Proteolytic and Peptidolytic Activities in Commercial Pancreatic Protease Preparations and Their Relationship to Some Whey Protein Hydrolysate Characteristics

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Endoproteinase and exopeptidase activities in the commercially available pancreatic protease preparations Corolase PP, PTN 3.0S, pancreatin, PEM 2500S, PEM 2700S, and PEM 800S were quantified using synthetic peptide substrates. These preparations were generally found to be low in aminopeptidase and dipeptidase activity. Trypsin and chymotrypsin, albeit in different ratios, were present in all pancreatic preparations. Elastase was present only in Corolase PP and pancreatin. The ability of these protease preparations to hydrolyze the insoluble heat denaturted whey protein, lactalbumin, was compared and contrasted with that of crystalline trypsin, chymotrypsin, and elastase in addition to a commercial exopeptidase preparation, Debitrase DBP.20. When the source, number, or ratio of endoprotease activity changes, there are distinctive differences in products produced with respect to percentage degrees of hydrolysis, gel permeation profile, solubility, and free amino acids present in the hydrolysate.

Keywords: *Pancreatic proteases; proteolytic activity; whey protein; enzymatic hydrolysis*

INTRODUCTION

Proteolytic enzyme preparations are economically the most important group of enzymes and their use is well established in the food industry (Godfrey and Reichert, 1983). Commercial protease preparations for use in large scale processes may consist of mixtures of proteinases and peptidases that are isolated from animal tissues, plants, or microorganisms. Proteinases split protein molecules into peptides, while exopeptidases remove amino acids either from the N terminus, i.e., aminopeptidases (Sanderink *et al.*, 1988), or from the C terminus, i.e., carboxypeptidases (Pétra, 1970), of peptides. The purification and physical and chemical properties of pancreatic proteinases have been reviewed: trypsin (Northrop and Kunitz, 1948; Desnuelle, 1961; Hakim *et al.*, 1962; Vestling *et al.*, 1990); chymotrypsin (Wilcox, 1970); elastase (Hartley and Shotton, 1971; Gerthler *et al.*, 1977); and protease E (Kobayashi *et al.*, 1981). The exopeptidases of the pancreas include carboxypeptidases A and B (Puigserver, 1986) in addition to a number of aminopeptidases and dipeptidases.

Enzymatic modification of proteins using selected proteases to hydrolyze specific peptide bonds is widely used (Adler-Nissen, 1986; Fox *et al.*, 1991; Arai and Fujimaki, 1991). Whey proteins enzymatically hydrolyzed with trypsin and chymotrypsin showed superior solubility and in-vitro digestibility as compared to their chemically treated counterparts (Lakkis and Villota, 1992). The choice of substrate and proteases employed and the degree to which the protein is hydrolyzed are factors known to affect the physicochemical properties of resulting hydrolysates. Manipulating the reaction conditions during enzymatic hydrolysis of milk proteins produces hydrolysates with different solubility and emulsifying characteristics (Turgeon and Gautier, 1992), foaming properties (Kuehler and Stine, 1974), or taste characteristics (Murray and Baker, 1952; Poulsen, 1987; Vegarud and Langsrud, 1989).

The degree to which milk proteins are hydrolyzed depends on the intended use for the hydrolysate. Low degrees of hydrolysis are desirable for maintaining functional properties, while extensive hydrolysis is necessary when the hydrolysate is to be used in, for example, chemically defined or hypoallergenic infant formulas (Merritt *et al.*, 1990; Thibault, 1991). Mixtures of proteases having complementary specificities may be used to attain the required degree of hydrolysis. The proportions of different proteinases, such as trypsin to chymotrypsin, and the presence or absence of exopeptidases, such as carboxypeptidase B, may be significant for production of specific hydrolysates such as hypoallergenic hydrolysates (Thibault, 1991) and/or hydrolysates with reduced bitterness (Fullbrook *et al.*, 1987; Plainer and Sprossler, 1990).

To date little information has been published that links the proteinase/peptidase activity of commercial proteases used for hydrolysis of milk proteins to the physicochemical characteristics of the final hydrolysate. Although many groups have devised processes for the production of milk protein hydrolysates using commercial pancreatic proteases for clinical nutrition (Grimble and Silk, 1989; Maubois and Léonil, 1989) and hypoallergenic and special dietetic products (Jost *et al.*, 1988; Asselin *et al.*, 1988, 1989; Thibault, 1991), the choice of enzyme is usually based on empirical screening or by random selection.

The specificity of the pancreatic proteolytic enzymes dictates the nature of peptides and free amino acids found in the hydrolysate. The tissue source, bovine or porcine, in addition to the methods of purification used, influences the composition and stability of protease preparations. A knowledge of the proteinase and exopeptidase activities in commercial protease prepara-

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tions would be a useful guide in the selection of a particular protease to generate a desired hydrolysate. The objective of this study was to determine the proteinase and peptidase activities in pancreatic protease preparations and to characterize the physicochemical properties of lactalbumin hydrolysates produced using these preparations.

MATERIALS AND METHODS

Enzymes. Commercial pancreatic proteases were received as gifts from manufacturers. Corolase PP (porcine) was supplied by Rohm GmbH, Darmstadt, Germany, and pancreatin (porcine) by Chemicon Ltd., Dublin, Ireland. PEM 2500S (porcine/bovine), PEM 800S (bovine), PEM 2700S (bovine), and PTN 3.0S (porcine) were supplied by Novo Nordisk A/S, Bagsvaerd, Denmark, and Debitrase DBP.20 (*Lactococcus lactis*, *Aspergillus oryzae*) was supplied by Imperial Biotechnology Ltd., London, U.K. Bovine trypsin (type XII), bovine chymotrypsin (type II), porcine carboxypeptidase A, porcine carboxypeptidase B (type I), and porcine elastase (type I) were obtained from Sigma Chemical Co., Poole, Dorset, U.K.

Substrates. Aminomethylcoumarin (AMC) and fluorogenic substrates alanine-AMC, asparagine-AMC, arginine-AMC, glycine-AMC, histidine-AMC, proline-AMC, Arg-Arg-AMC, Gly-Arg-AMC, Gly-Pro-AMC, Lys-Ala-AMC, acetyl-Ala-Ala-Pro-Ala-AMC, *N*-Suc-Leu-Leu-Val-Tyr-AMC, and *N*-benzoyl-L-Arg-AMC were obtained from Bachem, Bubendorf, Switzerland. Carboxypeptidase substrates, hippuryl-L-lysine, hippuryl-L-phenylalanine, and *N*-tosyl-L-lysine chloromethyl ketone (TLCK) were supplied by Sigma. Lactalbumin (Alatal 560) was from New Zealand Dairy Board, Wellington, New Zealand; it is an insoluble heat denatured whey protein, and its manufacture has been outlined by a number of groups (Robinson *et al.*, 1976; Mulvihill, 1992). Reagents for amino acid analysis were obtained from Beckman Instruments, High Wycombe, U.K. All other reagents were of analytical grade unless otherwise specified.

Quantification of Enzyme Activity. Enzyme activities were assayed using a modification of the fluorogenic (AMC) assay (Zimmerman *et al.*, 1977) as follows. Proteases (10 mg mL^{-1}) were centrifuged at 14400*g* (Microcentaur, MSE, U.K.) for 10 min, and 20 μL of supernant was added to 980 μL of 0.01 M Tris-HCl, pH 7.0, containing 0.02 mM specific substrate. The substrate–enzyme mixture was incubated at 37 °C for 1 h. The reaction was stopped by the addition of 1 mL of 1.5 M acetic acid, and fluorescence developed was measured on a spectrofluorometer (Perkin-Elmer 1000, Beaconsfield, Bucks, England) at excitation and emission wavelengths of 360 and 440 nm, respectively. When the substrates Arg-Arg-AMC, Gly-Arg-AMC, and Arg-Arg-AMC were used, the protease preparation was incubated with the trypsin inhibitor TLCK (1 mg mL^{-1}) in the ratio 1:5 (v/v) at 37 °C for 10 min, before the assay. One unit of activity is defined as that amount of enzyme which will give 1 μmol of AMC min^{-1} (mg of protien)$^{-1}$.

For quantification of carboxypeptidase A and B activities, samples (10 mg/mL of powder) were filtered through a 0.45 μm PS Whatman syringe filter, to remove aggregated material, and assayed using procedures outlined by Worthington Diagnostic Systems Inc., Freehold, NJ. One unit of carboxypeptidase A activity was defined as that amount of enzyme which catalyzes the hydrolysis of 1 μmol of *N*-α-hippuryl-L-phenylalanine min^{-1} at pH 7.5 at 25 °C. One unit of carboxypeptidase B activity was defined as that amount of enzyme which catalyzes the hydrolysis of 1 μmol of *N*-α-hippuryl-L-lysine min^{-1} at pH 7.65 and 25 °C.

Preparation of Hydrolysates. A 25 mL solution of lactalbumin, 8% (w/v) protein, was hydrolyzed with individual proteases at 50 °C for 240 min. The pH was maintained at constant pH 8.0 by continuous addition of 0.5 M NaOH using a pH-stat (Metrohm Ltd., Herisau, Switzerland.). The degree of hydrolysis (DH%), defined as the percentage of peptide bonds cleaved, was calculated from the volume and molarity of NaOH used to maintain constant pH (Adler-Nissen, 1986). Following hydrolysis, proteases were inactivated by heating at 80 °C for 30 min, cooled, and stored at −20 °C for further analysis. The enzyme (E) to substrate (S) ratio (0.003) was calculated on the basis of total protein content in the enzyme preparations and lactalbumin. The DH% was calculated as

$$DH\% = B(Mb)(1/\alpha)(1/MP)(1/h_{tot}) \times 100$$

where B is the volume of NaOH consumed (mL), Mb is the molarity of NaOH, α is the average degree of dissociation of the a α-NH_2 groups at pH 8.0 and 50 °C, MP is the mass of protein (g), and h_{tot} is the total number of peptide bonds in the protein substrate (mequiv/g of protein). The h_{tot} value, 8.8 mequiv/g of protein, and values of $1/\alpha$ for various pH–temperature combinations were those given by Adler-Nissen (1986).

Characterization of Hydrolysates. *Protein (Nitrogen) Determination.* Total protein (N × 6.25) of pancreatic preparations and of whey protein (N × 6.38) was determined by micro-Kjeldahl (AOAC, 1980).

Protein (Nitrogen) Solubility. Hydrolysates were adjusted to pH 6.6 with 1 M HCl and centrifuged at 1300*g* (Mistral 6000, MSE Scientific Instruments, West Sussex, England) for 15 min at 20 °C. The supernatants were then filtered through Whatman No. 1 filter paper; N content was determined as described above and expressed as a percentage of total N in the hydrolysate.

Molecular Size Distribution of Peptides in Lactalbumin Hydrolysates. A fast protein liquid chromatograph (FPLC) fitted with a Superose 12 gel permeation column (Pharmacia LKB Biotechnology Ltd., Uppsala, Sweden) was used to monitor the size distribution of peptides in lactalbumin hydrolysates. The column was eluted at 1 mL min^{-1} with 0.1 M Tris-HCl buffer containing 0.1 M NaCl and 10% (v/v) methanol. Hydrolysates were diluted in elution buffer to 0.25% (w/v) protein and filtered through a Whatman 0.45 μm PS syringe filter, and 100 μL was applied to the column. Analyses were performed in duplicate. Eluate was continually assayed at 214 or 280 nm using two single-path monitors (UV-1, Pharmacia) and recorded using a Minichrom data handling system (VG, Data Systems, Altrincham, Cheshire, U.K.). A calibration curve was prepared from the average retention volume (R_v) of standard proteins and peptides.

Free Amino Acid Analysis of Hydrolysates. Hydrolysates were deproteinized by mixing equal volumes of 24% (w/v) TCA and sample, which was allowed to stand for 30 min before centrifuging at 14400*g* (Microcentaur, MSE, U.K.) for 10 min. Supernatants were removed and diluted with 0.2 M sodium citrate buffer, pH 2.2, to give approximately 25 nmol of each amino acid residue per 50 μL of injection volume and then analyzed on a 120 × 4 mm cation exchange column (Na^+ form) using a Beckman 6300 amino acid analyzer (Beckman Instruments Ltd., High Wycombe, U.K.).

RESULTS

Characterization of Proteolytic Activity in Commercial Pancreatic Proteases. *Aminopeptidase Activity.* Aminopeptidase activity in the bovine and porcine protease preparations tested was generally very low (Table 1). Corolase PP had activity against all of the aminopeptidase substrates except Asp-AMC. Interestingly, there was essentially no activity against Asp-AMC in any preparation except Debitrase DBP.20. Debitrase DBP.20 was used as a positive control, as it is known to contain high exopeptidase activity as shown in Table 1. Apart from Corolase PP and to a lesser degree pancreatin, there was no activity against Ala-AMC in any of the pancreatic preparations. As expected, the two crystalline proteinases, trypsin and chymotrypsin, showed the least aminopeptidase activity; however, activity was observed against His-AMC and Leu-AMC in both preparations. While activity toward Arg-AMC was detected in both crystalline trypsin and chymotrypsin, it was eliminated by addition of trypsin

Table 1. Aminopeptidase Activity (Nanomoles of AMC per Minute per Milligram of Protein), Carboxypeptidase Activity (Units per Minute per Milligram of Powder), Dipeptidase Activity (Nanomoles of AMC per Minute per Milligram of Protein), and Endopeptidase Activities Including Trypsin, Chymotrypsin, and Elastase Activities (Micromoles of AMC per Minute per Milligram of Protein)

	Corolase PP	PTN 3.0S	pancreatin	PEM 2500S	PEM 800S	PEM 2700S	Debitrase	trypsin	chymotrypsin
aminopeptidase substrates									
Ala-AMC	1764.00	0.00	9.49	0.00	0.00	0.00	501.25	0.00	0.00
Arg-AMC	2954.50	3705.85	1021.85	1056.90	616.23	1216.84	2976.20	28.57	30.00
Arg-AMC + TLCK	2090.00	3190.00	740.00	790.00	470.00	880.00	160.00	0.00	0.00
Asp-AMC	0.00	0.00	0.00	0.00	3.08	0.00	181.70	0.00	0.00
Gly-AMC	86.90	19.33	21.90	0.00	2.30	2.64	20.68	0.00	0.00
His-AMC	152.10	8.38	29.20	27.00	26.56	20.28	37.59	1238.10	34.79
Leu-AMC	2520.00	184.30	208.70	104.92	212.50	111.55	64223.00	38.10	330.55
Pro-AMC	34.76	61.87	70.05	12.34	12.40	7.10	25.06	7.62	6.09
carboxypeptidase substrates									
hippuryl-L-phenylalanine	6.73	3.37	4.21	–[a]	–	–	–	–	–
hippuryl-L-lysine	1.74	–	14.76	–	–	–	–	–	–
dipeptidase substrates									
Arg-Arg-AMC	9558.57	8700.70	6567.43	1974.94	2231.19	2433.68	4699.25	10952.38	34.79
Arg-Arg-AMC + TLCK	6600.00	6090.00	4600.00	1380.00	1560.00	1700.00	3290.00	7670.00	20.00
Gly-Arg-AMC	7038.58	5317.09	1824.28	1357.77	1460.90	1886.10	839.60	5142.86	26.10
Gly-Arg-AMC + TLCK	1880.00	3130.00	240.00	600.00	320.00	610.00	530.00	100.00	0.00
Gly-Pro-AMC	225.93	19.33	620.26	0.00	0.00	0.00	97117.79	0.00	0.00
Lys-Ala-AMC	391.03	0.00	0.00	0.00	0.00	0.00	313.28	0.00	0.00
endoproteinase substrates									
N-benzoyl-L-arg-AMC	58.65	70.89	6.75	51.84	15.67	41.27	0.00	89.05	0.00
N-Suc-Leu-Leu-Val-Tyr-AMC	157.80	172.40	226.21	279.27	449.78	185.57	2.19	1.98	555.56
acetyl-Ala-Ala-Pro-Ala-AMC	189.43	0.70	80.27	0.49	0.52	0.11	0.01	0.01	0.03

[a] –, not determinable [i.e. activity values <1 mmol of AMC min^{-1} (mg of protein)$^{-1}$ using flurogenic substrates of <0.0001 unit min^{-1} (mg of protein)$^{-1}$ for carboxypeptidases].

inhibitor, TLCK. The activity against Arg-AMC was decreased to 70–75% of total activity in all preparations in the presence of TLCK.

Carboxypeptidase Activity. Carboxypeptidase A was present in the porcine preparations Corolase PP, PTN 3.0S, and pancreatin but was absent in the bovine preparations (Table 1). Carboxypeptidase B activity was detected in Corolase PP [1.74 units min^{-1} (mg of preparation)$^{-1}$] and pancreatin [14.76 units min^{-1} (mg of preparation)$^{-1}$]. Carboxypeptidase A or B activity was not detected in Debitrase DBP.20.

Dipeptidase Activity. Corolase PP was the only pancreatic preparation having dipeptidase activity for all four of the dipeptidase substrates tested (Table 1). Pancreatin, Corolase PP, and PTN 3.0S were the only pancreatic preparations showing Gly-Pro-AMC activity, which decreased in the order pancreatin > Corolase PP > PTN 3.0S. There was activity in all preparations toward Gly-Arg-AMC and Arg-Arg-AMC, but inhibitor studies using TLCK showed this activity to be largely due to trypsin (Table 1). Addition of TLCK reduced tryptic activity for Arg-Arg-AMC by ~33% in the case of crystalline trypsin. Crystalline chymotrypsin had little or no dipeptidase activity. The dipeptidase activities of PTN 3.0S toward Gly-Arg-AMC or Arg-Arg-AMC were reduced by 41 and 30%, respectively, in the presence of TLCK.

Debitrase DBP.20 had activity on all dipeptidase substrates tested; highest levels of activity were obtained with Gly-Pro-AMC as substrate [97.12 μmol min^{-1} (mg of protein)$^{-1}$].

Endoproteinase Activity. Trypsin activity, measured by hydrolysis of *N*-benzoyl-L-Arg-AMC, for bovine crystalline trypsin and pancreatic proteases is given in Table 1. The specific activities (micromoles of AMC per minute per milligram of protein) for each of the preparations in order of decreasing magnitude were as follows: trypsin > PTN 3.0S > Corolase PP > PEM 2500S > PEM 2700S > PEM 800S > pancreatin. Crystalline chymotrypsin showed no activity against this substrate, as expected.

All of the commercial preparations tested showed chymotrypsin activity as measured by hydrolysis of *N*-Suc-Leu-Leu-Val-Tyr-AMC (Table 1). Specific activities (micromoles of AMC per minute per milligram of protein) in order of decreasing magnitude were as follows: PEM 800S > PEM 2500S > pancreatin > PEM 2700S > PTN 3.0S > Corolase PP. Crystalline bovine chymotrypsin showed the highest specific activity [555.56 μmol of AMC min^{-1} (mg of protein)$^{-1}$], as expected. Low elastase activities were observed in PTN 3.0S, PEM 2500S, and PEM 800S; however, Corolase PP and pancreatin displayed much higher elastase activities, having values of 187.43 and 80.27 μmol of AMC min^{-1} (mg of protein)$^{-1}$, respectively, as measured by hydrolysis of acetyl-Ala-Ala-Pro-Ala-AMC.

Characterization of Lactalbumin Hydrolysates. *Degrees of Hydrolysis*. The hydrolysis curves (DH% vs time, min) obtained by incubation of lactalbumin with commercial proteases are shown in Figure 1. Debitrase DBP.20, as expected, had low activity on lactalbumin as seen from the low degree of hydrolysis obtained after incubation for 240 min. The curves obtained for crystalline chymotrypsin and trypsin began to plateau after ~60 min at DH% 2.77 and 5.23, respectively, while the DH% values after 240 min were 3.93 and 6.04, respectively. The curve obtained for crystalline elastase indicated low activity in the early stages of the hydrolysis reaction, but activity continued to increase over the incubation period. Protease preparations that contained more than one activity (Table 1) hydrolyzed lactalbumin to higher DH% values than the crystalline preparations, as seen for PEM 2500S, PEM 800S, PEM 2700S, Corolase PP, and pancreatin. The DH curves obtained for Corolase PP and pancreatin were distinctly different from those for all other proteases and indicated high activity toward lactalbumin. Unlike the other preparations, Corolase PP and pancreatin continued to hydro-

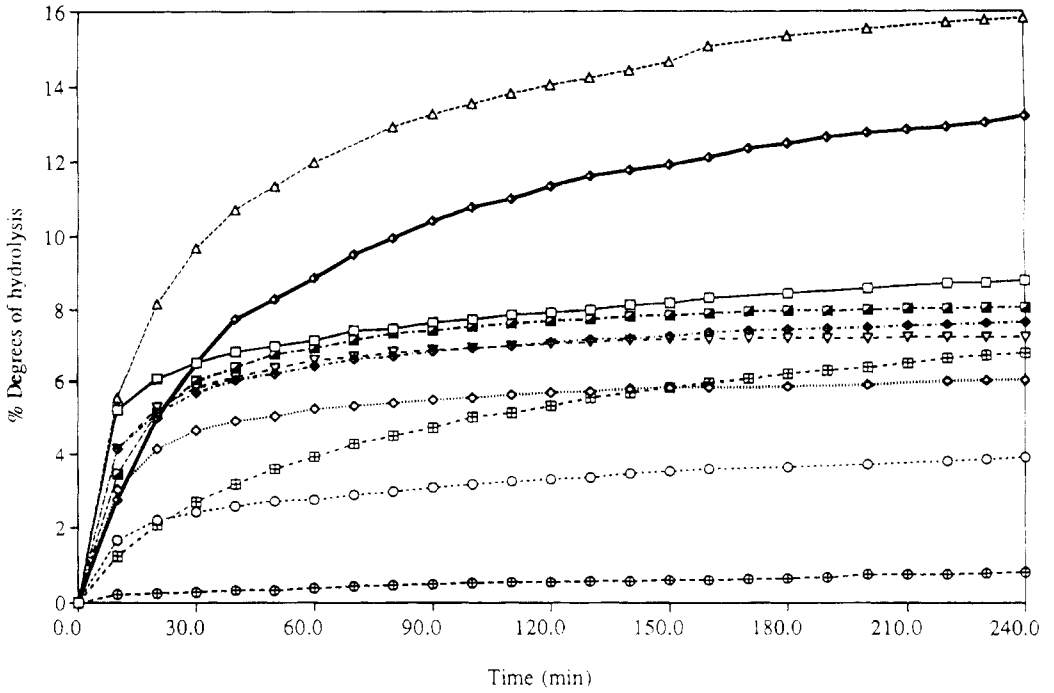

Figure 1. Enzymatic hydrolysis of lactalbumin by commercial proteases as a function of time: (△) Corolase PP; (tilted square right solid) pancreatin; (□) PTN 3.0S; (◆) PEM 2500S; (▽) PEM 2700S; (◪) PEM 800S; (◇) trypsin; (○) chymotrypsin; (⊡) elastase; (⊕) Debitrase. Reaction conditions: substrate concentration, 8% (w/v); E:S, 0.003; pH 8.0; 50 °C; time, 240 min.

Table 2. Soluble Nitrogen as Percent of Total N (pH 6.6) and Degree of Hydrolysis (DH%) in the Lactalbumin Hydrolysates after Incubation with Commercial Proteases for 240 min

protease	soluble N	DH%
PTN 3.0S	89.00	8.83
trypsin (bovine)	100.00	6.04
chymotrypsin (bovine)	93.00	3.93
elastase (porcine)	78.00	6.78
Corolase PP	81.00	15.82
PEM 2500S	78.00	7.66
Debitrase DBP .20	0.15	0.83
PEM 2700S	78.50	7.22
PEM 800S	78.70	8.08
pancreatin	100.00	13.22

lyze lactalbumin as incubation continued, giving DH% values of 15.82 and 13.22, respectively, after 240 min.

Nitrogen Solubility of Lactalbumin Hydrolysates. Incubation of lactalbumin with bovine crystalline trypsin, chymotrypsin, or pancreatin produced hydrolysates with high solubility at pH 6.6, i.e., ~90–100% of total N (Table 2), while protease preparations such as PEM 2500S, PEM 800S, and PEM 2700S produced hydrolysates that had similar nitrogen solubility values, i.e., in the range 78–81% of total N. The hydrolysate produced using Debitrase DBP.20 had a very low degree of solubility, i.e., 0.15% of total N.

Production of Free Amino Acids. The concentrations of amino acids in free solution produced by incubation of lactalbumin with commercial proteases are shown in Table 3. Concentrations less than 0.005 mg/mL were not included in the table. All of the commercial pancreatic protease preparations produced by hydrolysates with Lys, Arg, Ala, and Leu in free solution with the exception of the hydrolysates produced by PTN 3.0S, which did not contain Lys, and PEM 800S, which did not contain Arg. The hydrolysates produced by Corolase PP, pancreatin, PEM 2500S, and elastase contained Tyr

Table 3. Free Amino Acids (Milligrams per Gram of Protein) Present in Lactalbumin Hydrolysates Produced by Commercial Protease Preparations

amino acid	Corolase PP	PTN 3.0S	pancreatin	PEM 2500S	PEM 800S	PEM 2700S	trypsin	chymotrypsin	elastase	debitrase
Asp	–[a]	–	–	–	–	–	–	–	–	0.09
Thr	1.11	–	1.49	–	–	–	–	–	–	0.76
Ser	2.33	–	2.80	0.11	–	–	–	–	0.08	0.63
Glu	–	–	–	–	–	–	–	–	–	0.46
Pro	–	–	–	–	–	–	–	–	–	–
Gly	–	–	–	–	–	–	–	–	–	0.07
Ala	1.38	0.29	2.04	0.28	0.30	0.02	0.30	0.30	0.29	1.01
Met	3.47	–	3.99	–	–	–	–	–	–	0.65
Val	8.82	–	9.45	–	–	–	–	–	0.79	0.73
Ile	5.00	–	5.49	–	–	–	–	–	0.04	1.33
Leu	24.07	0.49	25.71	0.60	0.44	0.01	–	–	0.27	1.95
Tyr	12.30	–	17.80	0.62	0.22	–	–	–	0.56	–
Phe	10.74	–	12.25	0.27	–	–	–	–	0.72	0.37
His	2.01	–	2.17	–	–	0.07	0.63	–	0.40	0.40
Lys	38.86	6.37	30.07	5.05	1.31	0.17	2.06	0.09	1.76	0.66
Trp	–	–	–	–	–	–	–	–	–	–
Arg	22.87	0.12	22.60	1.07	–	0.12	–	–	–	–

[a] –, not detected.

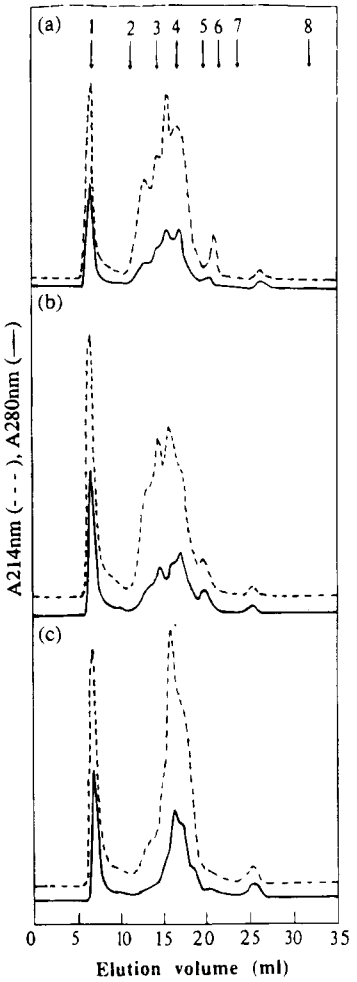

Figure 2. Chromatograms from FPLC Superose 12 column, assayed by absorbance at 280 (—) and 214 nm(– – –) of (a) elastase, (b) chymotrypsin, or (c) trypsin. Conditions: elution buffer (pH 7.0), 0.1 M Tris-HCl–0.1 M NaCl–10% methanol; flow rate, 0.5 mL min^{-1}. (1) Blue dextran (2 000 000 Da); (2) immunoglobulin G (150 000); (3) β-lactoglobulin (36 000); (4) α-lactalbumin (14 700); (5) insulin chain B (3494); (6) L-arginine (174); (7) L-tyrosine (181); (8) DL-tryptophan (204).

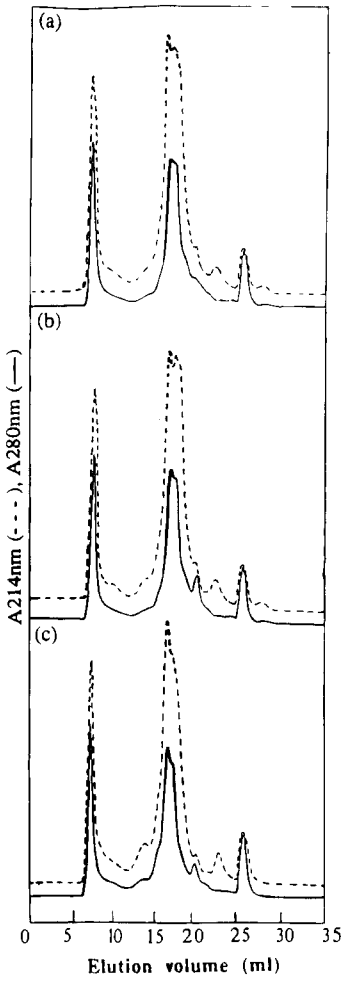

Figure 3. Chromatograms from FPLC Superose 12 column, assayed by absorbance at 280 (—) and 214 nm (– – –) of (a) PTN 3.0S, (b) PEM 2500S, or (c) PEM 2700S.

and Ser free in solution. All of the hydrolysates with the exception of Debitrase DBP.20 are deficient in residues of Asp, Glu, and Gly. Tryptophan was not detected. Proline was not found free in solution for any of the hydrolysates examined. The elastase hydrolysate had Ala, Val, Leu, Phe, Ser, and Tyr, in common with the hydrolysates produced by Corolase PP and pancreatin. Phenylalanine appeared to be produced only by Corolase PP, pancreatin, and PEM 2500S. Both Corolase PP and pancreatin produced hydrolysates that contained relatively high levels of free amino acids. The amino acids present in hydrolysates produced from both of these proteases were lysine > leucine > arginine > tyrosine > phenalanine > valine > isoleucine > methionine > serine > histidine > alanine > threonine.

Debitrase, as an exopeptidase, showed only limited hydrolysis of the lactalbumin substrate (Figure 1). However, in the hydrolysate produced, all amino acids were found.

Molecular Size Distribution of Peptides in Lactalbumin Hydrolysates. The chromatograms obtained on Superose 12, assayed at 280 or 214 nm, of lactalbumin hydrolysates are shown in Figures 2–4. The void volume (V_0) of the column corresponds to 2 × 10^6 Da, while the total column volume (V_t) corresponded to 174 Da. The molecular size distributions of peptides in hydrolysates produced by crystalline elastase or chymotrypsin (Figure 2a,b, respectively) were similiar, i.e., between 150 000 and 14 700 Da, while crystalline trypsin (Figure 2c) produced a distinctly different profile, particularly in this region. There was a strong similarity in the chromatograms of hydrolysates produced by PTN 3.0S, PEM 2500S, PEM 2700S, and PEM 800S (Figures 3 and 4a). However, hydrolysates produced by Corolase PP or pancreatin (Figure 4b,c, respectively) were substantially different, showing increased peak area corresponding to low molecular weight peptides, and reduced peak area corresponding to V_0.

DISCUSSION

There were low levels of aminopeptidase and dipeptidase activity in PEM 2500S, PTN 3.0S, PEM 800S, and PEM2700S, unlike Corolase PP and pancreatin which had higher levels of these activities. However, for all of the pancreatic preparations examined the ratios of the principal endoproteinases trypsin, chymotrypsin, and elastase were different. Limited hydrolysis of the lactalbumin substrate resulted when Debitrase DBP.20 was used, as is seen by the low percentage degrees of hydrolysis; this is because it contains only aminopeptidase and dipeptidase activities (Figure 1). The influence the detected activities may have on

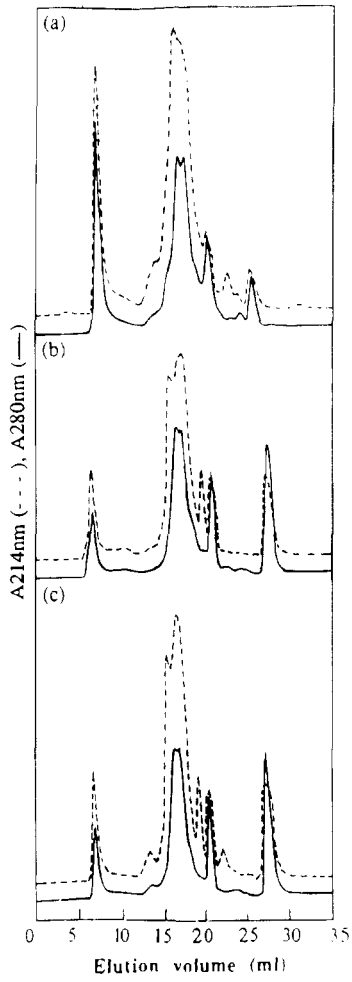

Figure 4. Chromatograms from FPLC Superose 12 column, assayed by absorbance at 280 (—) and 214 (nm (– – –) of (a) PEM 800S, (b) pancreatin, or (c) Corolase PP.

hydrolysis of lactalbumin was determined by characterizing hydrolysates obtained after incubation with individual protease preparations.

The ratio of proteinases in a pancreatic enzyme preparation is related not only to the degree of purification but also to the source of pancreatic tissue (Marchis-Mouren, 1965). In this study, trypsin-like activity was highest in preparations of porcine origin, i.e., Corolase PP, PTN 3.0S, and PEM 2500S, and may be attributed to the superior stability of porcine trypsin over the bovine counterpart (Buck *et al.*, 1962; Lazdunski and Delaage, 1965; Vithayathil *et al.*, 1961). PEM 2500S is sourced from porcine and bovine pancreas, although it is not clear at what stage of the purification that the two sources were mixed or if the bovine and porcine tissues were extracted separately and the proteases then mixed in a defined ratio. In this study the bovine trypsin preparations hydrolyzed lactalbumin to a lower degree than those preparations that contained porcine trypsin (Table 2 and Figure 1). The most obvious reason for this may be the better thermostability of porcine trypsin in comparison to bovine trypsin (Buck *et al.*, 1962), particularly in the presence of a whey protein substrate (Jost and Monti, 1977).

The low specific activity for chymotrypsin in Corolase PP, PTN3.0S, and pancreatin, which are of porcine origin, may be due to the labile nature of the porcine chymotrypsins B and C during acidic extraction (Desnuelle and Rovery, 1961; Northrop and Kunitz, 1948). Chymotrypsin also had activity toward Leu-AMC, which may be attributed to the presence of chymotrypsin C in porcine preparations (Folk, 1970). All preparations displayed arginine aminopeptidase activity, although TLCK inclusion had shown this to be largely associated with tryptic activity. There may, however, be a specific arginine aminopeptidase associated solely with porcine preparations which would explain the high levels of arginine found in hydrolysates produced by such preparations (Table 3). The levels of tyrosine and phenylalanine in hydrolysates produced by pancreatic preparations could be attributed to carboxypeptidase A activity, particularly in preparations of porcine origin (Tables 1 and 3) as this carboxypeptidase acts on the products of chymotryptic digestion (Guash *et al.*, 1992). Carboxypeptidase B acts on the products of trypsin digestion, and therefore the high levels of arginine and lysine found in hydrolysates are not unexpected, particularly in hydrolysates produced by Corolase PP or pancreatin which had high levels of carboxypeptidase B activity (Table 1).

Similarities in the peptidase activites between Debitrase DBP.20 and the pancreatic preparations Corolase PP and pancreatin are evident both from activity analysis using fluorogenic substrates and also from the type and concentration of amino acids liberated in the hydrolysates produced by these preparations. Debitrase DBP.20 is a protease preparation from *L. lactis* and *A. oryzae* and had significantly more aminopeptidase activity than the pancreatic preparations (Table 1). The increase in area and number of peaks corresponding to peptides of low molecular weight in chromatograms of Corolase PP and pancreatin hydrolysates probably reflects the contribution of aminopeptidase, dipeptidase, and elastase activities in these preparations (Figures 1 and 4).

Characteristic products of hydrolysis are obtained following treatment of lactalbumin with crystalline pancreatic enzymes. These characteristics are related to the specificities of the enzyme toward lactalbumin and can be seen in the degrees of hydrolysis (Figure 1), molecular mass profile of the peptides, within the range 150 000–14 700 Da (Figure 2), and free amino acid profiles (Table 3). To maintain or improve functionality, generally low degrees of hydrolysis are necessary (Kilara, 1985). Trypsin is specific for lysine and arginine residues; therefore, characteristic peptides and concentration of amino acids are expected after a specific hydrolysis period. In the case of chymotrypsin and elastase, the existence of a number of families of each enzyme increases the range of specificities of these enzymes, as is evident from the existence of a number of peaks corresponding to peptides, within the same range of 150 000–14 700 Da, of intermediate chain length in the gel permeation profiles at 280 and 214 nm (Figure 2a,b). Trypsin, because of its narrow specificity, produces larger peptides than chymotrypsin, and these peptides are reported to have greater emulsifying properties (Jost and Monti, 1982; Turgeon *et al.*, 1991, 1992).

There were very distinct differences in molecular mass profiles of peptides in lactalbumin hydrolysates produced using Corolase PP or pancreatin (Figure 4b,c) in comparison to the hydrolysates produced using PTN 3.0S, PEM 2500S, PEM2700S, and PEM 800S (Figures 3 and 4a). Generally, PTN 3.0S, PEM 2500S, PEM2700S, and PEM 800S produced hydrolysates of similar molecular mass profiles, and with low DH

Table 4. Commercial Pancreatic Protease Preparations, Principal Activities, and Potential Uses

	commercial protease	principal activities	potential uses	ref
group I	Corolase PP	trypsin	enteral feeds	Lalasidas et al. (1978)
	pancreatin	chymotrypsin	microbial growth media	Jost et al. (1988) (patent)
		elastase	dietetic foods	Poulsen (1987) (patent)
		leucine aminopeptidase	removal of bitterness	Sameulsson and Poulsen (1986) (patent)
		diaminopeptidases		
group II	PTN 3.0S	trypsin	functional food ingredients	Perea et al. (1993)
	PEM 2500S	chymotrypsin	dietetic foods	Kahn et al. (1990) (patent)
	PEM 800S		hypoallergenic foods	Thibault et al. (1991) (patent)
	PEM 2700S			

values, while Corolase PP and pancreatin gave more extensive hydrolyses.

Pancreatic preparations PTN 3.0S, PEM 2500S, PEM2700S, and PEM 800S, which contain only trypsin and chymotrypsin as the principal endoproteinase activity, produce hydrolysates with products within a narrow molecular mass range (Figures 3 and 4a) seen by lower percentage degrees of hydrolysis than that obtained with Corolase PP and pancreatin (Table 2). Interestingly, a similiar DH value (~8%) was obtained after 240 min of hydrolysis for the enzymes PTN 3.0S, PEM 2500S, PEM2700S, and PEM 800S, even though the ratio of trypsin to chymotrypsin was different in each (Table 1). The lactalbumin hydrolysates produced with these preparations had very similiar molecular mass profiles as determined by absorbance at 280 and 214 nm. The differences between preparations exist mainly in the molecular mass range 150 000–36 000 Da. It appears that as the ratio of chymotrypsin to trypsin decreased, there was less breakdown of large molecular mass peptides such that the size of peak 2 eluted within the molecular mass range of 150 000 Da is less substantial. Evidence of this can be seen when PTN 3.0S is compared with PEM 2500S, PEM 800S, and PEM 2700S. This peak does not exist in PTN 3.0S, but as the ratio of chymotrypsin is increased, the peak become more prominant. This may be attributed to the broader specificity of chymotrypsin (Folk, 1970), the low levels of exopeptidase activities (Table 1), or the presence of carboxypeptidase A or B in Corolase PP, pancreatin, and PTN 3.0S. It is known that the levels of carboxypeptidases are higher in porcine tissues (Desnuelle and Rovery, 1961). The chromatograms of hydrolysates produced using PTN 3.0S, PEM 2500S, and PEM 2700S were similiar; this may be due to the presence of only two endoproteolytic activities, trypsin and chymotrypsin, with minimal elastase activity (Table 1). Thibault (1991) suggested that a specific ratio of trypsin to chymotrypsin was necessary for hydrolysis of whey proteins to achieve a particular FPLC molecular mass profile and reduction in antigenicity, while the individual enzymes did not produce the desired product characteristics. Kahn *et al.* (1990) also used a specific ratio of trypsin, chymotrypsin, and porcine elastase to reduce the allergenicity of whey protein. Therefore, incubation of substrates with proteinases containing different ratios of endoproteolytic activities influences the hydrolysate characteristics.

There was no correlation between final degree of hydrolysis and solubility at pH 6.6 for the hydrolysates produced in this study. Elastase produced a hydrolysate that had a high solubility and was comparable to hydrolysates produced by commercial preparations containing mixtures of trypsin and chymotrypsin (Table 2). Preparations such as Corolase PP and pancreatin produced hydrolysates of higher solubility than hydrolysates produced by PTN 3.0S, PEM 2500S, PEM2700S, and PEM 800S. This may be explained by the presence of an additional endoproteolytic activity, elastase, and also to the additional exopeptidase activities in the former preparations. Debitrase DBP.20, being a preparation that had predominantly exopeptidase activities, produced a hydrolysate of low solubility.

The presence or absence of exopeptidases in commercial proteases may be of consequence for enzymatic hydrolysis of food proteins; for instance, in some applications, the level of free amino acids (Table 3) should be controlled so as to maintain low osmolality (Duke *et al.*, 1977) in the hydrolysate. Proteolytic preparations that contain exopeptidase activities produce less bitter hydrolysates than specific proteinases, such as trypsin (Clegg and McMillan, 1974; Cogan *et al.*, 1981; Vergarud and Langsrud, 1989; Moll, 1990). Interestingly, analysis of amino acids in free solution in the hydrolysates produced using pancreatic preparations (Table 3) did not reflect all of the aminopeptidase activities detected using fluorogenic substrates. The apparently low levels of free amino acids (Table 3) observed in hydrolysates from preparations containing exopeptidase activity (Table 1) may be due to the thermolability of the exopeptidases at 50 °C for 240 min. Furthermore, endoproteolytic digestion of lactalbumin may not result in peptides having N- or C-terminal sequences suitable for subsequent digestion by specific exopeptidases.

Pancreatin and Corolase PP, like Debitrase DBP.20, showed some activity toward the Gly-Pro-AMC substrate in addition to high activity for leucin aminopeptidase. Post proline dipeptidylaminopeptidase (PPDA), detected using Gly-Pro-AMC, has been used in synergy with leucine aminopeptidase (LAP) in the removal of bitterness from casein hydrolysates (Whitty, 1993). It could be suggested, therefore, that Corolase PP and pancreatin could have the capacity to produce hydrolysates of reduced bitterness as shown by other groups (Samuelsson and Poulsen, 1987; Lalasidas *et al.*, 1978; Chiang *et al.*, 1982). It may be that the cruder pancreatic preparations such as Corolase PP and pancreatin retain some of the dipeptidase activity, as they may have not been subjected to multiple purification steps such as acid extraction, which is used in the preparation of chymotrypsin (Northrop and Kunitz, 1948). The TCA soluble hydrolysates produced by Corolase PP and pancreatin are high in hydrophobic amino acid residues, free in solution, such as valine, leucine, and phenylalanine. The removal of these amino acids from peptides is often correlated with debittering of protein hydrolysates (Umetsu *et al.*, 1983).

From this study, it can be shown that depending on the number and ratio of activities within a protease preparation, the molecular characteristics such as molecular mass of the peptides, DH values, and concentration of amino acids in hydrolysates, differ. As the range of enzymatic activities within commercial preparations is increased, the hydrolysate becomes more complex.

The commercial proteases in this study can be classified into two distinct groups (Table 4). Group II proteases contain only trypsin and chymotrypsin, whereas group I preparations contain exopeptidases, in addition to endoprotease activities.

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