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Enzyme-Induced Gelation of Extensively Hydrolyzed Whey Proteins by Alcalase: Peptide Identification and Determination of Enzyme Specificity

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Extensive hydrolysis of whey protein isolate by Alcalase was shown to induce gelation mainly via hydrophobic interactions. The aim of this work was to characterize the peptides released in order to better understand this phenomenon. The apparent molecular mass distribution indicated that aggregates were formed by small molecular mass peptides (<2000 Da). One hundred and thirty peptides with various lengths were identified by reversed-phase high-performance liquid chromatography coupled with electrospray ionization mass spectrometry. Alcalase was observed to have a high specificity for aromatic (Phe, Trp, and Tyr), acidic (Glu), sulfur-containing (Met), aliphatic (Leu and Ala), hydroxyl (Ser), and basic (Lys) residues. Most peptides had an average hydrophobicity of 1−1.5 kcal/residue and a net charge of 0 at the pH at which gelation occurred (6.0). Therefore, an intermolecular attractive force such as hydrophobic interaction suggests the formation of aggregates that further leads to the formation of a gel.

KEYWORDS: Whey proteins; β-lactoglobulin; enzymatic hydrolysis; Alcalase; aggregation; peptides; hydrophobic interactions; net charge

INTRODUCTION

Improvements in ultrafiltration and chromatographic techniques have led to an increase in the production of whey protein ingredients. Enzymatic hydrolysis can be used to modify whey proteins and thus increase their value. Applications for whey protein hydrolysates (WPH) include, but are not limited to, reduced allergenicity (*1*), production of bioactive peptides (*2*), tailoring amounts and sizes of peptides for special diets (*3*), and altering the functional properties of gelation (*4*), foaming (*5*), and emulsification (*6*).

The specificities of the enzyme used to prepare WPH dictate the type of peptides produced and therefore its application. Enzymes with known specificity have been used to hydrolyze whey proteins. Peptides obtained after hydrolysis of whey proteins with trypsin (*7*, *8*), endoproteinase Arg-C (*9*), plasmin (*10*), and *Bacillus licheniformis* (BLP) (*11*) have been characterized. Amino acid sequence determination of the resulting peptides is made easier because it can be predicted from the protein sequence and the corresponding cleaving sites. On the other hand, the proteolytic specificity in most commercially available enzyme preparations is not very well-known. Smyth and FitzGerald (*12*) have determined endoproteinase and exopeptidase activities in nine commercially available enzyme preparations (four *Bacillus*, three *Aspergillus*, and two porcine pancreatic) using synthetic fluorogenic substrates. On the basis of their results, it was not possible to directly relate the characteristics of WPH to the enzyme complement present in crude commercial enzyme preparation. Furthermore, they found considerable variation in the enzyme complement, and the limited fluorogenic substrates used did not provide information for all of the possible cleaving sites.

Different methods are available to monitor protein hydrolysis and to characterize the resulting peptides. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) has been used to follow the proteolytic activity of different enzymes toward α-lactalbumin (α-LA) and β-lactoglobulin (β-LG) (*13*, *14*). Size exclusion high-performance liquid chromatography (*6*), reversed-phase high-performance liquid chromatography, and capillary electrophoresis (*15*) have also been used to characterize peptide profiles. More recently, mass spectrometry (MS) has emerged as an effective technique for the characterization of peptides and proteins. MS has become an indispensable tool because it is able to provide accurate molecular mass on low-picomole amounts of peptides. MS has been used in the

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identification of complex mixtures of peptides formed upon hydrolysis of whey proteins (*9*−*11*).

Other workers have shown that limited hydrolysis with a protease from BLP can induce the gelation of WPI (*4*). Several studies were conducted by the same group to elucidate the mechanism of gelation (*16*−*18*). They have shown that β-LG was the main protein from WPI involved in the gelation and that aggregates consisted of peptides of intermediate size (2−6 kDa) held together by non-covalent bonds (*19*). They also have tentatively identified by MS six to seven major peptides from β-LG responsible for aggregate formation, and the fragment f135−158 seemed to be the initiator of aggregation (*11*). In a previous study (*20*), we have shown that gelation occurs during extensive hydrolysis of whey protein isolate with Alcalase 2.4L (Alcalase). The resulting gel was light brown and opaque, suggesting the formation of aggregates. Even though only one step was required to induce gelation, similarities were identified with the plastein reaction and the gelation seemed to be caused by physical aggregation, mainly via hydrophobic interactions, with hydrogen bonding and electrostatic interactions playing a minor role (*21*). The objectives of this work were to characterize the peptides released during extensive hydrolysis of WPI by Alcalase and to confirm the importance of hydrophobic interaction in order to better understand this phenomenon.

MATERIALS AND METHODS

Materials. Whey protein isolate (WPI) was obtained from Davisco Foods International (Le Sueur, MN). The composition of the protein isolate in β-LG, α-LA, and bovine serum albumin (BSA) was 66, 22, and 4%, respectively, according to the manufacturer. Alcalase 2.4L (a liquid preparation from subtilisin Carlsberg) was obtained from Novozymes (Franklinton, NC). All other chemicals were of reagent grade quality.

β-LG (L-0130), α-LA (L-5385), ribonuclease A (R-5125), and BSA (A-2153) were purchased from Sigma Chemical CO. (St. Louis, MO). Chymotrypsinogen and ovalbumin were from Pharmacia Biotech (Baie d'Urfé, PQ, Canada). β-LG 142−148 and 41−60, α-LA 50−53, β-casein 193−202, and α_{s1}-casein 28−34 were synthesized by the Service de séquence de peptides de l'est du Québec (Sainte-Foy, PQ, Canada).

Hydrolysis of WPI. The 20% (w/v) WPI solution was prepared and hydrolyzed for 5 h with Alcalase, and the enzyme was inactivated according to the method described by Doucet et al. (*21*). WPI solutions (20% w/v) were heated to 45 °C and adjusted to pH 8.0 before the addition of Alcalase. The ratio of enzyme preparation to whey protein was 1:10 (v/w). The final pH of the hydrolysate was 6.0 (after 5 h of hydrolysis) because the pH was not controlled during the reaction. The enzyme-induced gel obtained after 5 h of hydrolysis was diluted with an equal volume of deionized water. Proteolysis was terminated according to the method of Adler-Nissen (*22*). The pH of the solution was lowered to 4.0 and the temperature was increased to 50 °C for 15 min to stop the enzyme reaction. The hydrolysate was then frozen, lyophilized, and stored at 4 °C before further analysis.

Molecular Mass Distribution Profiles. Molecular mass distribution profiles were determined by high-performance size exclusion chromatography (HPSEC) using a Waters HPLC system (Millipore, Milford, MA) and a TSK-Gel G2000 SW_{XL} column (0.78 i.d. × 30 cm) from TosoHaas (Montgomeryville, PA) connected to a guard column (0.6 i.d. × 4 cm) filled with the same matrix. Samples were prepared (1.0% protein in 0.1% TFA in 70% aqueous acetonitrile), 20 μL was injected onto the column, and elution was performed isocratically in the same TFA−acetonitrile buffer at 28 °C with a flow rate of 0.6 mL/min over 30 min. Detection was at 214 nm. Protein standards used for the calibration were BSA (67000 Da), ovalbumin (43000 Da), β-LG (36000 Da), chymotrypsinogen A (25000 Da), α-LA (14000 Da), ribonuclease A (13700 Da), β-LG 41−60 (2314 Da), β-casein 193−202 (1156 Da), β-LG 142−148 (837 Da), α_{s1}-casein 28−34 (822 Da), and α-LA 50−53 (499 Da). The total area of the chromatograms was integrated and separated into four molecular mass ranges (>10000, 5000−10000, 2000−5000, and <2000 Da), expressed as a percentage of the total area.

Liquid Chromatography—Electrospray Mass Spectrometry (LC-ESI/MS). LC-MS was performed on a ThermoFinnigan Surveyor LC system and LCQDuo MS (ThermoQuest, San Jose, CA) using a Vydac C_{18} column (0.21 i.d. × 15 cm) (Hesperia, CA) connected to a guard column (0.21 i.d. × 5 cm) filled with the same matrix and directly interfaced with the mass spectrometer. Analysis was performed at a flow rate of 0.2 mL/min (40 °C) using solvent A (0.1% TFA in water) and solvent B (0.09% TFA in 90% aqueous acetonitrile). Elution was obtained as follows: linear gradient of solvent B from 0 to 40% over 50 min, and 90% B, 2 min. The column was then re-equilibrated in solvent A for 8 min. Detection was at 214 nm, and the total run time was 60 min. Powder samples of the enzyme-induced gel were made to 1 mg/mL in 5% formic acid and stirred gently for 1 h at room temperature. Samples were then centrifuged in a microfuge at 16000*g* for 5 min. The samples were also analyzed after reduction with dithiothreitol (DTT). Samples were treated overnight at ambient temperature with 10 mM DTT in 5 mM Tris-HCl buffer, pH 8.

The mass spectrometer was operated in positive ion mode and was scanned over an *m*/*z* range of 50−2000. The electrospray interface was operated using the following settings: capillary voltage, 30 V; capillary temperature, 250 °C; spray voltage, 4.5 kV; tube lens voltage, 10 V. Nitrogen was used as nebulizing and drying gas. Calibration was performed using leucine enkephalin. Molecular masses were determined from the multiple charge ions using Xcalibur software (ThermoQuest). The amino acid sequence of each peptide was determined using MS/MS. ExPaSy Molecular Biology Server and Bioworks software (ThermoQuest) were used to analyze the product ions.

Frequency of Amino Acids in the P_1 Position and Cleavage Frequency Percentage. Enzyme specificity can be represented using the subsite nomenclature from Schechter and Berger (*23*, *24*). According to this model, amino acid residues in a polypeptide chain undergoing cleavage are designated P_1, P_2, P_3, P_4, etc., in the N-terminal direction from the cleavage bond. The amino acid before the scissile peptide bond (the bond susceptible to cleavage) in the polypeptide chain is therefore in the P_1 position. The residues in C-terminal position are designated P_1', P_2', P_3', P_4', etc., as shown in the following model:

$$P_N\text{- - - -}P_4{-}P_3{-}P_2{-}P_1 \neq P_1'{-}P_2'{-}P_3'{-}P_4'\text{- - - -}P_C'$$

The scissile peptide bonds at the amino and carboxyl termini for each peptide were identified from the MS/MS analysis and the β-LG amino acid sequence. The frequency of every amino acid in the P_1 position was then calculated. The cleavage frequency percentage was also calculated for all of the amino acids in the P_1 position according to their respective proportion in the β-LG molecule. For example, the amino acid phenylalanine was found 19 times in the P_1 position, and there are 4 Phe residues in the β-LG molecule, so the cleavage frequency was 475% (19/4 × 100).

SDS-PAGE. The enzyme preparation was solubilized at 10 mg/mL in 2% SDS, 8 M urea, 4% β-mercaptoethanol, and 20 mM Tris-HCl buffer, pH 8.0. The solution was heated at 100 °C for 2 min, brought to room temperature, covered, stirred continuously for 24 h at room temperature, and then centrifuged at 10000*g* for 20 min. The enzyme solution was then studied by SDS-PAGE using a 4−20% (w/v) linear gradient acrylamide gel (Bio-Rad Chemical Division, Richmond, CA) at two concentrations (50 and 100 μg/lane). The molecular mass standard contained proteins of the following molecular masses: 66200, 45000, 31000, 21500, 14400, and 6500 Da. Gels were fixed and stained overnight with a NOVEX Colloidal Blue staining kit (Novex Experimental Technology, San Diego, CA) containing a Colloidal Coomassie G-250 stain. The stained gels were destained overnight with deionized water. Gels were then dried using the Dry Ease gel drying system (NOVEX).

RESULTS AND DISCUSSION

Peptide Characterization. WPH designed for nutritional applications (sports nutrition, enteral formulas, hypoallergenic

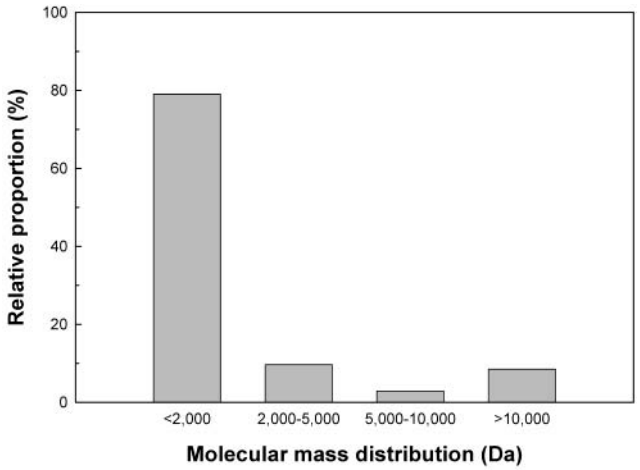

Figure 1. Molecular mass distribution of peptides in the enzyme-induced gel determined by HPSEC.

infant formulas, etc.) have generally a high degree of hydrolysis and therefore a high content of short peptides. Short-chain peptides are less antigenic due to the elimination of sequential epitopes (*1*). Furthermore, a product with a low content of free amino acids is absorbed more efficiently because of intestinal absorption differences between peptides (primarily di- and tripeptides) and free amino acids (*3*, *25*). To obtain that kind of profile, an extensive enzymatic hydrolysis is required, preferentially with an endoprotease having broad specificty, in order to avoid the presence of free amino acids in the final product. Alcalase represents a good candidate for the industrial production of WPH because of its endoprotease with broad specificity and its low cost. In a previous paper (*21*), we have shown that the average chain length of peptides forming an enzyme-induced gel after a 5 h hydrolysis of WPI with Alcalase was ~4.3 residues as determined by α-amino group analysis before and after acid hydrolysis. To better characterize the molecular mass distribution, the enzyme-induced gel was analyzed by HPSEC. **Figure 1** shows that >80% of the peptides have a molecular mass smaller than 2000 Da. This suggests that aggregates are made of small molecular mass peptides.

RP-HPLC/MS was performed to identify the peptides present in the enzyme-induced gel **(Figure 2**). Masses were determined, putative peptides were predicted by ExPasy Molecular Biology Server, and MS/MS was used to identify the amino acid sequence of the peptides (**Table 1**). Centrifugal experiments were also carried out to distinguish between peptides participating in the formation of the gel network (precipitate) and soluble peptides not included in the gel network (supernatant). Peptides identified in the precipitate were also found in the supernatant (results not shown); thus, results represent all of the peptides in the enzyme-induced gel. The RP-HPLC chromatogram was separated into 15 different fractions, and 130 peptides of various lengths were characterized by MS/MS. The MS/MS analysis showed that many peptides were present under each fraction of the chromatogram as seen in **Table 1**. It was interesting to note that protein components with molecular masses >2000 Da were observed from HPSEC analysis **(Figure 1)** but were not observed by LC-MS analysis **(Table 1)**. Visser et al. (*26*) have determined the molecular mass distribution of WPH by HPSEC and found that size exclusion is best suited for the comparison of apparent molecular mass distributions rather than for the precise determination of molecular mass. Toussavainen et al. (*27*) have characterized the peptide size range of extensively hydrolyzed whey proteins and found that small peptide standards gave incorrect molecular masses when analyzed by size exclusion. Size exclusion elution is based on hydrodynamic radius

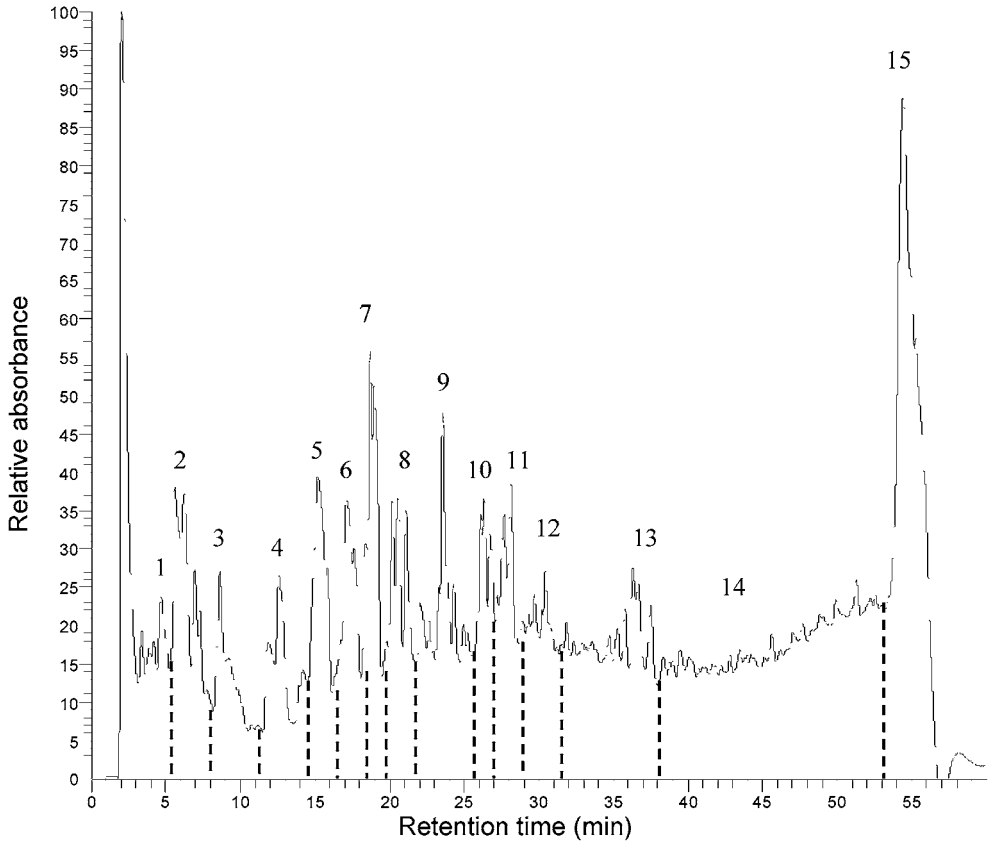

Figure 2. RP-HPLC chromatogram of the enzyme-induced gel. Detection was made at 214 nm. Fraction numbers refer to peptides described in **Table 1**.

Table 1. Identification by ESI-MS of Peptides in the Enzyme-Induced Gel As Separated by RP-HPLC (**Figure 2**)

fraction	exptl mass	theor mass[a]	amino acid sequence[b]	position in β-LG	isoelectric point[a]	charge at pH 6.0	Hf_{av}[c] (kcal/res)
1	260.1	260.2	(D)IQ(K)	12–13	5.52	0	1.48
	375.1	375.2	(F)KID(A)	83–85	6.09	0	1.48
	376.1	376.3	(E)KTK(I)	75–77	10.00	2	1.15
	425.3	425.3	(M)HIR(L)	146–148	9.76	2	1.23
	477.1	477.2	(E)VDDE(A)	128–131	3.80	–3	0.43
	749.2	749.3	(E)NGECAQK(K)	63–69	5.99	0	0.46
2	349.1	349.1	(M)ENS(A)	108–110	4.00	–1	0
	382.1	382.2	(W)YSL(A)	20–22	5.52	0	1.75
	532.1	532.2	(N)SAEPE(Q)	110–114	4.24	–2	0.67
	574.4	574.4	(F)DKALK(A)	137–141	8.59	1	1.23
	583.2	583.2	(F)CMENS(A)	106–110	4.00	–1	0.46
	646.2	646.3	(S)LLDAQS(A)	31–36	3.80	–1	0.93
3	203.0	203.1	(E)AL(E)	132–133	5.57	0	1.58
	219.0	219.1	(Y)SL(A)	21–22	5.24	0	1.2
	235.0	235.1	(D)TD(Y)	97–98	4.30	–1	0.23
	269.1	269.2	(M)HI(R)	146–147	6.74	0	1.48
	347.1	347.2	(I)AEK(T)	73–75	6.05	0	0.75
	433.1	433.2	(I)PAVF(K)	79–82	5.96	0	1.93
	635.0	635.3	(N)GECAQK(K)	64–69	5.99	0	0.54
4	189.0	189.1	(K)GL(D)	9–10	5.52	0	1.2
	294.1	294.2	(E)KF(D)	135–136	8.75	1	2.08
	317.2	317.2	(M)KGL(D)	8–10	8.75	1	1.3
	393.2	393.3	(A)VFK(I)	81–83	8.72	1	1.95
	446.2	446.3	(F)KIDA(L)	83–86	5.84	0	1.3
	618.4	618.4	(A)EKTKI(P)	74–78	8.69	1	1.28
5	332.1	332.2	(E)ALE(K)	132–134	4.60	–1	1.05
	389.1	389.2	(E)ELK(P)	45–47	6.10	0	1.3
	417.3	417.2	(K)GLDI(Q)	9–12	3.80	–1	0.6
	437.3	437.3	(L)RVY(V)	40–42	8.75	1	1.77
	462.2	462.2	(Q)KWE(N)	60–62	6.22	0	1.5
	551.2	551.4	(Y)KKYL(L)	100–103	9.70	2	2.06
6	231.1	231.2	(L)VL(D)	94–95	5.49	0	2.05
	333.2	333.2	(Q)KW(E)	60–61	8.75	1	2.25
	601.4	601.3	(L)VRTPE(V)	123–127	6.19	0	1.1
7	316.1	316.2	(K)IIA(E)	71–73	5.52	0	2.22
	363.1	363.2	(A)GTW(Y)	17–19	5.52	0	1.15
	372.1	372.2	(Q)CHI	160–162	6.73	0	1.32
	373.2	373.3	(K)KII(A)	70–72	8.75	1	2.47
	387.1	387.2	(Q)SAPL(R)	36–39	5.24	0	1.44
	478.2	478.2	(S)FNPT(Q)	151–154	5.52	0	1.43
	645.4	645.4	(F)DKALKA(L)	137–142	8.59	1	1.15
	684.3	684.4	(E)LKPTPE(G)	46–51	6.22	0	1.59
	721.4	721.4	(K)FDKALK(A)	136–141	8.59	1	1.47
	804.5	804.3	(R)TPEVDDE(A)	125–131	3.70	–4	0.68
8	359.2	359.3	(N)KVL(V)	91–93	8.75	1	1.87
	362.2	362.2	(P)EVD(D)	127–129	4.03	–2	0.57
	378.1	378.1	(V)DDE(A)	129–131	3.80	–3	0
	473.2	473.3	(E)NKVL(V)	90–93	8.75	1	1.4
	565.2	565.3	(L)SFNPT(Q)	150–154	5.24	0	1.14
	657.4	657.4	(E)KTKIPA(V)	75–80	10.00	2	1.63
	661.3	661.4	(Q)KVAGTW(Y)	14–19	8.75	1	1.23
	714.5	714.4	(C)LVRTPE(V)	122–127	6.22	0	1.32
	813.4	813.4	(E)ELKPTPE(G)	45–51	4.86	–1	1.36
9	245.1	245.1	(E)PE(Q)	113–114	4.60	–1	1.3
	368.1	368.2	(T)WY(S)	19–20	5.52	0	2.93
	373.2	373.3	(K)KII(A)	70–72	8.75	1	2.47
	549.3	549.2	(M)ENSAE(P)	108–112	4.24	–2	0.15
	573.3	573.4	LIVTQ(T)	1–5	5.52	0	1.5
	677.3	677.3	(P)EVDDEA(L)	127–132	3.43	–4	0.41
	694.3	694.3	(A)MAASDIS(L)	24–30	3.80	–1	0.82
	701.5	701.5	(Q)KKIIAE(K)	69–74	8.59	1	1.61
	716.4	716.4	(L)NENKVL(V)	88–93	6.00	0	0.93
	719.4	719.4	(S)FNPTQL(E)	151–156	5.52	0	1.35
	758.4	758.5	(F)DKALKAL(P)	137–143	8.59	1	1.33
	769.3	769.4	(L)DTDYKK(Y)	96–101	5.96	0	1.05
	773.3	773.4	(S)AEPEQSL(A)	111–117	3.80	–2	0.82
	806.3	806.4	(L)SFNPTQL(E)	150–158	5.24	0	1.16
	860.4	860.4	(N)SAEPEQSL(A)	110–117	3.79	–2	0.72
	974.4	974.4	(E)NSAEPEQSL(A)	109–117	3.79	–2	0.64
	1059.6	1059.5	(L)VRTPEVDDE(A)	123–131	4.16	–3	0.8

Table 1. (Continued)

fraction	exptl mass	theor mass[a]	amino acid sequence[b]	position in β-LG	isoelectric point[a]	charge at pH 6.0	Hf_{av}[c] (kcal/res)
10	247.1	247.1	(L)PM(H)	144–145	5.96	0	1.95
	559.3	559.3	(L)KALPM(H)	141–145	8.75	1	1.71
	791.5	791.4	(S)LAMAASDI(S)	22–29	3.80	–1	1.11
	817.5	817.4	(D)TDYKKY(L)	97–102	8.10	1	1.53
	838.4	838.4	(V)LVLDTDY(K)	93–99	3.56	–2	1.3
	861.2	861.4	(E)NSAEPEQS(L)	109–116	3.79	–2	0.42
	1041.6	1041.6	(A)LNENKVLVL(D)	87–95	6.00	0	1.34
	1098.7	1098.6	(E)LKPTPEGDLE(I)	46–55	4.43	–2	1.2
11	279.1	279.2	(L)LF(C)	104–105	5.52	0	2.53
	400.1	400.2	(P)MHI(R)	145–147	6.49	0	1.42
	526.2	526.3	(D)TDYK(K)	97–100	5.50	0	1.2
	560.3	560.3	(D)ISLLD(A)	29–33	4.30	–1	1.55
	658.3	658.3	(K)YLLFC(M)	102–106	5.52	0	2.26
	855.5	855.5	(F)DKALKALP(M)	137–144	8.59	1	1.49
	856.4	856.4	(L)KPTPEGDL(E)	47–54	4.37	–1	1.19
	1144.8	1144.5	(V)RTPEVDDEAL(E)	124–133	3.92	–3	0.87
	1227.7	1227.6	(E)ELKPTPEGDLE(I)	45–55	4.24	–3	1.09
	1305.6	1305.7	(L)DTDYKKYLLF(C)	96–105	5.96	0	1.66
12	318.1	318.2	(L)LDA(Q)	32–34	3.80	–1	1.05
	336.1	336.2	(P)AVF(K)	80–82	5.57	0	1.7
	344.1	344.3	LIV(T)	1–3	5.52	0	2.35
	345.1	345.2	(N)PTQ(L)	153–155	5.96	0	1.02
	358.2	358.3	(E)ILL(Q)	56–58	5.52	0	2.58
	538.4	538.3	(M)HIRL(S)	146–149	9.76	1	1.53
	678.3	678.3	(S)LAMAASD(I)	22–28	4.30	–1	0.85
	792.5	792.4	(L)ACQCLVR(T)	118–124	8.11	1	1.09
	900.5	900.5	(D)ALNENKVL(V)	86–93	6.05	0	1.09
	999.8	999.5	(T)QLEEQCHI	155–162	4.51	–2	0.79
	1045.7	1045.5	(E)NSAEPEQSLA(C)	109–118	3.79	–2	0.65
	1275.8	1275.7	(Q)SAPLRVYVEEL(K)	36–46	4.53	–1	1.38
	1372.8	1372.7	(L)VRTPEVDDEALE(K)	123–134	4.04	–4	0.86
	1455.7	1455.7	(Y)VEELKPTPEGDLE(I)	43–55	4.12	–4	1.05
13	568.3	568.3	(T)WYSL(A)	19–22	5.52	0	2.06
	610.5	610.3	(A)LPMHI(R)	143–147	6.74	0	1.85
	674.5	674.4	(T)KIPAVF(K)	77–82	8.75	1	2.03
	688.4	688.4	(D)ALNENK(V)	86–91	6.05	0	0.78
	903.6	903.6	(E)KTKIPAVF(K)	75–82	10.00	2	1.76
	1032.5	1032.6	(A)EKTKIPAVF(K)	74–82	8.69	1	1.56
14	392.2	392.3	(Y)LLF(C)	103–105	5.52	0	2.48
	560.2	560.3	(D)ISLLD(A)	29–33	4.30	–1	1.55
	647.3	647.4	(A)SDISLL(D)	27–32	3.80	–1	1.29
	673.5	673.4	(F)KIDALN(E)	83–88	5.84	0	1.27
	681.3	681.4	(K)ALPMHI(R)	142–147	6.79	0	1.67
	718.3	718.4	(A)ASDISLL(D)	26–32	3.80	–1	1.21
	726.3	726.3	(W)YSLAMAA(S)	20–26	5.52	0	1.26
	775.5	775.5	(K)TKIPAVF(K)	76–82	8.41	1	1.8
	783.3	783.4	(K)VAGTWYS(L)	15–21	5.49	0	1.25
	833.4	833.4	(A)ASDISLLD(A)	26–33	3.89	–2	1.06
	905.5	905.5	(K)FDKALKAL(P)	136–143	8.59	1	1.49
	1034.6	1034.6	(E)ALEKFDKAL(K)	132–140	6.11	0	1.33
	1165.5	1165.7	(L)KALPMHIRLS(F)	141–150	11.00	2	1.47
15	664.5	664.4	(Y)KKYLL(F)	100–104	9.70	2	2.13
	857.4	857.4	(K)PTPEGDLE(I)	48–55	3.91	–3	1.01
	933.5	933.5	(A)QSAPLRVY(V)	35–42	8.75	1	1.38
	986.5	986.6	(F)DKALKALPM(H)	137–145	8.59	1	1.47
	1001.7	1001.6	(K)GLDIQKVAGT(W)	9–18	5.84	0	0.98
	1114.6	1114.6	(L)PMHIRLSFN(P)	144–152	10.18	1	1.41
	1187.2	1186.8	(A)QKKIIAEKTK(I)	68–77	10.00	3	1.31
	1200.7	1200.7	(K)IPAVFKIDALN(E)	78–88	5.84	0	1.66
	1245.8	1245.6	(R)TPEVDDEALEK(F)	125–135	3.83	–4	0.85
	1330.0	1329.7	(A)SDISLLDAQSAPL(R)	27–39	3.56	–2	1.1
	1458.2	1458.7	(S)FNPTQLEEQCHI	151–162	4.51	–2	1

[a] Theoretical mass and isoelectric point were obtained from ExPASY Molecular Biology Server. [b] Amino acids before or after the peptidic sequence are shown in parentheses. [c] Average hydrophobicity was calculated according to the method of Bigelow (*42*).

and is best suited for globular proteins. Peptides deviate from the globular shape, and their molecular mass can be incorrect. Sylvestre et al. (*28*) have observed that electrostatic interactions can occur with small peptides and amino acids, thus causing deviations from size exclusion elution. Irvine et al. (*29*) have also reported that deviation can be caused by electrostatic and hydrophobic interactions between the solute and the matrix due to the anionic character of the stationary phase. This may explain why HPSEC results showed peptides with molecular masses >2000 Da.

According to the manufacturer, the WPI used in this study contained approximately 66% β-LG and 22% α-LA. Surprisingly, only peptides from β-LG were identified (**Table 1**). We are currently investigating the hydrolysis of α-LA alone and in the presence of different concentrations of β-LG to determine why α-LA peptides are not identified in this WPI hydrolysate. Furthermore, β-LG peptides identified were only from the B variant, even though the WPI used contained approximately the same proportions of A and B variants. The B variant can be considered as the parent form, and the A variant differs only by substitution of a Gly side chain at position 64 with an Asp and substitution of an Ala side chain with a Val at position 118 (*30*). Another possible explanation is that fragments of β-LG were not identified due to their low concentration and the complexity of the peptide mixture. Ion suppression effects can happen with electrospray ionization even though this technique has been applied successfully to peptide analysis (*31*).

Only 8 of the 130 peptides identified contained a cysteine residue that can participate in thiol/disulfide exchange. The enzyme-induced gel was treated with DTT, and peptides were identified by LC-MS to study disulfide-linked peptides (results not shown). The same peptides were present, and no others were identified. This reinforces results from a previous study (*21*) and confirms that disulfide bonds are not involved in the formation of the network. Otte et al. (*9*) have shown that hydrolysis of β-LG by other enzymes such as bromelain, papain, pepsin, or endoproteinase Arg-C, does not to lead to disulfide-linked peptides. The use of broad specificity enzymes has been suggested to produce a large number of peptides of which many lack a cysteyl residue that can participate in the SH/SS exchange (*10*).

Alcalase Specificity. Alcalase is an enzymatic preparation that has previously been used to produce soluble hydrolysates for soy protein (*32*) and fish protein (*33*). However, as mentioned before, extensive hydrolysis of WPI at high solid content with Alcalase produces gelation. Alcalase is an industrial food grade enzyme often used because of its low cost. It is a serine alkaline protease produced by a selected strain of *B. licheniformis*. Its main enzyme component, subtilisin Carlsberg (also called subtilisin, subtilisin A, subtilopeptidase A, and Alcalase Novo), was prepared and crystallized for the first time by Guntelberg and Ottesen (*34*). Subtilisin Carlsberg consists of a single peptide chain of 274 amino acid residues with no disulfide bonds, and it has a molecular mass of 27277 Da with an isoelectric point of 9.4. The enzyme has broad specificity, hydrolyzing most peptide bonds, preferentially those containing aromatic amino acid residues. The enzyme has a pH optimum for activity between pH 8 and 9 and broad pH stability, being inactivated quickly below pH 5.0 and above pH 11.0 (*35*). Although Alcalase has been widely used in many different applications such as detergents, little is known about its specificity and there are only a few scattered reports in the literature.

Figure 3 shows the distribution of amino acids in the P_1 position for the 130 peptides obtained after hydrolysis of WPI with Alcalase (**Table 1**). The cleavage frequency percentage for all of the amino acids in the P_1 position, according to their respective proportion in the β-LG molecule, was also calculated (see Materials and Methods for example) and is indicated above each bar.

Peptide bonds on the carboxyl side of Phe, Tyr, and Trp were hydrolyzed, confirming the specificity of subtilisin Carlsberg for aromatic amino acid residues (*35*). **Figure 3** shows that many peptides have a Phe residue in the P_1 position, but only a few

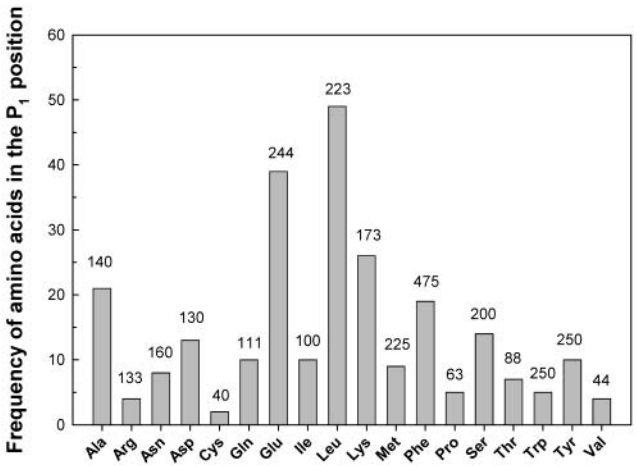

Figure 3. Distribution of amino acids in the P_1 position for the 130 peptides identified (**Table 1**) in the enzyme-induced gel. The cleavage frequency percentage for each amino acid according to their respective proportion in the β-LG molecule is indicated above the bar.

have Trp or Tyr. However, the cleavage frequency percentages for Trp and Tyr are the second highest (250%) after Phe (475%). This shows the importance of accounting for the number of amino acid residues in a particular protein when the specificity of an enzyme is described. The aliphatic residues Leu and Ala were found in high proportion in the P_1 position (**Figure 3**). β-LG contains 22 Leu and 15 Ala residues, which explains why so many peptides in this WPH are cleaved after these residues (*30*). Johansen et al. (*36*) have shown that when the oxidized B-chain of insulin was incubated for 4 h with subtilisin Carlsberg, peptide bonds between Gln^4-His^5, Ser^9-His^{10}, $Leu^{11}-Val^{12}$, $Leu^{15}-Tyr^{16}$, and $Tyr^{26}-Thr^{27}$ were cleaved. The $Leu^{15}-Tyr^{16}$ bond was found to be cleaved more rapdily than any other bond. Peptide bonds on the carboxyl side of all those residues were also cleaved in this work (**Figure 3**). Casein phosphopeptides were also prepared using Alcalase, and the enzyme specificity was determined after peptide analysis (*37*). Peptide bonds on the carboxyl side of Glu, Met, Leu, Tyr, Lys, and Gln residues were cleaved. Furthermore, of the 26 different cleavage sites in casein phosphopeptides, 17 contained a Glu in the P_1 position and most of them contained a Glu in the P_1 position with a hydrophobic residue in either P'_2 or P'_3. The specificity of the enzyme for Glu in the P_1 position was also identified in this work as one of the most frequent cleaving sites (**Figure 3**). The specificity for the sulfur-containing residue Met was also observed when the cleavage frequency percentage was evaluated (**Figure 3**).

Sukan and Andrews (*38*) have observed that a greater degree of hydrolysis could be achieved with Alcalase as compared with enzymes such as pepsin and chymotrypsin. They suspected Alcalase to contain several different proteinases, each with different specificities, because it is a relatively crude bacterial extract of *B. licheniformis*. **Figure 4** shows an electrophoretic gel pattern of Alcalase under denaturing and reducing conditions at two different concentrations. At least four different bands can be identified in this gel, which confirms the presence of different proteins. The top band appears as the most predominant and represents subtilisin Carlsberg. The second band from the top has a molecular mass of ~24000 Da. Svendsen and Breddam (*39*) have isolated and characterized a Glu-specific endopeptidase from a commercial preparation of Alcalase, and they have found a molecular mass of 23589 Da. The enzyme had an optimum pH at 7.5−8.0 that corresponds to the conditions used in this study. It appeared to be essentially specific for Glu and

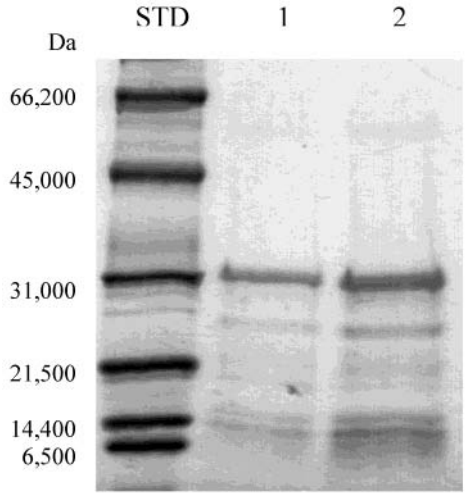

Figure 4. SDS-PAGE gel pattern of Alcalase 2.4L under denaturing and reducing conditions at 50 (lane 1) and 100 μg/lane (lane 2). Molecular mass marker is represented by STD.

Asp residues in the P_1 position with strong preference for the former. The presence of a Glu-specific endopeptidase in the Alcalase preparation used in this study explains why so many peptides contain Glu in the P_1 position and the high cleavage frequency percentage (**Figure 3**). Svendsen and Breddam (*39*) also found that nonspecific cleavages were observed after prolonged hydrolysis when a high enzyme concentration was used. This might explain why peptide bonds on the carboxyl side of so many different amino acids are cleaved but not always at a high cleavage frequency percentage (**Figure 3**). Two other minor bands were also identified between the molecular markers 14400 and 6500 Da, but it was not possible to identify the nature of these proteins (**Figure 4**).

It was surprising that many peptides contained a Lys residue in the P_1 position with a high cleavage frequency percentage (**Figure 3**). Subtilisin Carlsberg has a rather hydrophobic pocket with no particular affinity for Lys or Arg. The preference for aromatic and other hydrophobic amino acids comes from the presence of a hydrophobic binding area on the enzyme molecule (*40*). Peptides with a Lys residue in the P_1 position were also observed when casein phosphopeptides were prepared with Alcalase (*37*), but the possibility for different proteases in the enzymatic preparation was not discussed. Zuidweg et al. (*41*) have identified three components with different isoelectric points, pH optima, and thermostabilities in a commercial preparation of Alcalase. They also showed that the three different components were not the result of autodigestion. The enzymatic preparation used in this study was incubated under different conditions, and electrophoretic gels confirmed that bands were not the result of autodigestion (results not shown). As discussed previously, subtilisin Carlsberg has broad specificity, and the presence of other proteases in the commercial preparation of Alcalase also explains why most peptide bonds are hydrolyzed (**Figure 3**). This work presents cleaving sites that have never been reported before. We are currently investigating the time course hydrolysis of β-LG in the presence of Alcalase to determine if peptides identified in this work result from nonspecific cleavage.

Gelation Mechanism. Previous work showed that hydrophobic interactions were the main forces involved in the enzyme-induced gelation of WPI with Alcalase (*21*). The presence of peptides with a high content in hydrophobic amino acids was investigated to determine if the gelation was caused by aggregation of these fragments of the β-LG molecule. **Figure 5A** shows a distribution of the different peptides present in the enzyme-induced gel according to their average hydrophobicity using the method proposed by Bigelow (*42*). According to this method, peptides with a high average hydrophobicity were not the main component of the enzyme-induced gel, and most peptides had an average hydrophobicity between 1 and 1.5 kcal/residue **(Figure 5A)**. This suggests that another phenomenon could be contributing to the aggregation by hydrophobic interactions.

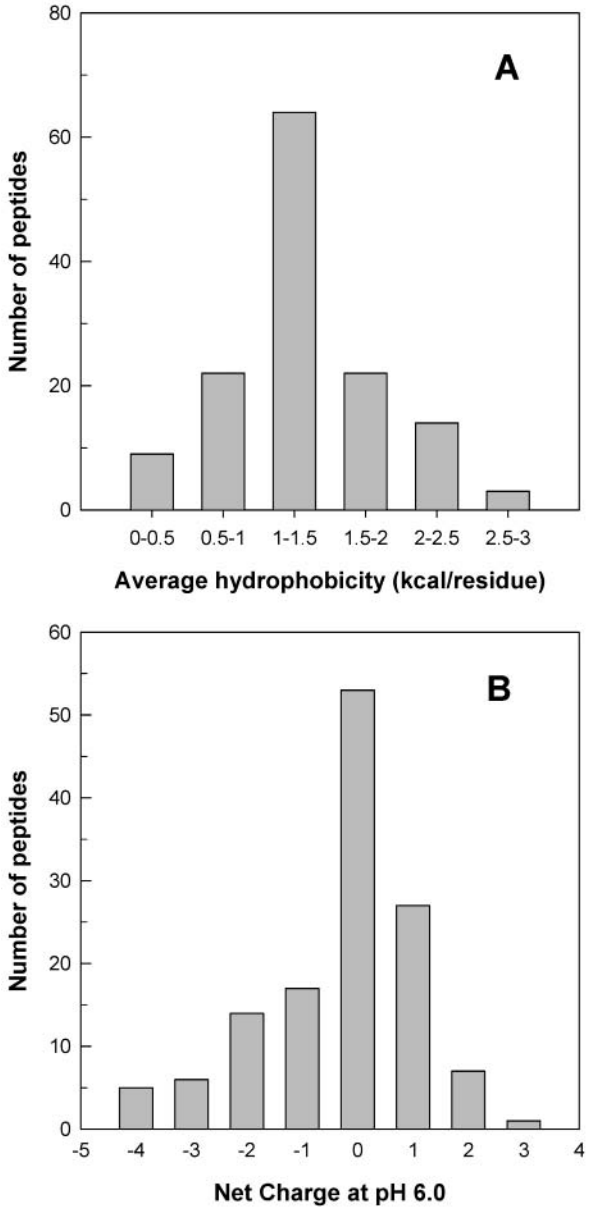

Figure 5. Distribution of the 130 peptides identified (**Table 1**) in the enzyme-induced gel according to their average hydrophobicities (A) calculated using the method proposed by Bigelow (*42*) and according to their net charge at pH 6.0 (B).

The net charge at pH 6.0, which is the pH when gelation occurs, was also investigated for all of the peptides (**Table 1**). **Figure 5B** indicates that >55 of the 130 peptides had an isoelectric point close to pH 6.0 and consequently had no charge. Near the isoelectric point, electrostatic forces are weak. In this state, an intermolecular attractive force such as hydrophobic interaction can induce the formation of aggregates. This might explain why gelation occurs and reinforces our observation of a physical aggregation driven by hydrophobic interactions.

Leonil et al. (*43*) have also reported that precipitation of peptides from a trypsin casein hydrolysate occurred as a result of a reduction of ionization leading to aggregation through hydrophobic interactions.

Plastein reaction has been reported to take place between pH 4 and 7 (*44*), and hydrophobic interactions were identified as the main forces involved (*45*). However, peptides involved in the plastein reaction have never been characterized. To our knowledge, this is the first complete peptide characterization of extensively hydrolyzed proteins with a broad specificity microbial protease leading to the formation of a gel. The abundance of peptides with no charge in the pH range 4−7 may explain why peptides aggregate by hydrophobic interactions and provides a better understanding of the plastein reaction. We are currently investigating the same phenomenon with other proteases and substrates to determine if the same mechanism is involved.

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