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Determination of key enzyme activities in commercial peptidase and lipase preparations from microbial or animal sources

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Abstract

The enzyme complement of a selection of commercial food-grade peptidase and lipase preparations was investigated. Each preparation was assayed for protein content, proteinase activity at pH 5.5 and 7.0 at 37 °C using azocasein and semi-quantitatively assayed for lipase, peptidase, proteinase, phosphatase and glycosidase activity by the API-ZYM system. Each peptidase preparation was also assayed for various endo-, carboxy-, amino- and di-peptidase activities at pH 5.5 and 7.0 at 37 °C, using chromogenic or fluorogenic substrates, while each lipase preparation was assayed for esterase and lipase activity at pH 7.0 at 37 °C using *p*-nitrophenol substrates. All enzyme preparations were found to contain enzyme activities in addition to their stated main activity. According to the API-ZYM system the peptidase preparations contained varying levels of lipase, proteinase, peptidase, phosphatase and glycosidase activity, with the lipase preparations containing lipase, phosphatase and glycosidase activity. Only two peptidase and two lipase preparations contained significant amounts of proteinase activity as measured by azocasein. The peptidase and lipase activities of the preparations appeared to be dependent upon source. Most peptidase preparations had significantly more activity at pH 7.0 than at 5.5.
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1. Introduction

Peptidases and lipases are used in the dairy industry for a number of applications, including cheese production, accelerated cheese ripening [1], enzyme-modified cheese (EMC) production [2,3] and the manufacture of protein (casein and whey) hydrolysates [4–6].

Commercial peptidases are generally derived from lactic acid bacteria (LAB) or from *Aspergillus oryzae* and are very important in flavour development and debittering in cheese and protein hydrolysate production. A number of specific peptidase activities are known to be critical for these purposes [7,8]. Endopeptidases, such as Pep O, cleave large peptides internally, creating more substrate molecules for other peptidase activities. Aminopeptidases, such as Pep N and Pep M, remove a wide range of amino acids from the N-terminus of peptides. For the purpose of this study, lysyl aminopeptidase was abbreviated Pep N, while membrane alanyl aminopeptidase was abbreviated Pep M; both of these enzymes have a low substrate specificity, but can be distinguished on the basis that Pep N preferentially releases lysine, whilst Pep M preferentially releases phenylalanine from a peptide. Specific aminopeptidases, such as aminopeptidase A (Pep A), preferentially remove aspartic or glutamic acid from the N-terminus of peptides. Carboxypeptidases cleave amino acids from the C-terminus of peptides and therefore may also have a role in debittering and flavour generation, although few are available commercially. Peptidases which specifically remove proline from peptides are particularly important in debittering applications, because the removal of proline alters the three-dimensional structure of peptides and increases their susceptibility to hydrolysis [9–12]. Key proline-specific peptidases are post-prolyl dipeptidyl aminopeptidase (Pep X), aminopeptidase P (Pep P) and proline iminopeptidase (Pep I).

Commercial lipases are generally produced from animal (pancreatic and pregastric tissues of ruminants) or fungal sources (*Penicillium* spp., *Aspergillus* spp., *Rhizopus* spp., *Rhizomucor* spp., *Mucor* spp. or *Candida* spp.). Animal lipases are highly specific for the liberation of free fatty acids (FFA) from the sn-1 and -3 positions [13,14], while those derived from microbial sources tend to have a much wider range of activity and specificity [15–17]. The distinction

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between lipases and esterases remains unclear, however it was suggested that a preferable definition for lipases is that they are esterases which can hydrolyse long-chain tri-aclyglycerides [15]. So therefore, in this study, lipase activity describes the preferential release of medium and long-chain FFA, while esterase activity refers to the preferential release of short-chain FFA from tri-acylglycerides.

Generally, commercial enzyme preparations are not pure due to high costs involved in purification and may contain several enzyme activities, which may negatively influence their overall reaction characteristics, e.g., by generating unwanted off-flavours, such as bitterness or rancidity. Thus, it is important that the complete enzymatic complement of commercial preparations is known prior to their use.

In this study, a selection of commercial peptidase and lipase preparations were assayed for a range of enzyme activities to determine their overall suitability for various processing applications used in the dairy industry, such as accelerated cheese ripening, enzyme-modified cheese and protein hydrolysate production. Each preparations was assayed semi-quantitatively for esterase, lipase, trypsin-like, chymotrypsin-like, general aminopeptidase, phosphatase and glycosidase activities using the API-ZYM system and for proteinase activity on azocasein at pH 7.0 and 5.5. In addition, a number of key peptidase activities useful for flavour development and debittering were quantified in each of the commercial peptidase preparations at pH 5.5 and 7.0. Each lipase preparation was assayed for esterase activity using the synthetic substrate, *p*-nitrophenol butyrate, and for lipase activity using *p*-nitrophenol palmitate at pH 7.0.

2. Materials and methods

2.1. Commercial enzyme preparations

Seven peptidase preparations, were obtained as gifts from five manufacturers:

Amano Enzyme Europe Ltd. (Roundway House, Cromwell Park, Chipping Norton, Oxfordshire, OX7 5SR, UK).
Biocatalysts Ltd. (Main Avenue, Pontypridd, CF 37 5UT, UK).
Novozymes A/S (Krogshoejvej, 36 2880 Bagsvaerd, Denmark).
Rhodia Food (Poleacre Lane, Stockport, Cheshire, SK6 1PQ, UK).
Rohm Enzyme GmbH (Kirschenallee, D-64275, Darmstadt, Germany).

Seventeen lipase preparations, were obtained as gifts from six manufacturers:

Amano Enzyme Europe.
Biocatalysts Ltd.
Chr Hansen's, Ireland Ltd. (Rohan Ind. Est, Little Island, Cork, Ireland).
DSM—Gist-Brocades (1, Wateringsweg, NL 2600, Delft, The Netherlands).
Novozymes A/S.
Stern-Enzym GmbH (Kornkamp 40, D-22926 Ahrensburg, Germany).

3. Enzyme and protein assays

3.1. Determination of total protein content

The protein content of all enzyme preparations was determined using a Sigma protein assay kit (Procedure No P 5656; Sigma Chemical Company Ltd., Fancy Road, Poole, Dorset, UK), which is based on a modified Lowry procedure. Absorbance at 750 nm was determined using a Hitachi U-1100 UV–Vis spectrophotometer (Hitachi Ltd., Tokyo, Japan) and the protein content estimated by reference to a standard curve for bovine serum albumin (BSA) in the range 50–400 $\mu g\,ml^{-1}$. All samples were analysed in triplicate and the results were expressed on a dry weight basis.

3.2. Determination of proteinase activity on azocasein at pH 7.0

Proteinase activity at pH 7.0 was determined by a modification of the method as described by Park et al. [18]. The reaction mixture consisted of 1 ml of 0.5% azocasein dissolved in 50 mM citrate-phosphate buffer, pH 7.0 (22.2 ml of 0.1 M citric acid and 27.8 ml of 0.2 M Na_2HPO_4 made up to 100 ml with distilled water) and 100 μl of proteinase preparation containing 0.05 mg protein ml^{-1} buffer. After incubation for 30 min at 37 °C, the reaction was terminated by addition of 100 μl of 2 M TCA, mixed and centrifuged at 15,000 × *g* for 5 min. A sample (750 μl) of supernatant was transferred to a cuvette to which 250 μl of 0.5 M NaOH were added. Absorbance was read at 440 nm on a Hitachi U-1100 UV–Vis spectrophotometer. Each assay was performed in triplicate. Activity was expressed as the change in absorbance $min^{-1}\,mg^{-1}$ protein under the assay conditions.

3.3. Determination of proteinase activity on azocasein at pH 5.5

Proteinase activity at pH 5.5 was determined essentially as described above with the following modifications: since azocasein dissolves slowly below pH 7.0, 0.5 g of azocasein were pre-dissolved in 20 ml of distilled water and then mixed with 80 ml of 50 mM citrate-phosphate buffer at pH 5.2 (23.3 ml of 0.1 M citric acid and 26.7 ml of 0.2 M Na_2HPO_4 made up to 100 ml with distilled water) to achieve a final pH of 5.5. NaOH 1.0 M was used to raise the pH to 7.0, as azocasein is colourless below pH 7.0. Absorbance was determined and activity expressed as above.

Table 1
Summary of manufacturer's technical data on commercial peptidase and lipase preparations

Code	Manufacturer	Trade Name	Source	IUB number	Name and Class	Application	Form	Optima		Activity
								pH	Temperature (°C)	
1	Biocatalysts	Flavorpro 192	*Aspergillus* sp.	EC 3.4.11.1	General aminopeptidase, carboxypeptidase[a]	Debittering	Powder	7.0	50	Aminopeptidase, 500 u g^{-1}[b]; carboxypeptidase, 250 u g^{-1}[c]
2	Novo	Flavourzyme MG/A	*A. oryzae*	EC 3.4.11.1	General aminopeptidase	Debittering and flavour development	Powder	5.0–7.0	50	1000 LAP u g^{-1}[d]
3	Rhodia Food	DBS50	*A. oryzae*	EC 3.4.11.1	General aminopeptidase	Debittering	Powder	7.0	30	650 LAP u g^{-1}[e]
4	Rohm	Corolase LAP	*A. sojae*	EC 3.4.11.1	General amin20opeptidase	Debittering	Liquid	6.0–9.0	30–70	350 LAP u g^{-1}[f]
5	Rhodia Food	DBP20	*A. oryzae, L. lactis*	EC 3.4.11.1, EC 3.4.14.11	General aminopeptidase, post-proline dipeptidyl aminopeptidase	Debittering and EMC production	Powder	7.0	20–30	220 LAP u g^{-1}[e]
6	Rhodia Food	DBL100	*L. lactis*	EC 3.4.11.1, EC 3.4.14.11	General aminopeptidase, post-proline dipeptidyl aminopeptidase	Debittering and cheese production	Powder	7.0	20–30	24 LAP u g^{-1}[e]
7	Amano	Peptidase R	*R. oryzae*	EC 3.4.11.1	General aminopeptidase, endoprotease[a]	Debittering	Powder	7.0	40–45	Peptidase, 420 u g^{-1}[g]; endoprotease, 4000 u g^{-1}[h]
8	Amano	Lipase A 6	*A. niger*	EC 3.1.1.3	Triacylglycerol hydrolase	Foods	Powder	6.5	45	60000 u g^{-1}[i]
9	Stern	Sternzyme LP6063	*R. arrhizus*	EC 3.1.1.3	Triacylglycerol hydrolase	Foods	Powder	7.0	37–50	10000 u g^{-1}[j]
10	Amano	Lipase F-AP15	*R. oryzae*	EC 3.1.1.3	Triacylglycerol hydrolase	Foods	Powder	7.0	35–45	150000 u g^{-1}[j]
11	Amano	Lipase M 10	*M. javanicus*	EC 3.1.1.3	Triacylglycerol hydrolase	Foods	Powder	7.0	40	10000 u g^{-1}[k]
12	Novo	Palatase 2000	*R. miehei*	EC 3.1.1.3	Triacylglycerol hydrolase	Cheese and EMC	Liquid	7.5	40	2000 LU g^{-1}[l]
13	Amano	Lipase AY 30	*C. rugosa*	EC 3.1.1.3	Triacylglycerol hydrolase	EMC and foods	Powder	7.0	45	30000 u g^{-1}[k]
14	Amano	Lipase G 50	*P. camemberti*	EC 3.1.1.23	Acylglycerol hydrolase	Fats and oil processing	Powder	5.0	45	50000 u g^{-1}[m]
15	Amano	Lipase R	*P. roqueforti*	EC 3.1.1.3	Triacylglycerol hydrolase	EMC and foods	Powder	7.0	30	900 u g^{-1}[n]
16	Biocatalysts	Lipomod 338P	*P. roqueforti*	EC 3.1.13	Triacylglycerol hydrolase	Blue EMC	Powder	5.0–7.0	40–50	4500 u g^{-1}[o]
17	Biocatalysts	Lipomod 187	Fungal spp.	EC 3.1.1.3	Triacylglycerol hydrolase	Cheddar EMC	Powder	5.0–7.0	40–50	11000 u g^{-1}[o]
18	Chr Hansen	Calf lipase	Calf pregastric esterase	EC 3.1.1.3	Triacylglycerol hydrolase	Cheese production	Powder	–	–	17–23 LFU g^{-1}[p]

19	Gist-Brocades	Piccantase C	Calf	EC 3.1.1.3	Triacylglycerol hydrolase	Cheese production	Powder	6.0–6.5	27–38	20 RU kg^{-1}[p]
20	Gist-Brocades	Piccantase K	Kid	EC 3.1.1.3	Triacylglycerol hydrolase	Cheese production	Powder	6.0–6.5	27–38	20 RU kg^{-1}[p]
21	Gist-Brocades	Piccantase L	Lamb	EC 3.1.1.3	Triacylglycerol hydrolase	Cheese production	Powder	6.0–6.5	27–38	20 RU kg^{-1}[p]
22	Chr Hansen	Lamb lipase	Lamb pregastric esterase	EC 3.1.1.3	Triacylglycerol hydrolase	Cheese production	Powder	–	–	17–23 LFU g^{-1}[p]
23	Biocatalysts	Lipomod 224	Pancreatic lipase with proteinase	EC 3.1.13, EC 3.4.21.4	Triacylglycerol hydrolase, trypsin	Cheddar EMC	Powder	7.0–8.0	45–50	21000 u g^{-1}[o]
24	Biocatalysts	Lipomod 299	Pancreatic lipase with proteinase	EC 3.1.13, EC 3.4.21.4	Triacylglycerol hydrolase, trypsin	Cheddar EMC	Powder	7.0–8.0	45–50	2000 u g^{-1}[o]

[a] EC number not supplied.

[b] One leucine aminopeptidase (LAP) unit is the amount of aminopeptidase required to liberate 1 μmol of leucine per minute from leucine *p*-nitroanilide (24.0 mM) at pH 7.2 and at 37 °C.

[c] One unit of enzyme which catalyses the transformation of 1 μmol of substrate (3.3 mM Cbz-Phe-Ala) per minute at pH 7.0 and at 37 °C.

[d] One LAP unit is the amount of aminopeptidase required to liberate 1 μmol of leucine per minute from leucine *p*-nitroanilide (26.0 mM) at pH 8.0 and at 40 °C.

[e] One LAP unit is the amount of aminopeptidase required to liberate 1 μmol of leucine per minute from leucine *p*-nitroanilide (2.0 mM) at pH 7.0 and at 30 °C.

[f] One LAP unit is the amount of aminopeptidase required to liberate one μmol of leucine per minute from leucine *p*-nitroanilide (2.0 mM) at pH 7.5 and at 30 °C.

[g] One peptidase unit is defined as the amount of aminopeptidase required to liberate 1 μmol of leucine per minute from leucyl-glycyl-glycyl (0.25 mM) at pH 7.0 and at 37 °C.

[h] One unit is the amount of enzyme which produces from a casein solution a soluble amino acid solution with an absorbance at 660 nm equivalent to 100 μg of tyrosine in 1 ml of filtrate under specified conditions in 1 h at pH 7.0.

[i] One unit is equivalent to the amount of enzyme which releases 1 μmol of fatty acid per minute at pH 6.0 at 37 °C from an olive oil emulsion under the conditions of the assay (Amano method, pH 6.0).

[j] One FIP unit is equivalent to the amount of enzyme which releases 1 μmol of fatty acid per minute at pH 7.0 at 37 °C from an olive oil emulsion under the conditions of the assay (FIP method).

[k] One unit is equivalent to the amount of enzyme which releases 1 μmol of fatty acid per minute at pH 7.0 at 37 °C from an olive oil emulsion under the conditions of the assay (Lipase MAP method).

[l] One lipase unit (LU) is the amount of enzyme which liberates 1 μmol of butyric acid per minute at pH 7.0 at 30 °C from a tributyrin emulsion under the conditions of the assay (AF 95/5-GB).

[m] One unit is defined as the amount of enzyme which liberates 1 μmol of fatty acid per minute pH 5.6 at 40 °C from an olive oil emulsion under the conditions of the assay (LV method, pH 5.6).

[n] One unit is equivalent to the amount of enzyme which releases 1 μmol of fatty acid per minute at pH 7.0 at 37 °C from an olive oil emulsion under the conditions of the assay (Modified JIS method, pH 7.0).

[o] One unit is equivalent to the amount of enzyme which releases 1 μmol of butyric acid per minute at pH 6.9 at 30 °C from glycerol tributyrin under the conditions of the assay (Esterase Assay Procedure 017).

[p] One (RU, Ramsey unit or LFU, Lipase Forestomach unit) unit is the amount of enzyme which releases 1.25 μmol of butyric acid per minute at pH 6.20 at 45 °C from a hydroxylated lecithin and tributyrin substrate under the conditions of the assay Food Chemical Codex Standard Method).

3.4. Semi-quantitative assay for hydrolytic activities

A range of hydrolytic activities were determined colorimetrically on 20 naphtyl substrates using the API-ZYM kit system (API Laboratory Products Ltd., Biomerieux, Marcy-l'Etoile, France). To facilitate comparison of enzyme activities, wherever possible the protein content was standardised to a concentration of 10 mg ml^{-1} in distilled water. The assay was performed according to the manufacturer's instructions with the following modifications: 60 μl of enzyme preparation were added to each cupule and allowed to react for 15 min. The principle of the procedure is described in detail by Arora et al. [19]. All preparations were analysed in duplicate.

3.5. Determination of peptidase activities at pH 7.0 and 5.5

Lysyl aminopeptidase (Pep N, EC 3.4.11.1) and membrane alanyl aminopeptidase (Pep M, EC 3.4.11.2), post-proline dipeptidyl aminopeptidase (Pep X; EC 3.4.14.11), proline iminopeptidase (Pep I; EC 3.4.11.5), endopeptidase (not classified) and carboxypeptidase (not classified) activities were determined using the following 7-amino-4-methyl coumarin (AMC)-labelled substrates, H-Lys-AMC.acetate, H-Phe-AMC.TFA, H-Gly-Pro-AMC. HBr, H-Pro-AMC.HBr, N-Suc-Gly-Pro-Leu-Gly-Pro-AMC and CBZ-Gly-Pro-AMC (Bachem, UK Ltd., Walden, Essex, CB10 1AA, UK), respectively. The method used to assay for Pep N and Pep M activity was a modification of that described by Habibi-Najafi and Lee [20]. Each substrate was made up to 0.111 mM in 50 mM Tris–HCl buffer, pH 7.0. The reaction mixture consisted of 50 μl of sample of known protein concentration, 500 μl buffer and 450 μl 0.111 mM substrate; the mixture incubated for 15 min at 37 °C and the reaction terminated by the addition of 1 ml of 1.5 M acetic acid. Fluorescence was read at an excitation wavelength of 370 nm and an emission wavelength of 440 nm using a Kontron SFM 25 fluorimeter (Kontron Instruments, Watford, Herts, UK). Activity was quantified using a standard curve of AMC in the range 0–2 μmol ml^{-1} at pH 7.0. Each enzyme preparation was assayed in triplicate. Results were expressed as units of activity, where one unit was the amount of enzyme which released 1 μmol of AMC min^{-1} mg^{-1} protein under the assay conditions.

Pep N, Pep M, Pep X, Pep I, endopeptidase and carboxypeptidase were also quantified at pH 5.5 and using a citrate-phosphate buffer at pH 5.5 buffer 50 mM using conditions identical to those described above.

Aminopeptidase A (Pep A; EC 3.4.11.7) activity was determined essentially as described by Exterkate [21], using the *p*-nitroanalide of aspartic acid (Asp-*p*NA) (Bachem) as substrate; the release of *p*NA (molar extinction coefficient, 8800 M^{-1} cm^{-1}) is directly proportional to Pep A activity under specified conditions. The reaction mixture consisted of 400 μl of 50 mM potassium phosphate buffer (pH 7.0), 50 μl of enzyme solution of known protein concentration and 50 μl of substrate (20 mM Asp-*p*NA). The mixture was incubated at 37 °C for 30 min and the reaction terminated by adding 1 ml of 1.5 M acetic acid. Absorbance was measured using a Hitachi U-1100 UV–Vis spectrophotometer at 410 nm. Activity was expressed as mmol of *p*NA released min^{-1} mg^{-1} protein under the assay conditions. Each preparation was assayed in triplicate.

Pep A activity was also measured at pH 5.5 under the conditions described above except that a citrate-phosphate buffer 50 mM was used.

Endopeptidase O (Pep O, not classified) activity was determined using a coupled assay on CBZ-Ala-Ala-Phe-AMC (Bachem), which is cleaved at Ala-Phe by Pep O to release Phe-AMC. Pep M from porcine kidney microsomes (Sigma-Aldridge, Gillingham, Dorset, UK) which cleaves Phe-AMC, was added to the reaction mixture, the fluorescence of AMC was quantified and is directly related to Pep O activity. A second sample was prepared without the addition of Pep M; any fluorescence measured in the latter sample was subtracted from that of the first sample to determine fluorescence due to Pep M activity. The reaction mixture, which consisted of 50 μl of enzyme preparation of known protein concentration, 50 μl of Pep M (1 μg protein), 450 μl buffer and 450 μl substrate, was incubated for 15 min at 37 °C and the reaction terminated by the addition of 1 ml of 1.5 M acetic acid; results were expressed as the amount of enzyme which released 1 mmol of AMC min^{-1} mg^{-1} protein. Each enzyme preparation was assayed in triplicate.

Pep O and Pep M activity were also measured at pH 5.5 as described above, except that a 50 mM citrate-phosphate buffer was used.

Aminopeptidase P (Pep P; EC 3.4.11.9) was assayed essentially as described by Doi et al. [22], using Arg-Pro-Pro (Bachem, UK Ltd.) as substrate. The reaction mixture consisted of 50 μl of sample of known protein concentration, 20 μl of 0.2 M bicine-NaOH buffer (pH 7.0), 10 μl of 10 mM $MnCl_2$ and 10 μl of 5 mM aqueous Arg-Pro-Pro substrate. The mixture was incubated for 30 min at 37 °C, after which the reaction was stopped by adding 700 μl of cadmium-ninhydrin reagent. Samples were heated at 84 °C for 5 min, followed by centrifugation at 15,000 × *g* for 5 min. Absorbance at 506 nm was measured using a Hitachi U-1100 UV–Vis spectrophotometer. Pep P activity was quantified from a standard curve generated using 0–800 nM arginine. Each preparation was assayed in triplicate. Activity was expressed as nmol Arg released min^{-1} mg^{-1} protein under the assay conditions.

3.6. Determination of esterase ($C_{4:0}$) and lipase ($C_{16:0}$) activity at pH 7.0

The method used was a modified version of the Sigma lipoprotein lipase assay (Sigma in-house procedure). The

reaction mixture consisted of 900 μl of buffer (100 mM sodium phosphate—150 mM NaCl—0.5% (v/v) Trition-X 100, adjusted to pH 7.0 with 1 M NaOH), 100 μl of proteinase preparation of known protein concentration and 10 μl of 50 mM substrate in acetonitrile (*p*-nitrophenol butyrate for esterase activity and *p*-nitrophenol palmitate for lipase activity). The mixture was incubated for 5 min at 37 °C and the absorbance read at 400 nm on a Hitachi U-1100 UV–Vis spectrophotometer. Each assay was performed in triplicate. Activity was quantified using the molar extinction coefficient of *p*-nitrophenol (14800) at 400 nm. Activity was expressed in units, where one unit will release 1 nmol of *p*-nitrophenol min^{-1} mg^{-1} protein under the assay conditions.

4. Results and discussion

4.1. Technical data on enzyme preparations

Technical details of the seven peptidase and seventeen lipase preparations, as provided by manufacturers, are given in Table 1. Each peptidase preparation was purported to contain general aminopeptidase activity (EC 3.4.11.1) with some preparations also containing endoproteinase, carboxypeptidase or Pep X (EC 3.4.14.11) activity. These preparations are generally used in dairy applications for debittering and for cheese flavour development. The activity of each peptidase preparation was given in LAP units; however, only preparations from the same supplier could be compared as individual suppliers used different units of activity, substrates, substrate concentrations or assay conditions (see Table 1). Preparations 3, 5 and 6, from the same supplier, contained 50, 220 and 24 LAP units of activity, respectively.

The lipase preparations were derived from either microbial or animal sources and all except preparation 14 were purported to have tri-acylglycerol hydrolase activity (EC 3.1.1.3). Preparation 14 was purported to contain acylglycerol hydrolase activity (EC 3.1.1.23). Most of the animal lipases are used in natural cheese production, mainly for Italian-type cheeses, e.g., Harboe [23]. According to manufacturer's data, preparations 23 and 24 are used exclusively for the production of Cheddar EMC. It is apparent from Table 1 that most manufacturers use their own method to determine lipase or esterase activity, making it difficult to compare the activities of preparations from different manufacturers. However, some preparations from different suppliers could be compared, e.g., 9 and 10, where the latter is claimed to have 15 times the lipase activity of the former; preparations 18–22 which were stated by the manufacturer to have similar levels of esterase activity. Some preparations derived from the same supplier could also be compared, i.e., preparations 11 and 13 where the latter was purported to have three times the lipase activity of the former; and preparations 16, 17, 23 and 24 which were purported to have different esterase activities.

Table 2
Protein content and specific proteinase activities of commercial peptidase and lipase preparations at pH 7.0 or 5.5

Code	Protein (%)[a]		Specific proteinase activity (mg^{-1} protein)[b]			
	Mean[c]	S.D.[d]	pH 5.5		pH 7.0	
			Mean[c]	S.D.[d]	Mean[c]	S.D.[d]
1	1.2	0.0	14.3	1.8	5.2	0.1
2	4.5	0.6	2.2	0.1	3.0	0.1
3	4.7	0.1	0.1	0.0	0.3	0.0
4	7.1	0.1	0.0	0.0	0.2	0.0
5	21.2	3.0	0.0	0.0	0.1	0.1
6	34.9	0.5	0.0	0.0	0.1	0.0
7	22.6	1.0	0.9	0.1	0.2	0.0
8	4.2	0.1	0.2	0.0	0.0	0.0
9	1.9	0.1	0.0	0.0	0.0	0.0
10	8.7	0.2	0.0	0.0	0.0	0.0
11	6.1	0.4	0.2	0.0	0.0	0.0
12	1.8	0.2	0.1	0.0	0.1	0.0
13	0.5	0.0	0.0	0.0	0.0	0.0
14	1.3	0.2	0.2	0.0	0.0	0.0
15	1.3	0.0	0.0	0.0	0.0	0.0
16	1.3	0.0	0.0	0.0	0.0	0.0
17	1.1	0.0	0.6	0.0	0.2	0.1
18	0.6	0.0	0.0	0.0	0.1	0.1
19	6.0	0.5	0.0	0.0	0.0	0.0
20	2.4	0.4	0.0	0.0	0.0	0.0
21	2.7	0.4	0.0	0.0	0.0	0.0
22	0.5	0.0	0.0	0.0	0.1	0.0
23	11.1	0.3	1.1	0.1	2.2	0.2
24	0.8	0.1	2.3	0.1	5.1	0.1

[a] On a dry weight basis.
[b] Change in absorbance at 440 nm on azocasein min^{-1} mg^{-1} of protein at 37 °C.
[c] Each preparation was assayed in triplicate.
[d] S.D., standard deviation.

4.2. Protein content and proteinase activity at pH 7.0 and 5.5

The protein content of each preparation on a dry weight basis is given in Table 2. The protein content of the peptidase preparations (1–7) ranged from 1.2 to 34.9% on a dry weight basis, while the protein content of the lipase preparations (8–24) ranged from 0.5 to 11.1% on a dry weight basis. These results indicate that other materials were present in these preparations, possibly to stabilise activity or facilitate spray drying, both of which are common in the commercial production of enzymes [9,24].

Proteinase activity of each preparation on azocasein at pH 7.0 and 5.5 are also given in Table 2. The proteinase activity of the peptidase preparations (1–7) at pH 5.5 or 7.0 was either very low or absent. Preparations 1 and 2 had the highest levels of proteinase activity.

All but two lipase preparations (23 and 24) possessed little or no activity on azocasein; according to manufacturer's data, preparations 23 and 24 contain a trypsin-like side activity, which most likely accounts the proteolytic activity as measured by the azocasein method.

Table 3
Semi-quantitative assay of enzyme activities (nmol) in commercial peptidase preparations

		Code						
		1	2	3	4	5	6	7
		Protein concentration (mg ml^{-1})						
		5	10	10	10	10	10	10
Esterase activity	Esterase ($C_{4:0}$)	0.0	10.0	0.0	5.0	2.5	2.5	2.5
	Esterase ($C_{8:0}$)	0.0	5.0	2.5	20.0	5.0	10.0	10.0
Lipase activity	Lipase ($C_{14:0}$)	0.0	0.0	0.0	2.5	0.0	0.0	10.0
Peptidase activity	Leucine amino-	0.0	30.0	5.0	40.0	30.0	30.0	20.0
	Valine amino-	0.0	30.0	2.5	40.0	30.0	5.0	20.0
	Cystine amino-	0.0	20.0	2.5	40.0	5.0	2.5	5.0
Proteinase activity	Trypsin-like	0.0	20.0	0.0	0.0	2.5	0.0	0.0
	Chymotrypsin-like	0.0	30.0	2.5	2.5	20.0	30.0	40.0
Phosphatase activity	Acid phosphatase	10.0	40.0	0.0	40.0	30.0	40.0	20.0
	Alkaline phosphatase	2.5	0.0	5.0	40.0	20.0	30.0	40.0
	Phosphohydrolyase	5.0	40.0	0.0	40.0	40.0	40.0	20.0
Glycosidase activity	α-Galactosidase	0.0	40.0	0.0	2.5	0.0	0.0	10.0
	β-Galactosidase	5.0	40.0	10.0	40.0	40.0	0.0	40.0
	β-Glucuronidase	0.0	10.0	0.0	0.0	0.0	0.0	0.0
	α-Glucosidase	10.0	40.0	0.0	40.0	0.0	2.5	20.0
	β-Glucosidase	5.0	20.0	0.0	40.0	0.0	0.0	10.0
	β-Glucosaminidase	10.0	40.0	10.0	40.0	30.0	0.0	30.0
	α-Mannosidase	0.0	10.0	0.0	0.0	0.0	0.0	20.0
	α-Fucosidase	0.0	5.0	0.0	0.0	0.0	0.0	40.0

Colorimetric reaction corresponds to extent of substrate hydrolysis.

4.3. Semi-quantitative analysis of hydrolytic activities using API-ZYM system

The results of the API-ZYM assay on the peptidase preparations are shown in Table 3. Using this technique each preparation was found to possess enzyme activities in addition to their stated main activity. Preparation 1 contained only phosphatase and glycosidase activities, preparations 2–7 contained varying levels of esterase, peptidase, proteinase, phosphatase and glycosidase activities. Only preparations 4 and 7 contained lipase activity. A significant amount of aminopeptidase activity was also present in preparations 2–7 (it was not possible to compare the levels of LAP activity using the API-ZYM method with the in-house methods of the manufacturers due to different assay conditions). Chymotrypsin-like activity was found in preparations 2–7 with trypsin-like activity present only in preparation 2. Preparations 1–7 possessed significant amounts of phosphatase activity, and all preparations, except 6, contained various glycosidase activities.

Lipolytic activities present in the various commercial preparations as measured by API-ZYM assay are shown in Table 4. All preparations except 8 showed activity towards both esterase and lipase substrates. Varying levels of side activities were also noted (data not shown); phosphatase and glycosidase side activities were found in all preparations, LAP activity was found only in preparations 9, 10, 17, 18, 23 and 24, preparations 23 and 24 had also trypsin-like and chymotrypsin-like activity. The latter results are in agreement with manufacturer's data and the results of the azocasein assay. Due to differences in the assay conditions, it is not possible to compare lipase and esterase activities

Table 4
Semi-quantitative assay of lipolytic activities (nmol) in commercial lipase preparations

Code	Protein concentration (mg ml^{-1})	Esterase $C_{4:0}$	Esterase $C_{8:0}$	Lipase $C_{14:0}$
8	10	0.0	2.5	0.0
9	5	0.0	20.0	10.0
10	10	0.0	5.0	5.0
11	10	0.0	10.0	20.0
12	5	10.0	20.0	20.0
13	1.25	0.0	10.0	10.0
14	5	0.0	5.0	2.5
15	5	0.0	10.0	5.0
16	5	0.0	5.0	10.0
17	1	10.0	10.0	20.0
18	10	10.0	20.0	20.0
19	10	20.0	30.0	2.5
20	10	10.0	10.0	5.0
21	10	10.0	10.0	5.0
22	1.22	20.0	20.0	20.0
23	10	5.0	20.0	10.0
24	5	5.0	10.0	5.0

Colorimetric reaction corresponds to extent of substrate hydrolysis.

as measured by the API-ZYM method with those described in Table 1.

4.4. Peptidase activities of commercial peptidase preparations

Specific activities of Pep N, Pep M, Pep A, Pep X, Pep I, Pep P, Pep O, endopeptidase and carboxypeptidase for peptidase preparations (1–7) quantified at pH 7.0 and 5.5 and at 37 °C are shown in Table 5. In general, activities were considerably higher at pH 7.0 than at 5.5, with many having little or no activity at pH 5.5 under the assay conditions used. These results imply that most of these preparations would have limited use in applications at or below pH 5.5, having important implications in the production of natural cheese and EMC, in which they are commonly used to accelerate

Table 5
Peptidase activities in commercial microbial peptidase preparations

Peptidase activity	pH		1	2	3	4	5	6	7
Pep N[a]	7.0	Mean[b]	66.8	55.1	64.5	71.1	5.3	1.4	1.7
		S.D.[c]	4.9	2.2	0.3	7.5	0.2	0.1	0.0
	5.5	Mean[b]	0.1	0.0	0.1	3.3	0.8	0.3	0.0
		S.D.[c]	0.0	0.0	0.0	0.1	0.0	0.0	0.0
Pep M[a]	7.0	Mean[b]	30.8	58.4	8.8	1.2	1.5	0.0	18.8
		S.D.[c]	4.5	4.5	0.2	0.0	0.1	0.0	0.0
	5.5	Mean[b]	0.0	0.0	0.2	0.1	0.0	0.0	1.7
		S.D.[c]	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Pep A[d]	7.0	Mean[b]	8.2	1.5	0.5	0.2	18.0	6.8	0.8
		S.D.[c]	0.8	0.3	0.1	0.0	0.0	0.7	0.1
	5.5	Mean[b]	1.6	0.3	0.3	0.2	0.0	0.0	0.0
		S.D.[c]	0.1	0.1	0.1	0.0	0.0	0.0	0.0
Pep X[a]	7.0	Mean[b]	8.7	0.7	4.1	0.1	43.4	51.2	0.2
		S.D.[c]	0.8	0.0	0.3	0.0	3.4	5.6	0.0
	5.5	Mean[b]	1.9	0.0	1.1	0.0	10.4	4.9	0.0
		S.D.[c]	0.0	0.0	0.1	0.0	0.0	0.1	0.0
Pep I[a]	7.0	Mean[b]	0.0	0.3	0.0	0.1	0.0	0.0	44.7
		S.D.[c]	0.0	0.0	0.0	0.0	0.0	0.0	2.3
	5.5	Mean[b]	0.0	0.0	0.0	0.0	0.0	0.0	12.3
		S.D.[c]	0.0	0.0	0.0	0.0	0.0	0.0	0.4
Pep P[e]	7.0	Mean[b]	17.8	318.0	0.0	31.0	0.0	56.0	2185.0
		S.D.[c]	124.3	133.6	0.0	94.7	0.0	20.5	227.2
	5.5	Mean[b]	0.0	328.0	0.0	354.0	24.0	0.0	610.0
		S.D.[c]	0.0	111.0	0.0	245.5	19.7	0.0	61.5
Pep O[a]	7.0	Mean[b]	264.0	183.5	9.9	1.4	1.7	0.2	18.4
		S.D.[c]	16.7	11.5	0.1	0.1	0.1	0.0	0.4
	5.5	Mean[b]	24.2	15.1	0.5	0.1	0.4	0.2	1.6
		S.D.[c]	1.3	0.7	0.1	0.0	0.0	0.0	0.0
Endopeptidase[a]	7.0	Mean[b]	0.3	0.0	0.0	0.0	0.0	0.0	0.0
		S.D.[c]	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	5.5	Mean[b]	0.8	0.0	0.0	0.0	0.0	0.0	0.0
		S.D.[c]	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Carboxypeptidase[a]	7.0	Mean[b]	0.2	0.0	0.0	0.0	0.0	0.0	0.0
		S.D.[c]	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	5.5	Mean[b]	0.3	0.0	0.0	0.0	0.0	0.0	0.0
		S.D.[c]	0.1	0.0	0.0	0.0	0.0	0.0	0.0

[a] Activity expressed as mmol AMC released min^{-1} mg^{-1} at pH 7.0 and 37 °C.
[b] Each preparation was assayed in triplicate.
[c] S.D., standard deviation.
[d] Activity expressed as μmol *p*-nitroanilide released min^{-1} mg^{-1} at pH 7.0 and 37 °C.
[e] Activity expressed as nmol arginine released min^{-1} mg^{-1} at pH 7.0 and 37 °C.

flavour development and/or to reduce bitterness. For example, in Cheddar cheese, the conditions throughout ripening are approximately pH 5.2 and 6–8 °C [25] and appear unsuitable for peptidase activity; however, as shown by Laan et al. [26] the NaCl and $CaCl_2$ content in the moisture phase of Cheddar cheese exert a positive influence on peptidase activity enabling cheese flavour development. In EMC production, the pH can decrease below pH 5.0, in part due to the accumulation of FFA [27], which can markedly reduce the efficiency of peptidases to eliminate bitterness and develop good flavour. Therefore, in EMC production, the pH must be maintained at a level optimal for peptidase activity to ensure good flavour development without bitterness.

Pep P was more active at pH 5.5 than at 7.0 in preparations 2, 4 and 5, however the accuracy of this method was limited, as evident by the standard deviations. Preparation 1 contained both endopeptidase and carboxypeptidase activities which were also higher at pH 5.5 than 7.0, however the overall levels of activity were quite low. Preparation 1 was the only preparation to contain both endopeptidase and carboxypeptidase activity; it had been stated by the manufacture to contain carboxypeptidase activity. All preparations were found to contain Pep O activity; with preparations 1 and 2, derived from *Aspergillus* spp. possessing the highest levels of activity.

All preparations had Pep N activity at pH 7.0, with the preparations derived from *Aspergillus* spp. (1–4) having the highest levels of activity. Pep N activity is an indicator of general aminopeptidase activity and the trends found with this assay mirrored the manufacturer's declared LAP activities of preparations 3, 5 and 6. All preparations, except 6, which was derived from *Lactococcus lactis*, showed Pep M activity at pH 7.0. Preparations 1, 2, 3 and 7, derived from *Aspergillus* spp., and *Rhizomucor oryzae* had the highest levels of Pep M activity. All preparations had Pep A and Pep X activity at pH 7.0; however, preparation 1, derived from a unidentified *Aspergillus* source, and preparations 5 and 6, derived from *L. lactis*, contained the highest levels of Pep A and Pep X activity. It is notable that the preparations derived from *L. lactis* had the highest levels of Pep X activity. Significant amounts of Pep I activity were found in preparation 7 only, which was derived from *R. oryzae*. All preparations, except 3 and 5, contained Pep P activity, with preparations 2 and 7 having significantly more than the other preparations.

4.5. Lipase and esterase activity of commercial lipase preparations at pH 7.0

The activity of each commercial lipase preparation (8–24) on $C_{4:0}$ (esterase) and $C_{16:0}$ (lipase) substrates at pH 7.0 is given in Table 6.

The microbial preparations (8–17) possessed varying levels of esterase and lipase activity. Preparations 8, 13, 15, 16 and 17 which were derived from *A. niger*, *Candida rugosa* (previously *C. cylindracea*), *Penicillium roqueforti* (15 and 16) and an unidentified fungal source, respectively, showed significantly higher esterase than lipase activity. However, according to manufacturer's data (not shown) preparation 8 from *A. niger* preferentially hydrolyses both medium and long chain fatty acids; Garcia et al. [28] have reported that *A. niger* lipase preferentially released short chain FFA and Godfrey and Hawkins [29] also reported that a *A. niger* lipase preferentially released both $C_{4:0}$ and $C_{16:0}$. Overall, there appears to be some disagreement in the literature regarding the chain specificity of *A. niger* lipases [30]. Godfrey and Hawkins [29] reported the preferential release of

Table 6
Specific esterase ($C_{4:0}$) and lipase ($C_{16:0}$) activities of selected lipase preparations

Code	Esterase activity[a]		Lipase activity[a]		Ratio of esterase to lipase activity
	Mean[b]	S.D.	Mean[b]	S.D.	
8	80	0.2	72	2.1	1.11
9	40	2.9	152	4.2	0.26
10	75	2.2	265	1.4	0.28
11	9	0.1	46	2.2	0.20
12	535	6.8	23514	810.8	0.02
13	516216	5405.4	354730	29054.1	1.46
14	96	0.7	1182	236.5	0.08
15	349	6.1	9	0.1	38.78
16	9304	168.9	6669	6.8	1.40
17	64122	67.6	43919	3513.5	1.46
18	81	2.0	29	0.8	2.79
19	24	0.2	24	1.4	1.00
20	21	2.9	7	1.1	3.00
21	16	0.5	14	2.3	1.14
22	170	3.1	50	1.5	3.40
23	551	14.9	60	14.2	9.18
24	47	4.3	17	0.5	2.76

[a] Activity units of *p*-nitrophenol released min^{-1} mg^{-1} at pH 7.0 and 37 °C.
[b] Each preparation was assayed in triplicate.

$C_{4:0}$ by *C. rugosa*, and according to manufacturer's data (not shown) this preparation (13) hydrolyses small, medium and long chain fatty acids. Villeneuve and Fogilia [15] reported that *P. roqueforti* lipase is specific for short-chain fatty acids, which also concurs with the findings in this study. According to manufacturer's data (not shown) preparation 17 preferentially releases $C_{4:0}$.

Preparations 9, 10, 11, 12 and 14, which were derived from *Rhizopus arrhizus*, *Rhizopus oryzae*, *Mucor javanicus*, *Rhizomucor miehei* and *P. camemberti*, respectively, possessed higher levels of lipase than esterase activity. In agreement with our study *R. arrhizus* has been shown to preferentially release long chain FFA [16] and according to manufacturer's data (not shown) and *M. javanicus* preferentially releases medium chain and long chain fatty acids. In contrast to our results, manufacturer's data (not shown) show that preparation 10 preferentially releases short and medium chain fatty acids and that preparation 12 preferentially releases short chain fatty acids. Also according to manufacturer's data preparation 14 is specific for mono- and di-acylglycerols only.

The animal lipase preparations (18–24) also showed different levels of esterase and lipase activity. Preparation 18, 20, 21, 22, 23 and 24 showed higher esterase than lipase activity while preparation 19 had similar levels of esterase and lipase activity. Preparations 18–22, pregastric esterases derived from oral glands of calves, kids or lambs [13,17] have a high specificity for $C_{4:0}$ [30]. The two pancreatic lipases, preparations 23 and 24, preferentially release $C_{4:0}$ [29,31].

It was difficult to compare manufacturer's data on esterase or lipase activity with the results in this study as different assay conditions were used. However, for preparations 9, 10, 11 and 13, manufacturers data were in agreement with the lipase activity found in this study. Also, the low level of esterase activity from preparations 18–22 found in this study was in agreement with manufacturing data. In contrast, manufacturer's data for preparations 16, 17, 23 and 24 were not reflected in the levels of esterase activity determined in this study. All of the manufacturer's expressed (see Table 1) results as either esterase or lipase activity and in most cases the assay conditions were different to those used in this study. In relation to the preferential release of fatty acids by each preparation, each manufacturer expressed their specificity (results not shown) based the actual fatty acid profile produced from a hydrolysed substrate such as butter oil or olive oil. This may give very different results to those obtained using chromogenic substrates as lipolytic action is very dependent upon the nature of the environment in which the reaction is taking place.

5. Conclusion

This study has compiled data from manufactures of a selection of commercial peptidase and lipase preparations with a study of their main and side enzyme activities. In general, all the preparations were found to possess enzyme activities in addition to their stated main activity and each also contained additional material added presumably to stabilise activity and or extend shelf life.

The peptidase preparations contained varying levels of lipase, proteinase, peptidase, glycosidase and phosphatase activities (API-ZYM); however, only two preparations had significant levels of proteinase activity on azocasein at pH 7.0 or 5.5. Most peptidase preparations were found to have significantly more peptidase activity at pH 7.0 than at pH 5.5 and in general, the preparations derived from *Aspergillus* spp. had high levels of Pep N activity; those from *L. lactis* had the highest levels of Pep X activity; the *R. oryzae* preparation had the highest level of Pep I and Pep P activity. The lipase preparations contained varying levels of lipase, glycosidase and phosphatase activities (API-ZYM) and only two preparations had proteinase activity on azocasein at pH 7.0 or 5.5. The fungal preparations differed considerably in their activity towards *p*-nitrophenol-butyrate and *p*-nitrophenol-palmitate and this specificity appeared to be source-specific, while most of the animal lipase preparations showed higher levels of activity on *p*-nitrophenol-butyrate.

This study has provided information on the enzyme complement of a selection of commercially-available peptidase and lipase preparations and highlighted their un-purified nature. Commercial users of these preparations should find this study valuable and allow them to make a more informed choice regarding the type of peptidase or lipase to use in dairy applications.

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