Two New Methods of Debittering Protein Hydrolysates and a Fraction of Hydrolysates with Exceptionally High Content of Essential Amino Acids

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Extraction of enzymatic protein hydrolysates with azeotropic secondary butyl alcohol (SBA) seems to be an efficient and generally applicable method for removal of bitter compounds. For complete debittering, approximately 5–10% of the hydrolysate is usually removed. The bitter peptides are concentrated in the SBA phase which has an extremely bitter taste. On extraction of aqueous solution of hydrolysates or extraction of dry powder hydrolysates with azeotropic SBA a concentration of 50–70% essential amino acids was reached in the SBA phase. Leucine, isoleucine, phenylalanine, and tryptophan were particularly increased. Experiments showed that bitterness of the SBA-soluble fraction could be reduced by applying the plastein reaction. A reduction in bitterness of protein hydrolysates could also be achieved by applying hydrophobic interaction chromatography. Of tested gels, hexylsepharose was found to be most effective for debittering of protein hydrolysates.

Great amounts of high quality protein are today used as animal fodder because of disagreeable odor or taste. Among substances which cause bad odor or taste are rancid fat and low molecular weight substances (in soy, e.g., 1-hexanol and 1-hexanal; in fish, e.g., trimethylamine and 2-butanone). Efforts have therefore been made to develop methods for removal of such undesirable components.

One method is to use proteolytic enzymes. Fujimaki et al. (1965), Arai et al. (1967), and Fujimaki et al. (1971) have shown that it is possible to remove beany odorants and fat by enzymatic hydrolysis of soy protein concentrate. But in spite of this, protein hydrolysates sometimes have an unacceptable taste. Carr et al. (1956) and Gordon and Speck (1965) have shown that when a protein is extensively hydrolyzed with proteolytic enzymes some bitter peptides are produced. According to Petrischek et al. (1972), the bitterness of a protein hydrolysate mainly depends on the protein raw material. Fujimaki et al. (1968), Yamashita et al. (1969), and Arai et al. (1970a) have isolated a number of bitter peptides from soy hydrolysates and identified their structures. Characteristic for most of these peptides is that they have a leucine at the C-terminal and a predominantly hydrophobic composition. According to Ney (1971), a peptide is bitter when its hydrophobicity exceeds a certain level. The importance of hydrophobicity for bitter taste was also pointed out by Wieser and Belitz (1975). They found that the threshold for bitter taste decreased with increasing number of hydrophobic side chains ≥3 in the peptide. Another work of Wieser and Belitz (1976) further supported these results. Schalinatus and Behnker (1974) published a review of bitter peptides from casein and cheese. Arai et al. (1970b) treated a bitter tasting hydrolysate with carboxypeptidases and found a reduction of the bitterness. Fujimaki et al. (1970) have reported that it is possible to debitter a protein hydrolysate by using the plastein reaction and obtain a water-insoluble product with a bland odor and taste. Fukumoto and Okada (1973) reduced the bitter taste of a hydrolysate by carrying out hydrolysis in the presence of glutamic acid, aspartic acid, alanine, glycine, serine, proline, hydroxyproline, threonine, or a mixture of these. These amino acids were incorporated at the C- or N-terminals of the peptides by transamination. Other suggested methods involve extraction with cyclodextrin (TEIJN) and treatment with copolymers of styrene and divinylbenzene or acrylic ester polymers (EISAI). In this paper we describe two new methods by which we were able to debitter or reduce bitterness of enzymatic hydrolysates. The methods involve extraction with azeotropic secondary butyl alcohol (SBA) and hydrophobic interaction chromatography.

A mixture of 72.8% SBA and 27.2% water v/v is called azeotropic SBA. When more than 36% water is added to SBA a two-phase system is formed. The SBA phase is composed of 64% SBA and 36% water. The aqueous phase is composed of 80% water and 20% SBA. In our work with SBA as a fat solvent, we found that, when a bitter fish protein hydrolysate was extracted with azeotropic SBA, the hydrolysate remaining in the aqueous phase became free from bitterness. This prompted us to investigate further the general use of SBA for debittering.

Hydrophobic interaction chromatography on alkyl-substituted sepharose derivatives of water-soluble protein is used to obtain a high degree of purification of biological material (Cuatrecases and Alfinsen, 1971; Blumberg et al., 1972; Hjertên, 1973; Hofstee, 1973; Broussard et al., 1976; Wilchek and Mirom, 1976). Since data in the literature indicated that hydrophobic peptides cause the bitter taste in protein hydrolysates, we tested the efficiency of some sepharose-substituted gels for debittering bitter-tasting protein hydrolysates. The investigation of reduction of the bitterness of bitter-tasting hydrolysates by hydrophobic chromatography is not yet completed, and the results we present in this paper should be regarded as preliminary.

MATERIALS AND METHODS

Substrates. The following raw materials were used for production of hydrolysates:

Soy protein isolate (SPI), commercial name "SOY 92". Analytical data: 90.4% protein (N × 6.25), 1.0% fat, 3.6% ash, and 5.7% water.

Fish protein concentrate (FPC, Astra Nutrition, Mölndal, Sweden). Analytical data: 78.4% protein (N × 6.25), 0.2% fat, 17.7% ash, and 5.6% water.

Deboned cod filleting offal (DCO, 17.5% w/w dry substance). Analytical data of freeze-dried sample: 86.3% protein (N × 6.25), 3.6% fat, 9.0% ash, and 5.6% water.

Fresh herring (29.8% w/w dry substance). Analytical data of freeze-dried sample: 51.5% protein (N × 6.25), 36.2% fat, 9.2% ash, and 0.6% water.

Enzymes. The following enzymes were used for hydrolysis: Alcalase, a nonspecific bacterial endopeptidase from *Bacillus licheniformis*, having a specific activity of 6 Anson units/g (Novo Industri A/S, Denmark) and pancreatine having a specific activity of 6 × NF (Scientific

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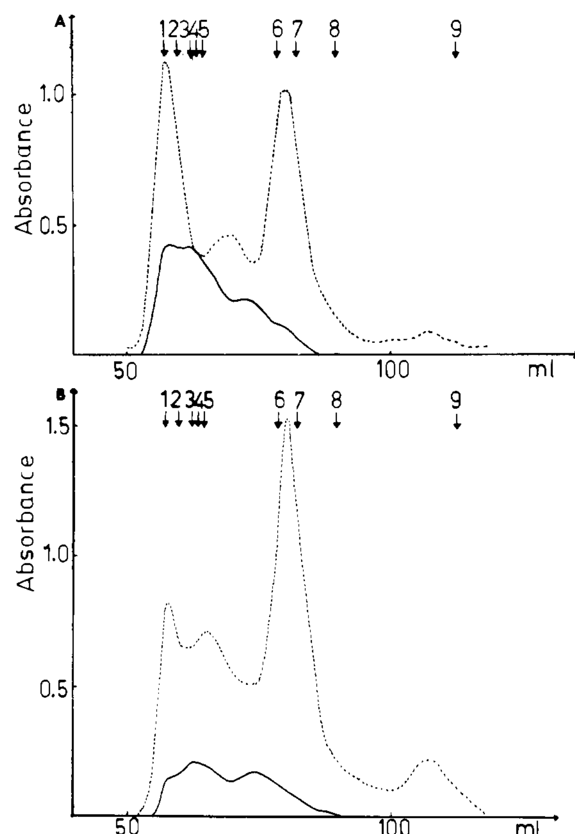

Figure 1. (A) Chromatography on Sephadex G:10 of 30 mg of FPH1 (fish protein hydrolysate). Solid lines: the material distribution in the eluate estimated by measuring the ninhydrin color at 570 nm. Dotted lines: the material distribution in the eluate estimated by measuring the absorbance at 280 nm. The numbers denote elution positions of the following compounds: (1) insulin (mol wt 5733) and β-chain of insulin (mol wt 3300), (2) H-Phe-Phe-Val-Arg-O-Isopropyl-2HCl, (3) H-Phe-Val-Arg-O-Met-2HCl, (4) H-Phe-Arg-NH_2-2HCl, (5) lysine, arginine, and histidine, (6) amino acid mixture except basic and aromatic amino acids, (7) phenylalanine, (8) tyrosine, (9) tryptophan. (B) Sephadex chromatography of 20 mg of SBA-soluble material from FPH1 (fish protein hydrolysate). Other symbols as in Figure 1A.

Protein Laboratories, USA).

General Procedure for Preparation of Protein Hydrolysates. Protein hydrolysis with Alcalase was carried out at pH 8.5 and 50 °C and with pancreatine, at pH 7.7 and 37 °C. Protein hydrolysates which were subsequently extracted with SBA, concentrated NaOH, and HCl were used to adjust the pH, while for protein hydrolysates which were used for subsequent hydrophobic chromatography $Ca(OH)_2$ and H_3PO_4 were used. The use of $Ca(OH)_2$ and H_3PO_4 gave hydrolysates with low mineral content. The pH was kept constant during the hydrolysis. After completion of hydrolysis, the solution was centrifuged and the supernatant freeze-dried.

Soy Hydrolysate. One kilogram of SPI was suspended in 9 L of water and 5 g of Alcalase was added. The hydrolysis continued for 4 h (SPH1). Its amino acid composition is shown in Table IA. At 5% w/w the hydrolysate has a bitter taste (3 on the five-point scale).

FPC Hydrolysate. One kilogram of FPC was suspended in 19 L of water and 5 g of Alcalase was added. The hydrolysis continued for 3 h (FPH1). The amino acid composition and molecular weight distribution of FPH1 are shown in Table IB and Figure 1A. At 5% w/w FPH1 has a weak bitter taste (2 on the five-point scale).

DCO Hydrolysate. Ten kilograms of frozen DCO was thawed and suspended in 10 L of water. The protein was

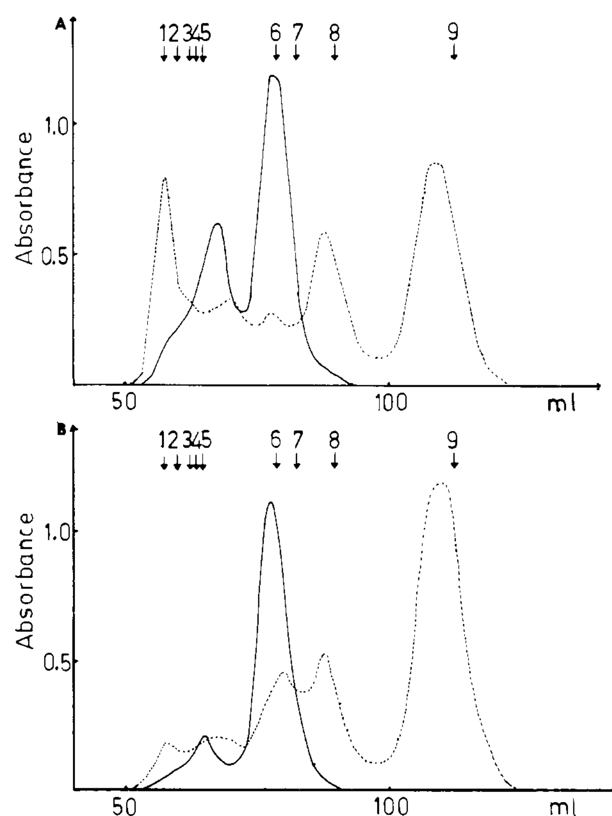

Figure 2. (A) Sephadex chromatography of 30 mg of FPH2 (fish protein hydrolysate). Other symbols as in Figure 1A. (B) Sephadex chromatography of 20 mg of SBA-soluble material from FPH2 (fish protein hydrolysate). Other symbols as in Figure 1A.

hydrolyzed first with 10 g of Alcalase for 3 h, followed by 4 h of hydrolysis with 15 g of pancreatine (FPH2). The amino acid composition and molecular weight distribution of FPH2 are shown in Table IC and Figure 2A. Total free amino acids accounted for about 39% of the total nitrogen, and the composition of the free amino acids is shown in Table ID. At 5% w/w the FPH2 was free from bitterness (0 on the five-point scale).

Herring Hydrolysate. Five kilograms of frozen herring was thawed, ground, and suspended in 5 L of water, whereafter 5 g of Alcalase was added. The hydrolysis continued for 2 h (FPH3). The amino acid composition of FPH3 is shown in Table IE. Total free amino acids accounted for about 14% of the total nitrogen, and the composition of free amino acids is shown in Table IF. At 5% w/w the hydrolysate was free from bitterness (0 on the five-point scale).

SBA Extraction. *General Description for Extraction with SBA.* To a fixed amount of freeze-dried water-soluble protein hydrolysate (usually 10 g) water was added. Then the pH of the solution was adjusted with concentrated NaOH or HCl to the desired value, and SBA was added in sufficient amount to give a two-phase system. The mixture was shaken for about 2 min. Experiments showed the equilibrium between peptides and amino acids in SBA and the aqueous phase to be reached in this time. All extractions were carried out at room temperature. The mixture was subsequently centrifuged to remove precipitated material and the two phases were separated, concentrated, and freeze-dried. Recovered precipitate was added to material recovered from the aqueous phase.

SBA Extraction for Removal of Bitter Compounds. Extractions were carried out with different concentrations of protein hydrolysates in water.

Example 1: Ten-gram samples of SPH1 and FPH1 were

Table I. Amino Acid (AA) Composition and Free Amino Acids (FAA) of Protein Hydrolysates from Soy (A), FPC (B), DCO (C, D), and Herring (E, F)[a] (g/16 g of N)

Amino acid	A	B	C	D	E	F
Asp	12.57	9.86	9.74	1.60	9.18	0.29
Thr	3.69	4.70	4.44	1.58	4.45	1.38
Ser	5.43	4.98	5.16	2.22	4.17	0.50
Glu	21.17	14.48	14.53	1.85	14.11	0.81
Pro	5.22	4.40	4.92	0.12	4.01	0.24
Gly	3.89	6.30	7.23	0.62	5.78	0.60
Ala	3.86	6.63	6.60	2.31	6.31	1.94
Val	4.82	4.66	5.23	2.16	4.92	1.01
Met	1.09	2.96	3.49	2.22	2.91	0.92
Ile	4.75	4.16	4.67	2.10	4.00	0.64
Leu	8.29	7.57	7.47	4.94	7.49	2.46
Tyr	4.00	3.06	3.60	2.45	2.80	0.70
Phe	5.30	3.56	3.99	2.56	3.62	1.18
Lys	6.45	9.09	8.91	4.56	9.15	1.60
His	2.20	2.60	3.27	2.05	1.72	0.55
Arg	7.74	5.67	6.19	4.36	4.84	1.01
Cys	1.40	0.87	1.12		1.18	
Trp	1.08	1.19	0.95	0.87	1.14	0.14
Total FAA				38.57		14.56
$\frac{\text{Ess. AA}}{\text{Total AA}} \times 100$	34.4	39.2	38.6	54.4	41.0	64.0

[a] A, Alcalase hydrolysate of soya; B, Alcalase hydrolysate of FPC (fish protein concentrate); C, Alcalase hydrolysate of DCO (deboned cod offal); D, free amino acids in hydrolysate C treated with Pancreatine; E, Alcalase hydrolysate of whole fresh herring; F, free amino acids in hydrolysate E.

dissolved in 190, 90, 40, and 23 mL of water to yield solutions of about 5, 10, 20, and 30% w/w. Two hundred milliliters of azeotropic SBA was added, and the solutions were extracted for 2 min at pH 8.

Extractions with varying amounts of SBA.

Example 2: To solutions of 10 g of SPH1 in 23 mL of water 50, 200, and 400 mL of azeotropic SBA was added. The mixture was shaken for 2 min at pH 8.

Extraction with SBA for Concentration of Essential Amino Acids. The efficiency of SBA for concentration of essential amino acids was tested by extraction of both dry powders and aqueous solutions of protein hydrolysates. Also, the effect of pH, concentration of the protein hydrolysate and amount of SBA was studied.

Effect of pH: Ten-gram samples of FPH1 were dissolved in 40 mL of water and 50 mL of azeotropic SBA was added. The pH of the solutions was adjusted to 6, 7, 8, or 9 and the solutions were extracted for 2 min. The SBA phase was separated and freeze-dried.

Effect of concentration of the protein hydrolysate: 10-g samples of FPH1 and FPH2 were extracted with SBA in the same manner as described in example 1. However, this time the SBA phase was recovered and freeze-dried.

Effect of Amount of SBA: Ten-gram samples of SPH1 and FPH3 were extracted with SBA in the same manner as described in example 2. However, this time the SBA phase was recovered and freeze-dried.

Extraction of Dry Powder: Ten-gram samples of FPH2 were extracted in the dry state with 50, 100, 200, 400, 600, 800, 1000, or 2000 mL of azeotropic SBA for 30 min at pH 8. The SBA phase was separated and freeze-dried.

Plastein Reaction. Fifty-gram samples of the freeze-dried SBA soluble fraction from FPH1 and FPH3 (representing 14.2 and 20.9%, respectively, of the total nitrogen in the hydrolysates) were dissolved in 92.8 mL of water (35% w/w). The pH was adjusted to 7 with HCl, 0.5 g of Alcalase was added, and the samples were incubated at 50 °C for 4 h. The treatment resulted in formation of plastein gels and the enzyme was inactivated by heating at 80 °C, whereafter the samples were freeze-dried. To a 10-g sample of freeze-dried plastein product, made from SBA-soluble material from FPH3, 50 mL of 95% ethanol was added. The material was extracted for 10 min at room temperatures, followed by centrifugation. The ethanol-soluble and -insoluble fractions were separated and freeze-dried.

Removal of Bitter Compounds by Hydrophobic Interaction Chromatography. Thirty-six grams of freeze-dried SPH1 was dissolved in water to obtain a 5% w/w solution and the pH adjusted to 7. A column (bed dimensions 19 × 0.9 cm) was packed with hexylsepharose 6B (gift from the Institute of Biochemistry, Uppsala, Sweden) and equilibrated with distilled water. The 5% w/w solution was applied to the column and passed through at a flow rate of 200 mL/h at room temperature. The eluate was collected as one fraction, fraction I. After completion of elution 200 mL of water was passed through the column in order to remove material which was not strongly bound to the gel. This eluate was combined with fraction I, and the whole volume was freeze-dried.

Recovery of Material Adsorbed on the Gel. Two hundred milliliters of 0.01 M NaOH was passed through the column. A strongly colored zone developed. When the colored zone was in the middle of the column a turbid solution started to elute from the column, fraction II (this occurred only with SPH1), and turbid material continued to flow until the colored zone of material reached the bottom of the column. Collection of fraction III was then started. After the elution with NaOH was completed, 100 mL of 50% v/v ethanol was applied to the column and the corresponding eluate collected as fraction IV. The column was then equilibrated with distilled water and was ready to be used again.

The same experiments as described above were repeated with materials adsorbed when 12, 24, and 36 g of SPH1 was applied to the column. In these experiments eluates with the adsorbed material were collected in only one fraction, fraction V, VI, and VII, respectively. The same experiment was also repeated with 36 g of FPH1.

Nitrogen content was determined by the Kjeldahl method using the Kjell Foss automatic apparatus (A/S N. Foss Electric, Denmark).

Lipid content was determined by extraction with chloroform–methanol 2:1 v/v.

Ash content was determined according to the AOAC method (AOAC, 1970).

Table II. Yield (Nitrogen × 6.25), Taste, and Amino Acid (AA) Composition of Water-Soluble Fraction (WSF) after SBA-Extraction of SPH1 and FPH1. Effect of Concentration of Water Solutions of Protein Hydrolysates

	WSF from SPH1				WSF from FPH1			
w/w concn	5%	10%	20%	30%	5%	10%	20%	30%
Yield, %	93.4	86.1	82.5	82.1	94.6	90.3	86.2	85.8
Taste at w/w concn 5%	1	0	0	0	0	0	0	0
10%	2	1	0	0	1	0	0	0
Amino acid	g/16 g of N				g/16 g of N			
Asp	11.56	11.87	11.62	12.68	10.92	10.82	10.84	11.15
Thr	3.52	3.41	3.20	3.34	4.89	4.84	4.60	4.66
Ser	5.53	5.06	4.66	4.97	4.42	4.53	4.21	4.44
Glu	20.90	21.92	21.31	23.68	17.97	17.86	18.20	18.32
Pro	4.92	5.04	4.66	4.79	4.90	4.91	4.80	4.73
Gly	4.04	3.88	3.65	3.79	7.05	7.03	6.79	6.91
Ala	3.78	3.69	3.85	3.50	7.14	7.10	6.73	6.94
Val	4.28	3.97	3.66	3.77	4.73	4.37	4.44	4.50
Met	1.02	1.01	0.96	1.04	3.17	2.91	2.85	2.76
Ile	4.29	3.98	3.64	5.46	4.81	4.39	3.60	3.58
Leu	7.07	6.25	5.45	5.83	7.44	6.89	6.63	6.52
Tyr	3.58	3.23	2.87	2.86	3.69	3.02	2.81	3.11
Phe	4.60	4.07	3.69	3.74	3.52	3.19	3.15	2.75
Lys	4.66	4.67	6.12	6.64	8.68	9.03	8.72	8.90
His	1.84	1.82	2.32	2.09	2.39	2.48	2.63	2.52
Arg	6.12	6.39	6.67	8.47	6.49	6.72	6.37	6.42
Cys	1.08	1.48	1.60	1.62	0.92	0.99	1.00	1.02
Trp	0.93	0.80	0.72	0.58	0.92	0.88	0.64	0.67
$\frac{\text{Ess. AA}}{\text{Tot. AA}} \times 100$	32.4	30.4	30.3	30.8	36.7	35.8	35.0	34.4

Amino acid composition was determined by hydrolysis in 6 M HCl under vacuum at 110 °C for 22 h, followed by ion-exchange chromatography essentially according to the method of Stein and Moore (1954). The free amino acid composition was determined in the same manner but without HCl treatment.

Cysteine–cystine was determined as cysteic acid and "total methionine" as methionine sulfone after oxidation with performic acid according to Moore (1963).

Tryptophan was determined according to Spies and Chambers (1948, 1949).

Sephadex Chromatography. The molecular weight distribution of protein hydrolysates and the SBA-soluble materials was investigated by means of Sephadex chromatography. A glass column (bed dimensions 57 × 0.87 cm) was packed with Sephadex G:10 (Pharmacia Fine Chemicals, Sweden), equilibrated with 50% v/v analytical grade acetic acid in water. Thirty-milligram samples of hydrolysates or 20 mg of SBA-soluble material were dissolved in 0.5 mL of the elution medium (50% acetic acid). The elution was carried out at room temperature and the eluent was collected in 2.5-mL fractions. The flow rate was 5 mL/h. The amino acid and peptide distribution in the eluates was determined by measuring the absorbance at 280 nm and by carrying out the ninhydrin reaction (Yemn and Cocking, 1955). The column was calibrated with a number of reference substances of known molecular weight dissolved in 50% acetic acid.

Taste Evaluation. All the taste evaluations were carried out by a panel of 12 persons. The panel was asked to grade the bitterness of the samples on a five-point scale (0, no bitterness; 1, weak bitter aftertaste, 2, weakly bitter; 3, bitter; 4, strongly bitter; 5, extremely bitter).

RESULTS AND DISCUSSION

Extraction of Protein Hydrolysates with SBA. In this laboratory SBA has been successfully used for defatting of fish protein products. On extraction of a water-soluble protein hydrolysate with SBA, we observed that not only fat but also nitrogenous compounds were transferred to the SBA phase and there was a simultaneous decrease in bitterness. We investigated the SBA-soluble material by Sephadex chromatography and found that it mainly consisted of free amino acids and low molecular weight peptides. Amino acid analysis showed a small relative decrease of the essential amino acid content in the aqueous phase and a strong concentration of essential amino acids in the SBA-soluble fraction.

Table III. Yield (Nitrogen × 6.25), Taste, and Amino Acid (AA) Composition of Water-Soluble Fractions (WSF) after SBA-Extraction of SPH1. Effect on Different Amounts of SBA

	WSF from SPH1		
mL of SBA	50	200	400
Yield, %	92.7	82.1	74.2
Taste at w/w concn 5%	1	0	0
10%	2	0	0
Amino acid	g/16 g of N		
Asp	13.11	12.68	12.24
Thr	3.54	3.34	3.06
Ser	5.41	4.97	4.77
Glu	21.39	23.68	23.60
Pro	4.58	4.79	4.65
Gly	4.32	3.79	3.53
Ala	3.75	3.50	3.20
Val	4.17	3.77	3.29
Met	0.98	1.04	1.03
Ile	4.22	5.46	3.38
Leu	6.95	5.83	4.80
Tyr	3.67	2.86	2.43
Rhe	4.71	3.74	2.96
Lys	6.35	6.64	6.41
His	2.16	2.09	2.03
Arg	7.71	8.47	8.44
Cys	1.42	1.62	1.51
Trp	0.79	0.58	0.47
$\frac{\text{Ess. AA}}{\text{Tot. AA}} \times 100$	32.0	30.8	27.7

These interesting results were further investigated in experiments where the effect of SBA on removal of bitter compounds and on concentration of essential amino acids were studied separately.

Extraction with SBA for Removal of Bitter Compounds. Tables II and III show the taste, yield, and amino acid composition of protein hydrolysates after extraction

Table IV. Amino Acid (AA) Composition of SBA-Soluble Fractions (SBASF) from FPH1. Effect of pH

Amino acid	SBASF from FPH1 pH 6	SBASF from FPH1 pH 9
	g/16 g N	
Asp	5.76	5.70
Thr	4.36	4.73
Ser	3.37	3.67
Glu	6.87	7.14
Pro	4.22	4.44
Gly	5.02	5.80
Ala	5.69	6.23
Val	6.96	8.15
Met	5.60	5.99
Ile	8.52	10.58
Leu	16.88	17.64
Tyr	6.21	6.30
Phe	10.76	10.92
Lys	4.43	5.86
His	2.40	3.10
Arg	4.53	6.07
Cys	0.87	0.57
Trp	3.45	3.43
$\frac{\text{Ess. AA}}{\text{Tot. AA}} \times 100$	57.6	57.8

with SBA. The results show that SBA efficiently removes bitter compounds from both SPH1 (a soya hydrolysate) and FPH1 (a fish hydrolysate). Higher concentrations of protein hydrolysates resulted in a greater loss of material (Table II). There was no difference between 20 and 30% w/w concentrations. However, with increased transfer of material to the SBA phase there was a gradual reduction of the content of essential amino acids, e.g., for SPH1 from originally 34.4 to 30.8% and for FPH1 from originally 39.2 to 34.4%.

Table III shows that when increasing amounts of SBA are used an increasing amount of peptides is extracted from the hydrolysates. Both Tables II and III illustrate a decreased content of essential amino acids after extraction with SBA. The greatest drops were observed for tryptophan, phenylalanine, leucine, tyrosine, valine, and isoleucine. All these amino acids, except for tyrosine, are hydrophobic. The results were similar for SPH1 and FPH1. A possible explanation for these results is the difference in polarity between the two phases. The SBA phase is less polar than the aqueous phase but sufficiently polar to attract some peptides and amino acids. When a protein hydrolysate is extracted with SBA the result is a selective transfer of hydrophobic peptides and amino acids to the SBA phase, while more hydrophylic peptides and amino acids remain in the aqueous phase. Since peptides containing these hydrophobic amino acids have a bitter taste, it is reasonable to assume that the reduction of bitterness is mainly or wholly due to removal of these compounds. The material recovered from the SBA phase has an exceptionally strong bitter taste.

To test the general validity of SBA extraction as a debittering method, we extracted bitter tasting hydrolysates of casein and whey (produced by hydrolysis with Alcalase) with azeotropic SBA. The bitter taste of casein and whey hydrolysates was reduced or abolished by extraction with SBA. We therefore conclude that SBA seems to be a generally applicable solvent for removal of bitterness from protein hydrolysates. Our experience is that, when 5–10% of the total nitrogen is removed, the hydrolysate is free from bitterness at a concentration of 5% w/w.

Extraction with SBA for Concentration of Essential Amino Acids. The observed relative increase in the concentration of essential amino acids in the material recovered in the SBA phase prompted us to investigate whether this interesting effect could be further accentuated. As the first parameter, the effect of pH was studied.

The effect of pH on the transfer of peptides from an aqueous phase to a SBA phase is shown in Table IV. Hydrophobic amino acids as leucine, isoleucine, phenylalanine, and tryptophan were strongly increased. The overall effect was an increase of essential amino acids from 39 to about 58%. However, the effect of pH in the range of 6 to 9 on the transfer of peptides was small. Therefore, we arbitrarily chose pH 8 for the continued work.

Effect of Concentration of Protein Hydrolysates and the Amount of SBA: Table V shows the effect of concentration of protein hydrolysates on the transfer of peptides and amino acids to the SBA phase. Higher concentration of protein hydrolysates resulted in greater transfer of material to the SBA phase and a trend toward a higher content of

Table V. Yield (Nitrogen × 6.25) and Amino Acid (AA) Composition of SBA-Soluble Fractions (SBASF) from FPH1 and FPH2. Effect of Concentration of Water Solutions of Protein Hydrolysates

	SBASF from FPH1			SBASF from FPH2		
w/w protein concn	5%	20%	50%	5%	20%	50%
Yield, %	5.4	13.9	14.3	4.1	11.1	10.8
Amino acid	g/16 g N			g/16 g N		
Asp	6.24	6.81	6.45	3.81	3.40	3.00
Thr	5.11	5.44	5.77	2.54	3.95	4.01
Ser	4.78	5.39	5.36	4.23	3.98	3.65
Glu	7.45	8.90	7.79	5.81	5.07	4.55
Pro	4.34	5.27	4.73	4.30	4.63	4.87
Gly	5.44	5.65	5.61	6.90	6.86	6.80
Ala	6.04	7.28	7.04	5.42	5.58	5.56
Val	7.68	8.45	8.75	6.82	7.74	8.39
Met	3.32	6.29	6.24	5.76	6.60	7.34
Ile	8.31	8.85	9.06	7.70	9.03	10.22
Leu	15.73	16.52	17.59	17.39	20.70	24.33
Tyr	6.31	6.54	6.52	6.82	4.34	3.94
Phe	9.51	9.83	9.61	11.46	12.09	13.85
Lys	3.15	5.66	5.85	4.04	3.21	2.68
His	3.32	3.59	3.60	2.23	1.79	1.81
Arg	5.10	4.99	5.09	4.05	3.77	3.19
Cys	0.70	0.58	0.68	1.22	1.00	1.18
Trp	3.12	2.56	2.48	2.52	2.70	2.65
$\frac{\text{Ess. AA}}{\text{Tot. AA}} \times 100$	52.8	53.6	55.3	56.5	62.0	65.6

Table VI. Yield (Nitrogen × 6.25) and Amino Acid (AA) Composition of SBA-Soluble Fractions (SBASF) from SPH1 and FPH3. Effect of Different Amounts of SBA

	SBASF from SPH1			SBASF from FPH3		
mL of SBA	50	200	400	50	200	400
Yield, %	7.3	17.9	25.8	4.1	12.6	20.9
Amino acid	g/16 g N			g/16 g N		
Asp	7.79	8.72	9.28	2.24	2.63	3.16
Thr	5.04	4.69	4.68	2.65	3.26	3.62
Ser	7.34	6.14	6.26	1.46	1.55	1.61
Glu	6.89	8.47	9.59	2.78	3.82	4.77
Pro	5.59	4.98	4.95	4.05	3.72	3.48
Gly	4.81	4.33	4.18	4.67	4.59	4.58
Ala	5.22	4.98	4.92	3.98	4.97	5.78
Val	8.10	7.30	6.90	6.23	6.76	6.99
Met	1.26	1.01	1.02	5.24	5.61	5.45
Ile	8.69	7.68	7.05	9.79	8.82	8.01
Leu	20.85	17.85	15.73	23.68	20.96	18.43
Tyr	7.57	7.48	6.85	2.25	2.29	2.96
Phe	13.28	12.24	10.88	12.36	10.81	9.16
Lys	4.11	5.84	4.72	2.09	2.62	3.36
His	2.96	2.47	2.42	0.91	1.03	1.09
Arg	5.62	5.39	5.45	1.03	1.48	1.94
Cys	0.54	0.58	0.61	0.38	0.36	0.45
Trp	2.45	2.18	1.71	2.93	2.80	2.28
$\frac{\text{Ess. AA}}{\text{Tot. AA}} \times 100$	54.0	52.3	49.2	73.2	70.0	65.8

Table VII. Yield (Nitrogen × 6.25) and Amino Acid (AA) Composition of SBA-Soluble Fractions from FPH2. Extractions Were Carried Out on Dry Powder Hydrolysates

mL of SBA	50	100	200	400	600	800	1000	2000
Yield, %	0.8	5.3	11.6	18.0	23.2	27.1	29.0	49.0
Amino acid				g/16 g N				
Asp	ND[a]	2.30	2.92	3.60	4.95	ND		
Thr		3.81	4.14	4.41	5.35			
Ser		3.27	3.85	4.26	4.92			
Glu		3.04	3.96	5.62	7.74			
Pro		5.43	5.55	4.92	4.64			
Gly		6.56	6.89	7.10	7.49			
Ala		4.75	5.48	6.27	7.01			
Val		7.61	9.05	8.44	7.43			
Met		7.49	7.59	7.21	5.86			
Ile		13.09	11.52	9.88	7.29			
Leu		29.50	26.19	21.05	14.70			
Tyr		3.09	3.72	4.46	4.92			
Phe		15.19	14.45	11.56	8.17			
Lys		2.21	2.71	3.15	4.01			
His		1.78	1.94	2.20	2.14			
Arg		2.81	2.74	3.64	4.58			
Cys		0.88	1.01	1.07	0.96			
Trp		3.06	2.85	2.32	1.45			
$\frac{\text{Ess. AA}}{\text{Tot. AA}} \times 100$		70.7	67.3	61.2	52.4			

[a] ND, not determined.

essential amino acids in the SBA phase. Despite an almost identical amino acid composition in FPH1 and FPH2, SBA extraction of FPH2, in comparison with extraction of FPH1, always resulted in transfer of material (calculated as nitrogen × 6.25) with a higher content of essential amino acids, but a lower total yield. We believe this is due to the reduced hydrophobicity caused by the exopeptidases, which give low molecular weight peptides of less hydrophobic composition and high amounts of free hydrophobic amino acids. Judged from results from Sephadex chromatography, FPH2 probably mainly contains di- and tripeptides in addition to a large amount of free amino acids, of which more than 50% are essential amino acids. FPH1, on the other hand, has an average molecular weight about three times that of FPH2 and contains almost no free amino acids.

As shown in Table VI, extraction with increasing amounts of SBA results in a higher yield of SBA-soluble material but the level of essential amino acids in the SBA phase decreases simultaneously.

Extraction of Dry-Powder Hydrolysates with SBA: For extraction of dry-powder hydrolysate it is necessary to use a ratio of azeotropic SBA to dry-powder hydrolysate of at least 10:1 v/w to get a two-phase system. In this case hydrophilic compounds evidently attract water from the azeotropic SBA (contains 27.2% v/v water) to form a two-phase system.

The results are presented in Table VII. The percentage of the material (calculated as nitrogen × 6.25) which was transferred to the SBA phase increased with increasing amounts of SBA. The amino acid composition (determined after acid hydrolysis) showed levels of essential amino acids around 67–70%, when low amounts of material were transferred to the SBA phase. These figures are somewhat higher than when aqueous solutions of protein hydrolysates are extracted. The high content of

Table VIII. Amino Acid (AA) Composition of Ethanol-Insoluble Nitrogen and Ethanol-Soluble Nitrogen after Plastein Reaction on SBA-Soluble Material, and Amino Acid Composition of Material from SPH1 Bound to Hexylsepharose Gel (C)

Amino acid	A g/16 g og N	B g/16 g of N	C g/100 g
Asp	3.38	3.04	3.57
Thr	3.19	3.50	1.62
Ser	2.83	2.88	2.63
Glu	3.64	3.75	4.23
Pro	2.28	4.47	2.79
Gly	3.73	5.49	1.87
Ala	5.09	3.17	1.89
Val	9.74	5.23	2.25
Met	9.13	2.29	0.61
Ile	13.00	4.92	3.05
Leu	30.03	8.66	4.66
Tyr	4.02	2.41	2.17
Phe	14.83	7.53	3.66
Lys	2.53	3.08	2.67
His	1.15	1.36	0.93
Arg	2.04	2.45	0.93
Cys	0.54	0.34	0.89
Trp	2.50	2.17	1.55
$\frac{\text{Ess. AA}}{\text{Tot. AA}} \times 100$	74.7	56.0	44.9

essential amino acids was partly due to a very high level of leucine.

Experiments showed that on extraction of dry-powder hydrolysates with 100% SBA almost no nitrogenous compounds in the protein hydrolysates were soluble.

Bitterness of SBA-Soluble Fractions and Efforts to Reduce It. As mentioned earlier, the SBA-soluble materials recovered from extraction of bitter hydrolysates (e.g., SPH1 and FPH1) are extremely bitter. At concentration as low as 0.5% w/w they have a bitter taste. Corresponding SBA-soluble material from nonbitter hydrolysates (e.g., FPH2 and FPH3) are less bitter. At 3.0% w/w they have a bitter taste. A greater transfer of material to the SBA-phase usually results in a slight decrease in bitterness of the SBA-soluble fraction. We believe that the main reason for the bitterness of the enzymatic protein hydrolysates is peptides which have more than three amino acids.

Chromatography on Sephadex G:10 of SBA-soluble material from SPH1 and FPH1 showed almost the same molecular weight distribution as the original hydrolysates (Figure 1B). It is interesting to note that even the fraction with the highest molecular weight was partly transferred to the SBA phase. This is in contrast to SBA extraction of a hydrolysate treated with exopeptidases, e.g., FPH2 or FPH3 (Figure 2B). Only small amounts of fractions with the highest molecular weight were transferred to the SBA phase and the same was true for di- and tripeptides. High amounts of free amino acids were found in the SBA phase.

Since bitterness due to peptides has been shown to be reduced by applying the plastein reaction (Fujimaki et al., 1970), this method was tried. As described under the Material and Methods section, the plastein reaction was applied to SBA-soluble material from FPH1 and FPH3. The plastein reaction considerably reduced the bitterness and at 5% w/w both products had a weakly bitter taste. When 10 g of the plastein produced from SBA-soluble material from FPH3 was extracted with 50 mL of ethanol, the ethanol-insoluble material (6.9 g) was completely free from bitterness at 5% w/w concentration. As shown in Table VIII (I, II), the ethanol-insoluble fraction had a particularly high content of essential amino acids (75%). A preliminary experiment indicated that treatment with exopeptidases (Pancreatine) also reduced the bitterness of SBA-soluble material from SPH1.

Removal of Bitter Compounds by Hydrophobic Interaction Chromatography. From the results achieved with SBA extraction and knowledge of the hydrophobicity of bitter peptides, we thought it might be of interest to test hydrophobic interaction chromatography as a tool for debittering of protein hydrolysates. Since the ionic strength influences the hydrophobic interaction chromatography (Hjertên, 1973), protein hydrolysates with low ash content were selected for this study.

As described under the Material and Methods section, chromatography on hexylsepharose was tried with solutions of SPH1 and FPH1. When a solution of 36 g of SPH1 was passed through a column 19 × 0.9 cm, a reduction in bitterness occurred (bitter aftertaste at 5% w/w) and the characteristic odor and taste of soy was strongly reduced. When less material (24 and 12 g) was passed through the column, an even more pronounced reduction in bitterness occurred, indicating that the gel had a limited capacity to bind the bitter compounds. This was supported by the finding that about the same amount of material had been trapped in the gel when 12, 24, and 36 g of SPH1 was passed through the column (1.23, 1.26, and 1.22 g, respectively). (These figures are based on recovery after freeze-drying and not on nitrogen analysis). The amino acid composition of material recovered from the column in the experiment with 36 g of SPH1 is shown in Table VIII (III). The content of essential amino acids in the adsorbed material was 10% higher than in the original hydrolysate. Material adsorbed from 12 and 24 g of SPH1 also had similar amino acid compositions. Fractions II (turbid), III (alkali), and IV (ethanol) weighed 0.21, 0.72, and 0.33 g, respectively. Sephadex chromatography of these fractions showed that the average molecular weight was almost the same for all fractions and similar to that for SPH1. In a similar experiment with 36 g of FPH1, 0.61 g of material was adsorbed to the gel and bitterness was reduced (bitter aftertaste at 5% w/w). These results show that hydrophobic chromatography on hexylsepharose can be used for debittering of protein hydrolysates, supporting the hypothesis that hydrophobic compounds are mainly responsible for the bitter taste of protein hydrolysates. Experiments carried out under identical conditions with gels of butyl, phenyl, and octyl sepharose gave no debittering.

Technical Aspects. SBA is not a commonly used solvent in food processing. Chronic studies in rats and multigeneration studies in rats, performed in the USA, have not shown SBA to have any toxic effects (unpublished results). Since experiments have also shown that the residual SBA can easily be reduced to very low levels in a hydrolysate, we believe that SBA extraction of protein hydrolysates is a realistic approach. Also, hydrophobic interaction chromatography should be technically applicable. A high flow rate was used in this study and also a batch procedure not involving column chromatography should be possible. However, we believe that other types of hydrophobic gels can be developed with improved capacity and better properties.

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Facial Tissue Paper as a Feedstuff for Lambs

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A ton of a pelleted ration was prepared containing 25% by weight facial tissue paper mixed with corn, soybean and alfalfa meals, molasses, minerals, and vitamins. The facial tissue paper consisted entirely of virgin cellulose fibers from which almost all lignin had been chemically removed during manufacture. The ration was fed to four 3-month-old wethers for 125 days. A similar pelleted ration containing proportionately more alfalfa meal to replace the facial tissue was fed to wethers as a control diet. The in vivo dry matter ration digestibilities averaged 74 and 71% for the replicated lambs fed the control and facial tissue paper-containing diets, respectively. No significant differences ($p > 0.05$) were observed in the average daily feed consumed or average daily weight gains among the two treatment groups.

The incorporation of waste paper as a substitute form of cellulose in farm animal rations has been studied by several investigators. Sherrod and Hansen (1973), Dinius and Oltjen (1971, 1972), Mertens et al. (1971), Daniels et al. (1970), and Kesler et al. (1967) investigated newspaper and magazines as feedstuffs. Nishimuta et al. (1969) studied office bond paper and Becker et al. (1975) and Coombe and Briggs (1974) investigated a variety of others.

The digestibility of various types of paper by ruminants depends upon their lignin content since lignin is not digestible. Newspaper typically contains largely mechanical fibers but also some fibers chemically treated to remove lignin. Magazine paper contains about one-third mechanical fibers (no lignin removed), one-third "chemical fibers" (lignin removed), and one-third fillers such as starch, clays, and alumina which are added to impart opacity to the paper. Magazine, office bond, and computer printout paper, all of which are partially delignified during manufacture, are therefore more digestible by ruminants than newspaper. Other delignified wood products which have been studied as additives to farm animal rations have included various wood pulp fines and residues (Dinius and Bond, 1975; Millett et al., 1973) and wood cellulose (Riquelme et al., 1975).

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Table I. Composition of the Complete Pelleted Animal Rations

Constituent	Percent dry weight in	
	Facial tissue paper ration	Control ration
Facial tissue paper	25	
Corn meal	34	40
Soy bean meal	25	12
Alfalfa meal	11	43
Molasses	4.7	4.7
Minerals	0.25	0.25
Vitamins (A, D, and E)	0.05	0.05

Table II. Content of Ash, Fat, Protein, Fiber, and Energy in the Pelleted Rations

Constituent	Percent (dry weight) in	
	Facial tissue paper ration	Control ration
Ash	4.9	6.3
Fat	1.9	3.8
Protein	16.3	19.0
Crude fiber	22.0	9.4
Energy	4309[a]	4453[a]

[a] Calories per gram (dry weight).

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