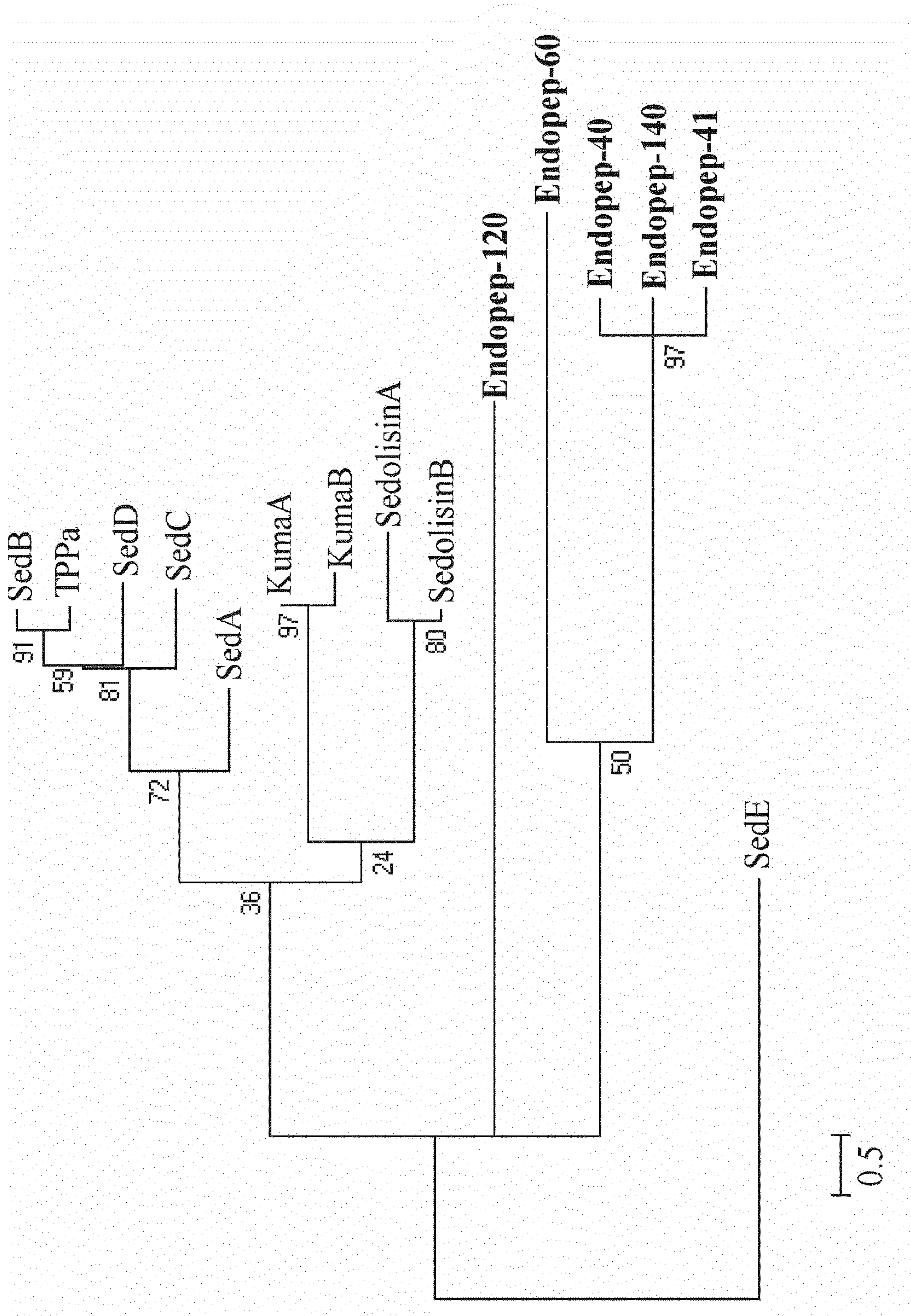
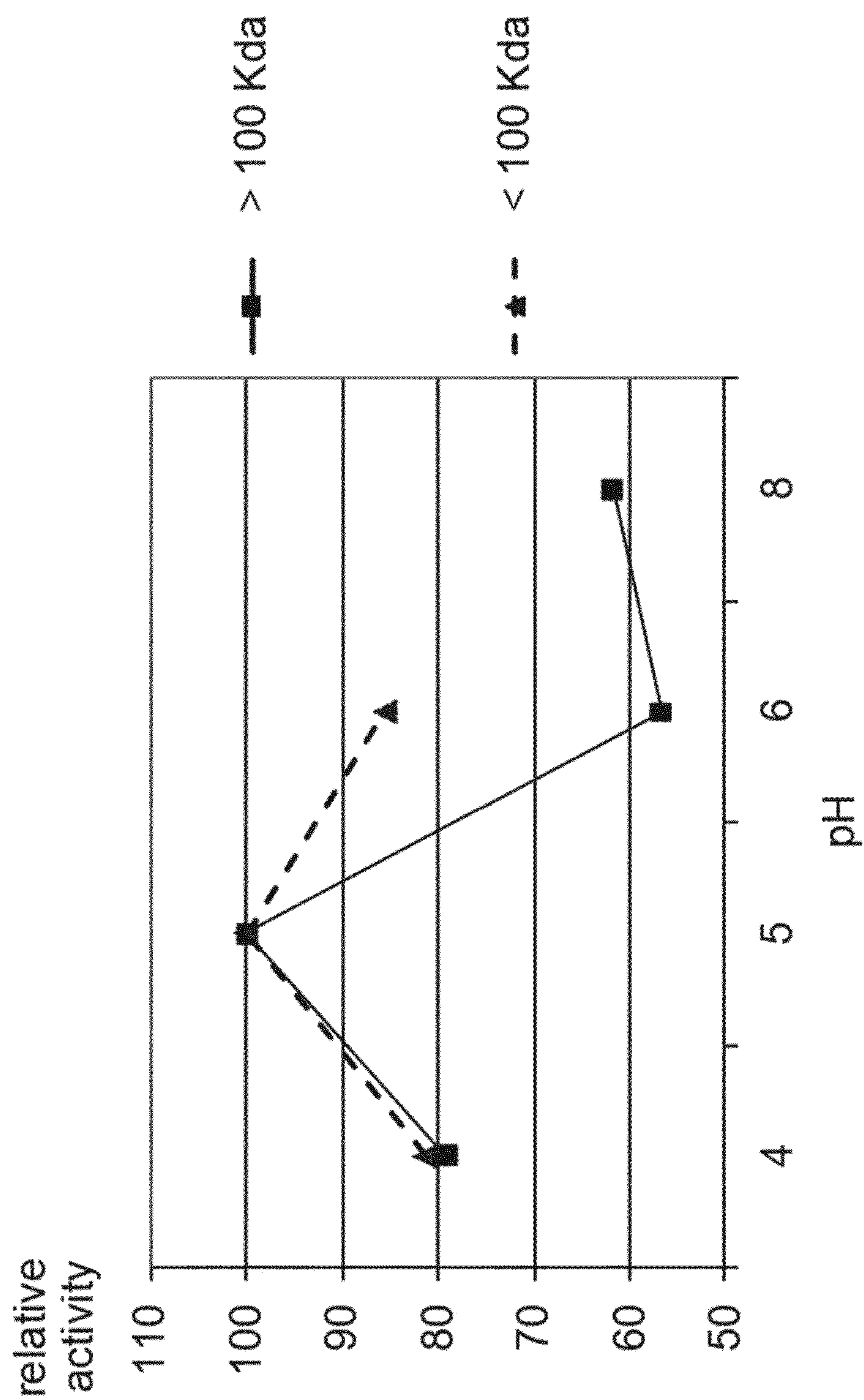
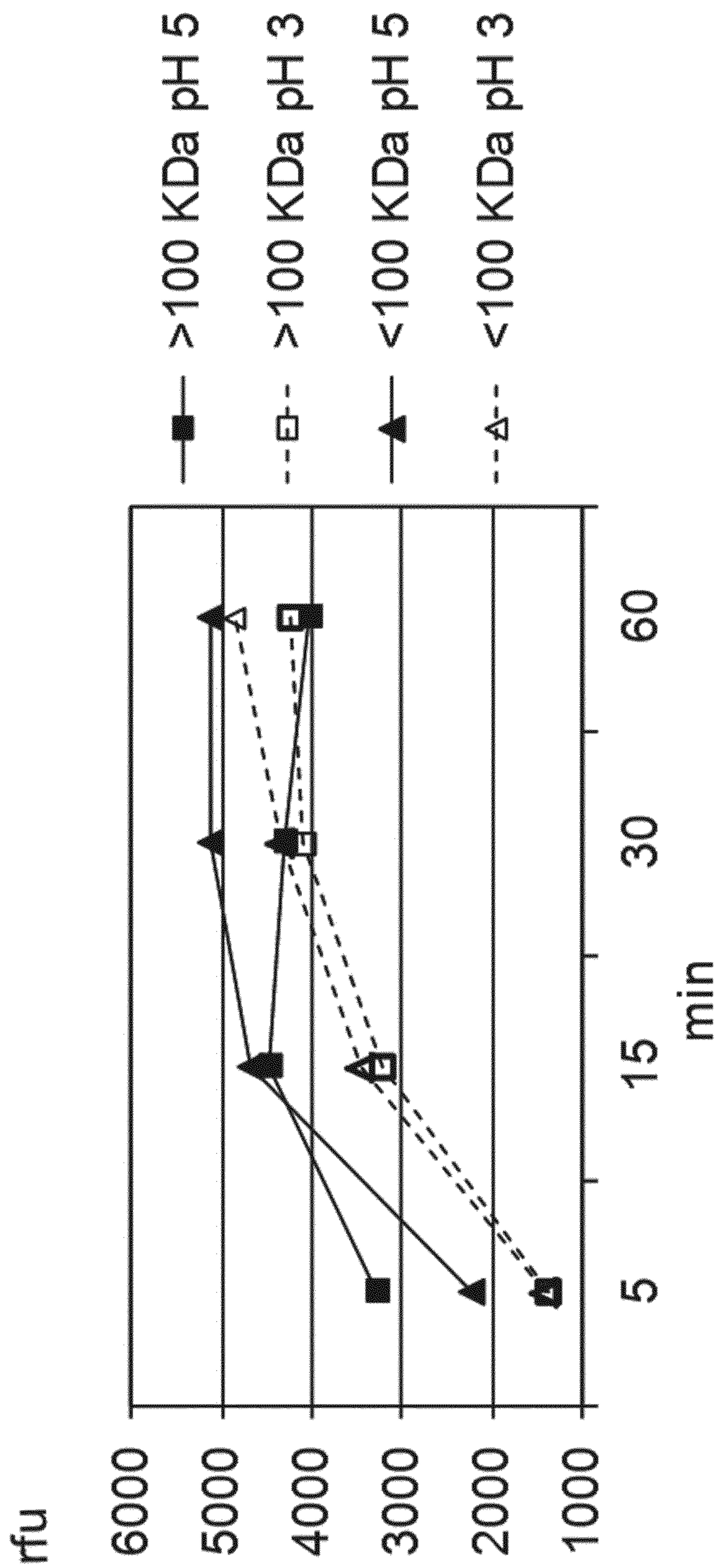


Fig. 1

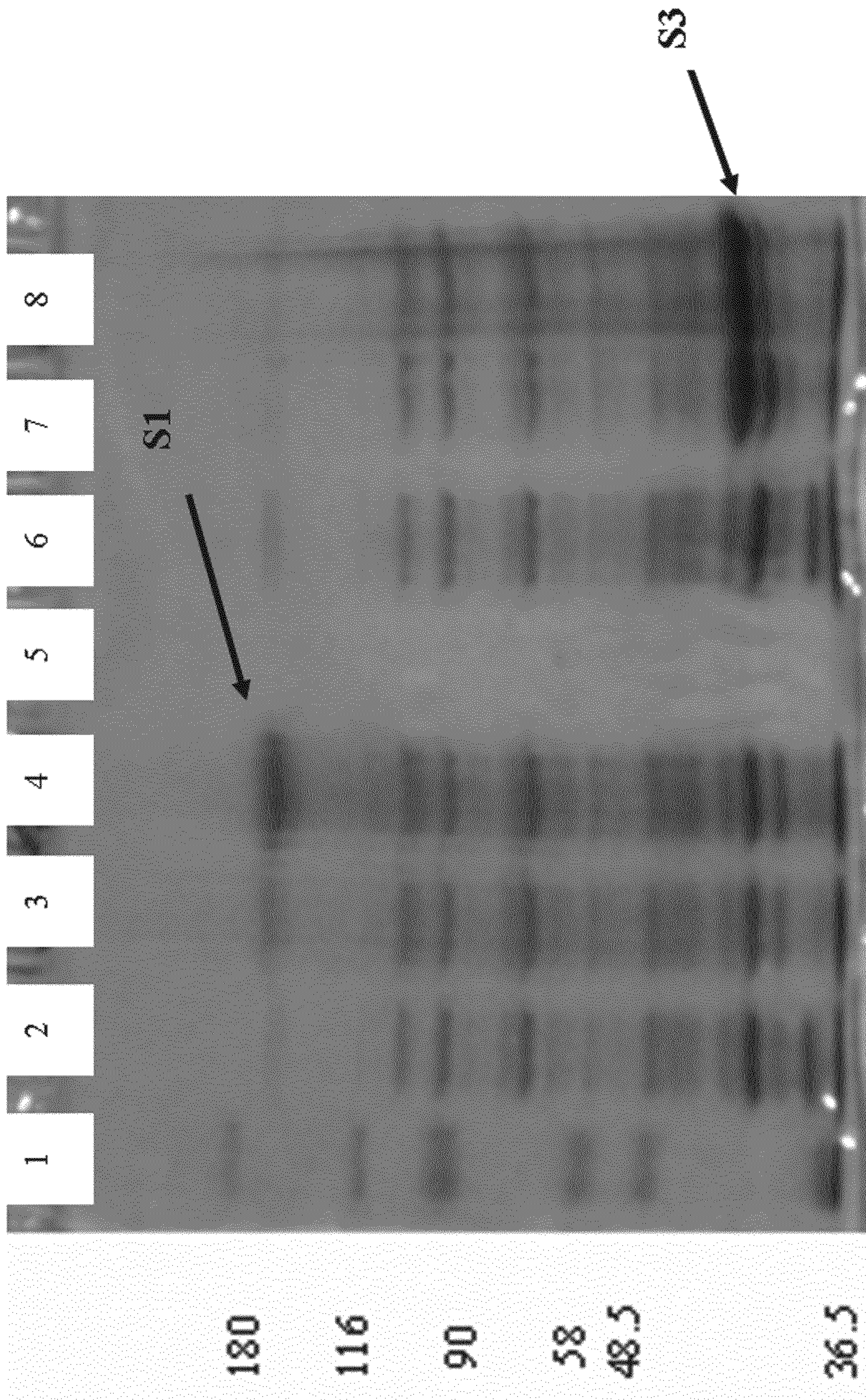
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*Fig. 2*

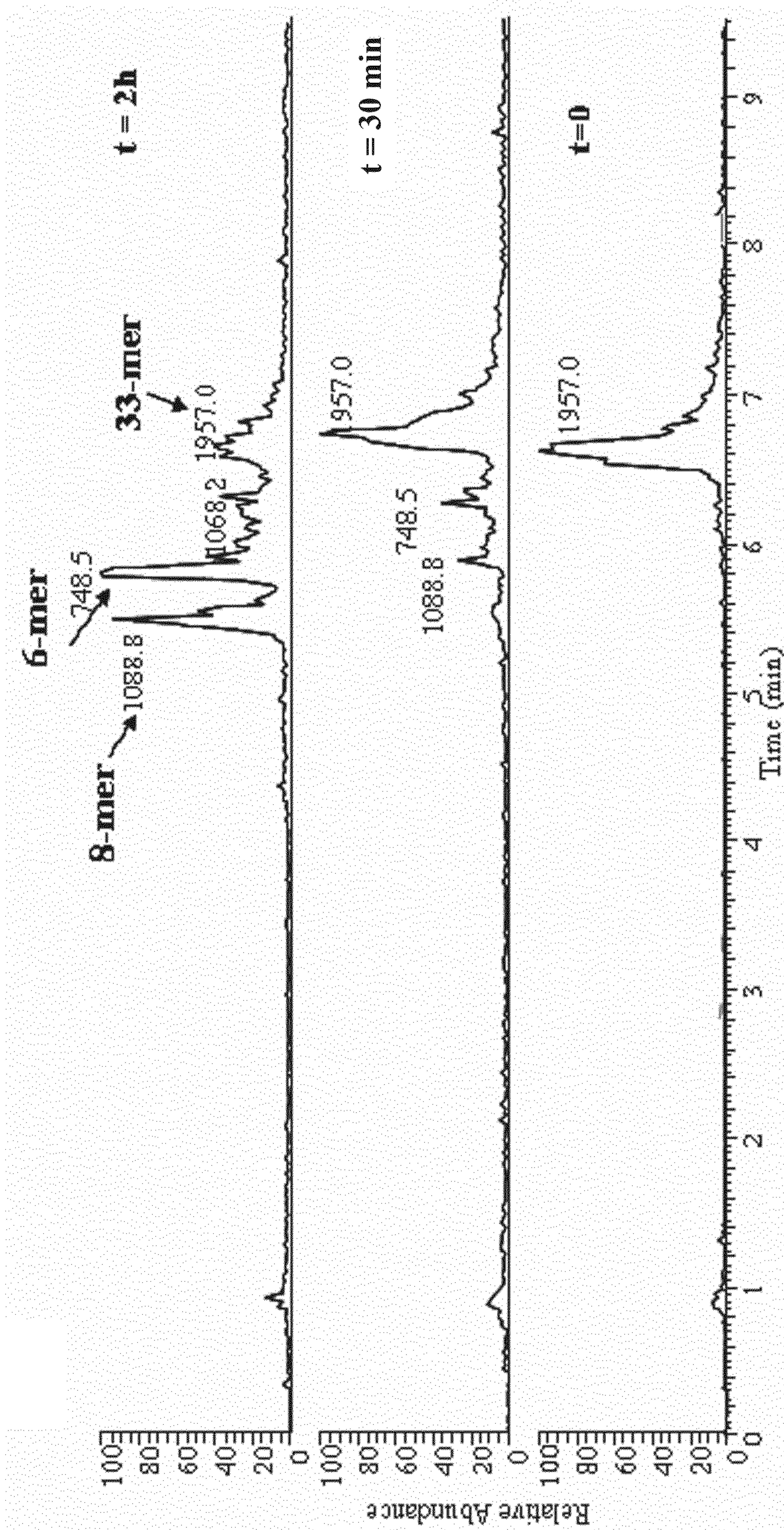
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*Fig. 3*

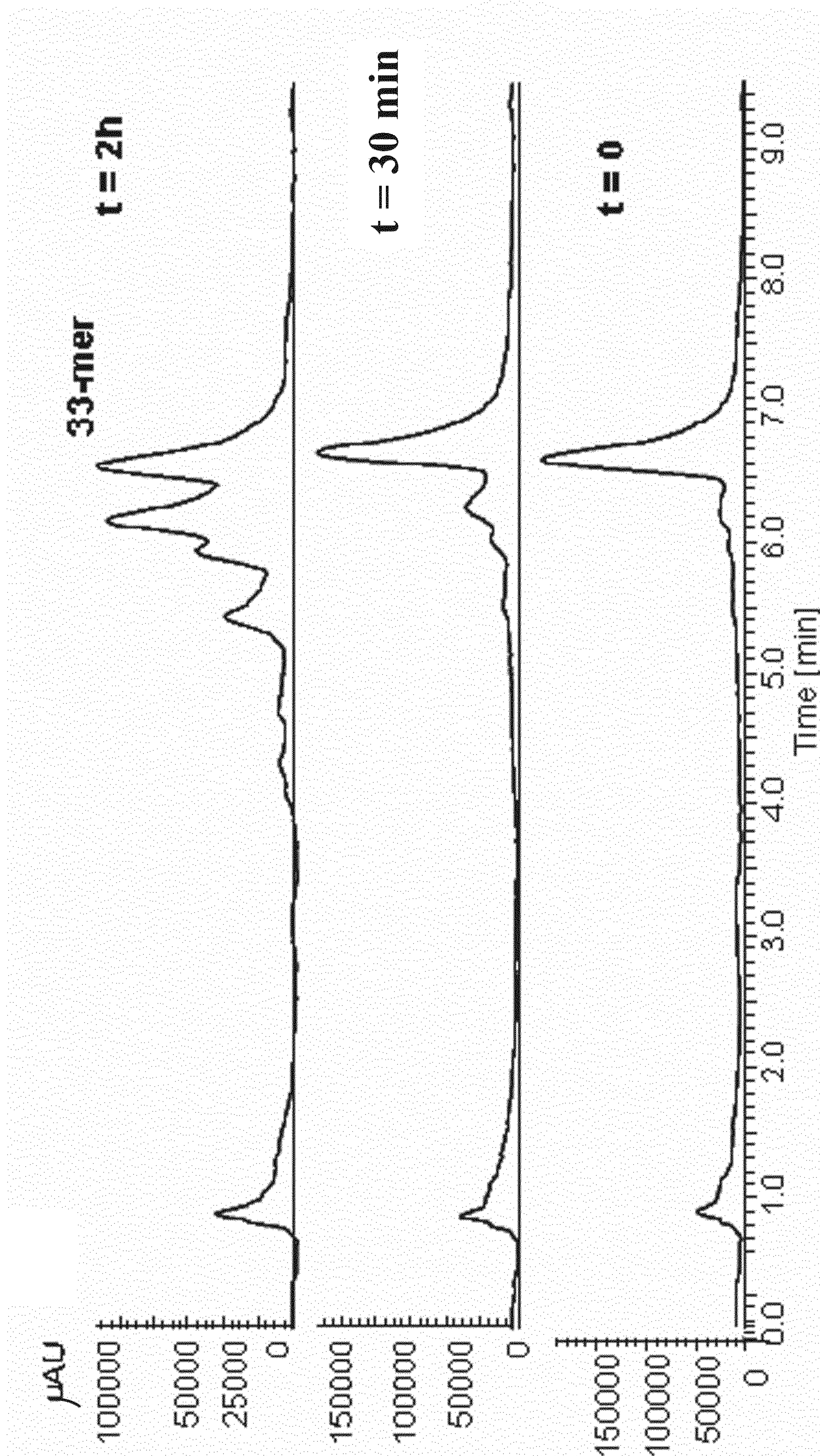
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**Fig. 4**

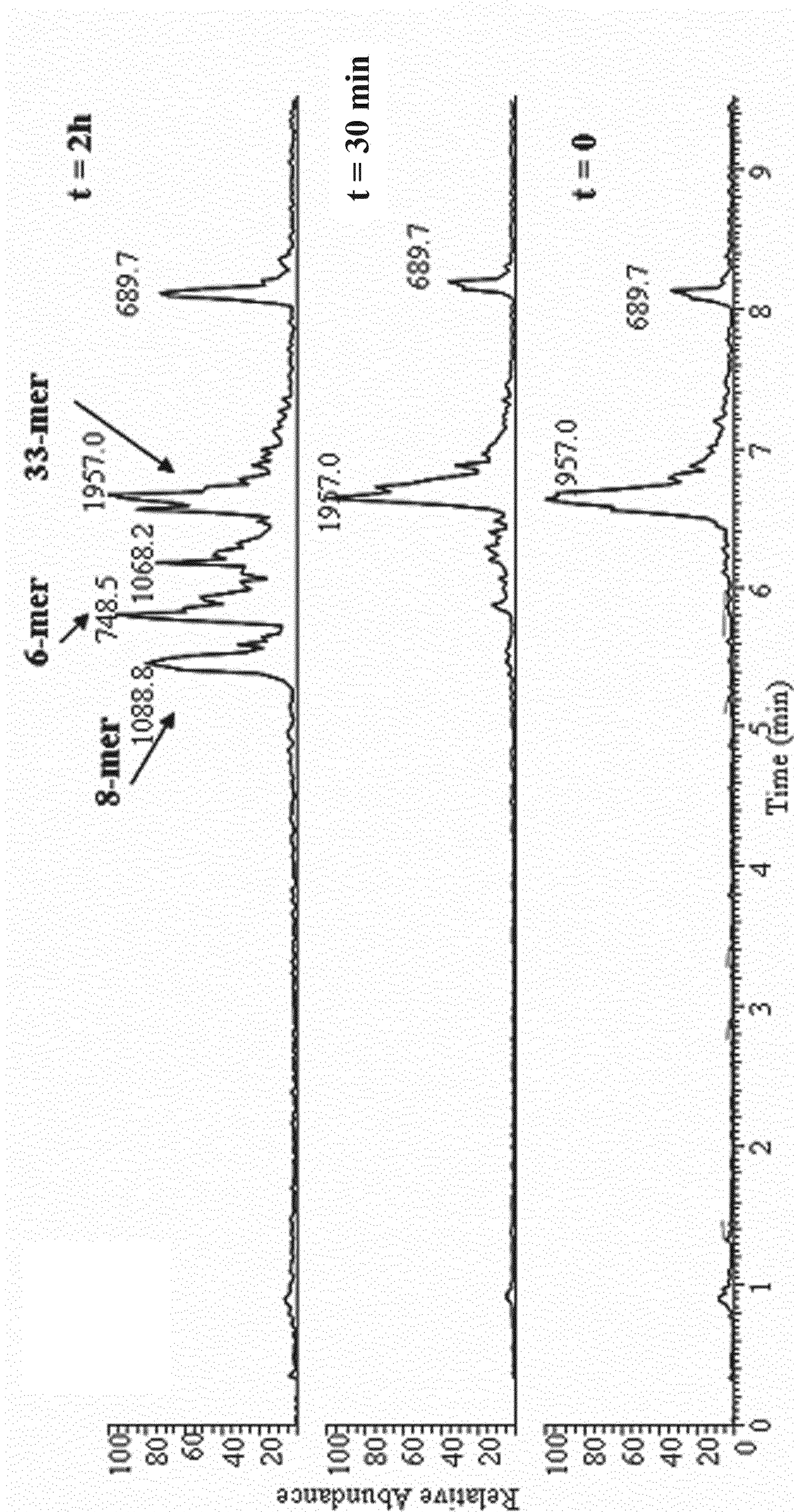
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**Fig. 5A**

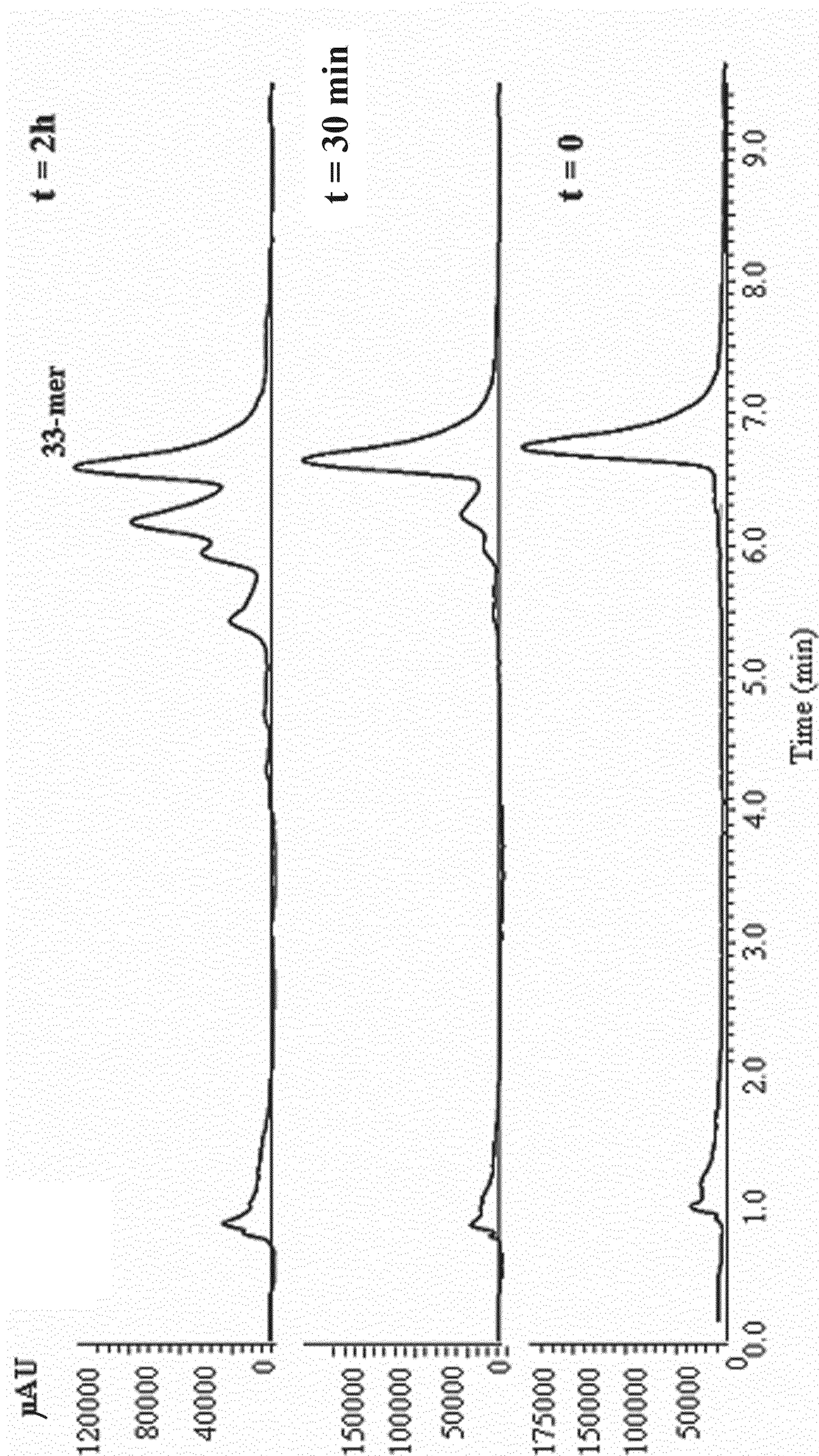
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*Fig. 5B*

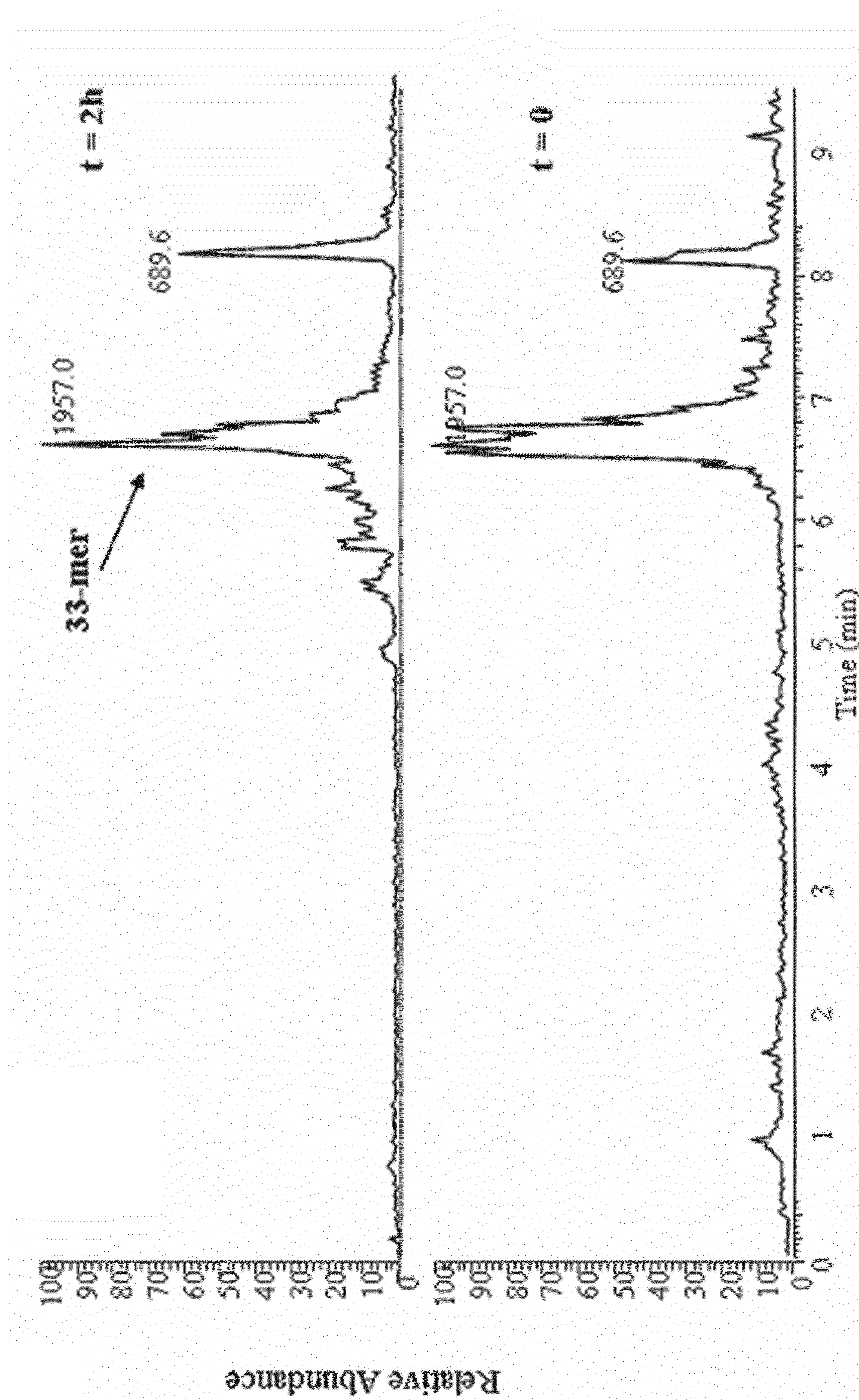
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*Fig. 6A*

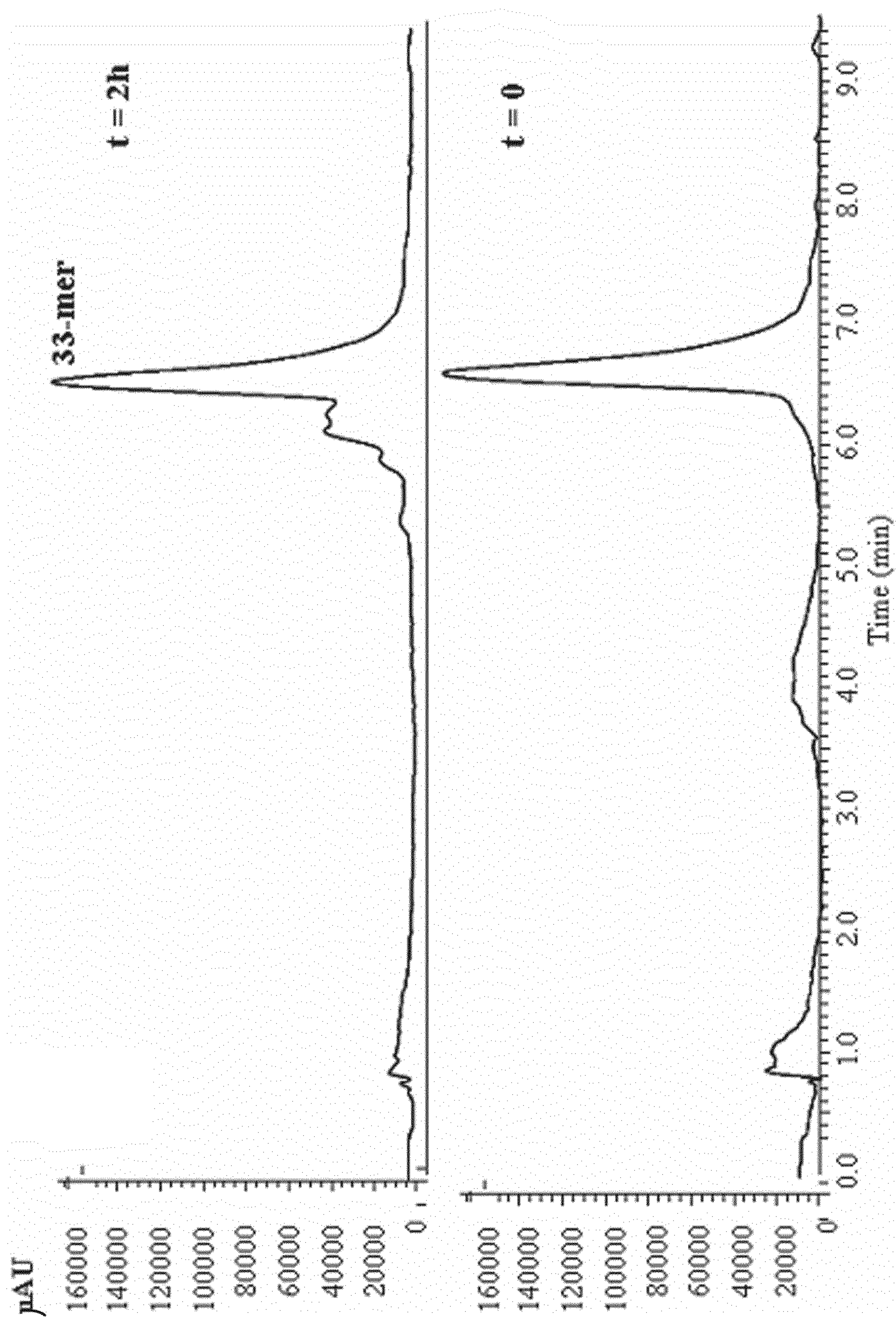
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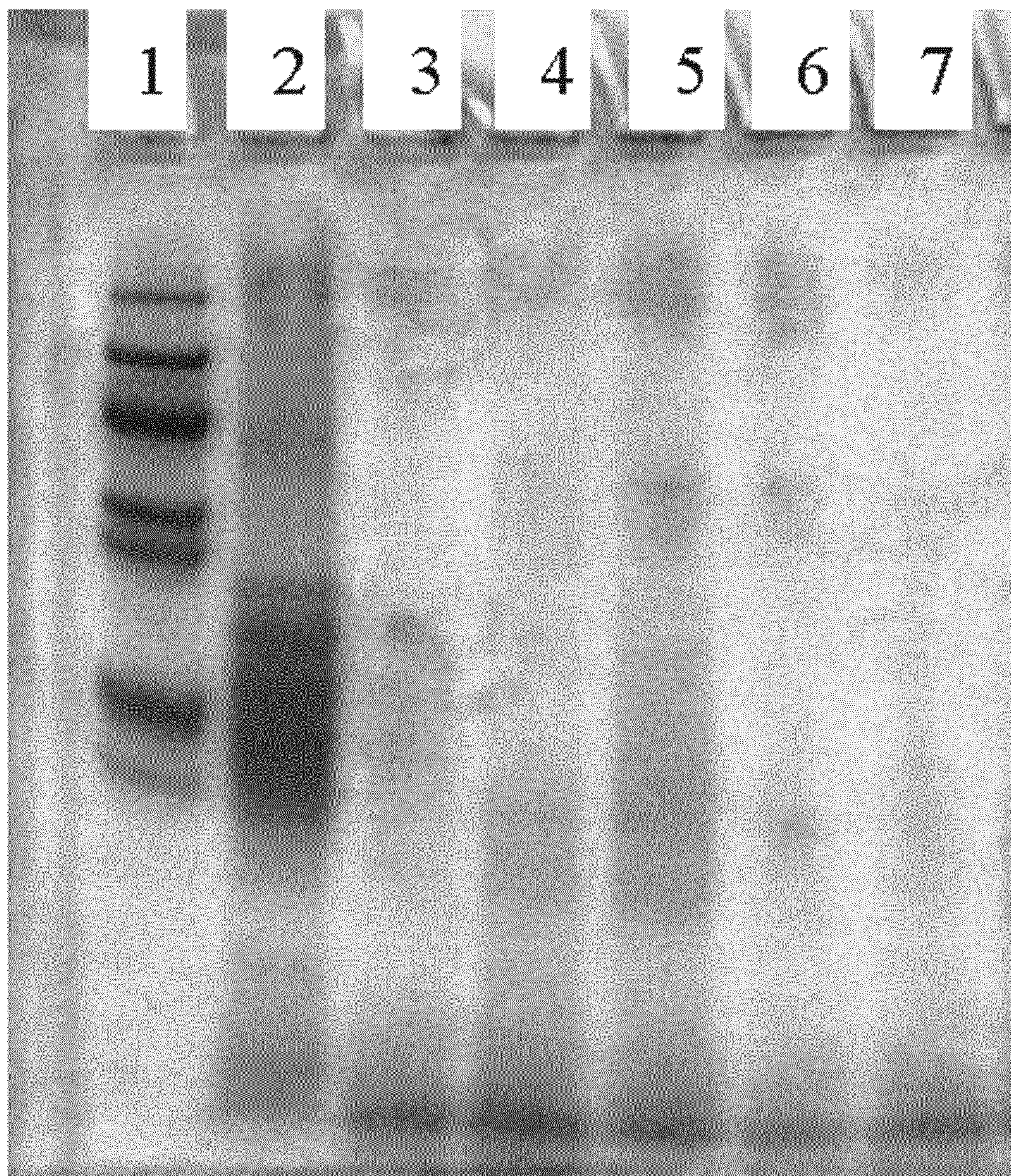
**Fig. 6B**

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**Fig. 7A**

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**Fig. 7B**

11/11***Fig. 8***