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Isolation of low-molecular-mass hydrophobic bitter peptides in soybean protein hydrolysates by reversed-phase high-performance liquid chromatography☆

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Abstract

The molecular mass distribution of peptides in isoelectric soluble soybean protein hydrolysates and their hydrophobic peptide fractions was determined by gel permeation HPLC on a Zorbax Bio Series GF-250 column. The hydrophobic bitter peptide fraction of isoelectric soluble soybean protein hydrolysate with degree of hydrolysis 15% was fractionated on a Sephadex G-25 Fine column. The most bitter low-molecular-mass fraction of the peptides was separated by reversed-phase HPLC on a Spherisorb ODS-2 column. Fourteen low-molecular-mass hydrophobic bitter peptides containing three to six amino acid residues were isolated. They are predominantly composed of hydrophobic amino acids and have leucine, valine or tyrosine at their C-terminal position

1. Introduction

It has been known for a long time that enzymatic hydrolysis of proteins frequently leads to the production of a bitter taste, which is due to the presence of strongly hydrophobic bitter peptides arising as natural degradation products of the proteolytic reaction [1]. Numerous bitter peptides have been isolated from enzymatic hydrolysates of casein [2,3], soybean protein [4,5], cheese [6,7] and some other foods, and their structures determined. The bitterness of peptides can be predicted by calculation of the Q value of a peptide, which depends on the amino acid composition. The Q value represents the average hydrophobicity of a peptide and is calculated by summing the Δf values of the amino acid residues of a peptide and dividing by the number of amino acid residues as described by Ney [8]. When the Q value is greater than 1.4 kcal mol^{-1}, bitterness is likely to appear [9]. To avoid bitterness in this case, the relative molecular mass of a peptide must be as high as possible and more than 6000 [10]. The results of enzymatic hydrolysis of casein show that for larger peptides, neither hydrophobicity nor size alone is responsible for bitter potency, but that conformational parameters must be of greater im-

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portance. Further, it was concluded that only a part of the structure is responsible for contact with the taste receptor [2,11].

Different types of chromatographic methods have been developed for the separation of proteins and their hydrolysates. Among the various HPLC systems, including gel permeation [12,13], reversed-phase (RP-HPLC) [14–16] and ion exchange [17–19] modes, RP-HPLC has been most extensively used. There have been many studies of the separation of milk proteins, especially casein digest [20,21], but there are no published studies documenting the RP-HPLC separation of hydrophobic bitter peptides in soybean protein hydrolysates.

The aim of this work was to isolate small hydrophobic bitter peptides and to determine the molecular mass distribution of peptides in soybean protein hydrolysates using gel permeation HPLC, gel chromatography and RP-HPLC.

2. Experimental

2.1. Materials

Soybean protein isolate, Purina 500 E, was purchased from Protein Technologies International (Ypres, Belgium). Alcalase 2.4 l (a commercial preparation of subtilisin Carlsberg from *Bacillus licheniformis*) was obtained from Novo Nordisk (Bagsvaerd, Denmark). The protein molecular mass calibration standards used were carbonic anhydrase, cytochrome *c*, angiotensin II, angiotensinogen fragment 11–14 (Sigma, St. Louis, MO, USA) and the B-chain and A-chain of insulin (Serva, Heidelberg, Germany). Ammonium acetate and sodium dodecyl sulphate (SDS) were also obtained from Sigma, acetonitrile from Rathburn Chemicals (Walkerburn, UK) and trifluoroacetic acid (TFA) from Applied Biosystems (Warrington, UK). All other chemicals were of analytical-reagent grade.

2.2. Methods

Preparation of isoelectric soluble soybean protein hydrolysates (ISSPHs)

Five isoelectric soluble soybean protein hydrolysates were produced in the laboratory according to the standard procedure [22]. Hydrolysis was carried out under the following standard conditions: substrate concentration $S = 8\%$ protein (calculated from Kjeldahl N × Kjeldahl conversion factor for soy protein isolate, f_N, 6.25); enzyme/substrate concentration ratio $E/S = 12$ AU kg^{-1} (Anson units per kg substrate), pH = 8.0, temperature 50°C. One Anson unit (AU) is the amount of enzyme which under the given reaction conditions digests haemoglobin at an initial rate such that there is liberated per 10 min an amount of trichloroacetic acid-soluble product which gives the same colour with phenol reagent as 1 μmol of tyrosine [23]. The course of the reaction was followed by the pH-stat technique, and when the desired degree of hydrolysis (DH value) had been reached, the enzyme was inactivated by lowering the pH to 4.0–4.2 for 30 min, and the separation of the isoelectric soluble soybean protein hydrolysates from the insoluble matter was carried out at the same pH by centrifugation (1 h at 8000 *g*). All the hydrolysates were then freeze-dried, analysed for Kjeldahl N and stored in closed containers.

Isolation of the hydrophobic bitter peptide fractions (HPFs)

The hydrophobic bitter peptide fractions (HPFs) were extracted from the isoelectric soluble soybean protein hydrolysates (ISSPHs) with 2-butanol according to the procedure of Adler-Nissen [24], freeze-dried, analysed for Kjeldahl N and stored in closed containers.

Gel permeation high-performance liquid chromatography

The molecular mass distribution of the hydrolysates and the hydrophobic peptide fractions (ISSPHs and their HPFs) were determined by gel permeation high-performance liquid chromatography (GP-HPLC) on a Zorbax Bio Series GF-250 column (250 × 9.4 mm I.D., particle size 6 μm) with a separation range of M_r 4000–400 000 using an LDC Analytical HPLC system. The mobile phase was 0.1 *M* phosphate buffer (pH 8.0) containing 0.1% SDS at a flow-rate of 1.0 ml min^{-1}. Peptides were monitored at 215 nm with an absorbance range of 0.02 AUFS. The

column was calibrated with six peptide standards (carbonic anhydrase, cytochrome *c*, A- and B-chains of insulin, angiotensin II and angiotensinogen fragment 11–14) and the molecular masses of peptides were determined from the calibration plots log M_r vs. t_r (min): log $M_r = 7.81 - 0.45t_r$ ($r = 0.98$). Samples were dissolved in 0.1 *M* phosphate buffer, (pH 8.0) containing 0.1% SDS and filtered through a 0.45-μm filter (Millipore). The injection volume was 20 μl.

Preparative gel chromatography of the hydrophobic bitter peptide fraction

The hydrophobic bitter peptide fraction of isoelectric soluble soybean protein hydrolysate (HPF-ISSPH-DH 15%, 70 mg) was dissolved in 1.5 ml of water and filtered through a 0.45-μm filter (Millipore). The filtrate was applied to a Sephadex G-25 Fine column (78 × 3 cm I.D.) and eluted with distilled water at 8°C at a flow-rate of 15 ml h^{-1}. The effluent was collected in 5-ml fractions. The absorbance was measured at 215 nm and the collected fractions were sensorially analysed for bitterness. The column was calibrated with cytochrome *c*, angiotensin II and L-leucine.

Reversed-phase chromatography

The most bitter low-molecular-mass peptide fraction obtained by preparative gel chromatography on the Sephadex G-25 Fine column was separated on a Spherisorb ODS-2 column (250 × 4 mm I.D., particle size 5 μm) using an LDC Analytical HPLC system. After equilibration of the column with 0.1% (v/v) TFA (solvent A) at a flow-rate of 1.0 ml min^{-1}, peptides were eluted by linearly increasing the concentration of solvent B [0.1% (v/v) TFA in acetonitrile] as follows: 0–2 min, 0% B; 2–5 min, 0–10% B; 5–65 min, 10–40% B; 65–70 min, 40–70% B; 70–71 min 70–0% B. The re-equilibration time was 10 min. Peptides were monitored at 220 nm with an absorbance range of 0.1 AUFS. The injection volume was 20 μl and the injection was repeated twenty times in order to obtain substantial amounts for further analysis. The eluates were divided into fractions 1–18 as shown in Fig. 3. The peptide fractions were concentrated under a nitrogen atmosphere, dried in a Speed-Vac concentrator and tasted for bitterness.

Peptide material that was not well-separated into individual peaks was subjected to chromatography with a second solvent system, consisting of 25 m*M* amonium acetate (pH 6.0) (A) and 60% (v/v) acetonitrile in 50 m*M* ammonium acetate (pH 6.0) (B), with the following gradient: 0–2 min, 0% B; 2–62 min, 0–100% B; 62–63 min, 100–0% B. All other conditions were the same as above.

The ammonium acetate was removed from the isolated peptides to facilitate amino acid and sequence analysis with an elution system consisting of 0.1% (v/v) TFA (A) and 0.1% (v/v) TFA in acetonitrile (B) with the following gradient: 0–6 min, 0–15% B; 6–26 min, 15–25% B; 26–36 min, 25–50% B; 36–37 min, 50–80% B; 37–38 min, 80–0% B. The isolated peptides were evaporated in a Speed-Vac concentrator.

Amino acid analysis

Amino acid analysis of peptide hydrolysates, obtained after hydrolysis for 24 h in 6 *M* HCl at 110°C, was performed on an Applied Biosystems 421A amino acid analyser.

Sequence analysis

An Applied Biosystems Model 475A liquid-pulsed protein sequencer, connected on-line to a Model 120A phenylthiohydantoin–amino acid analyser from the same manufacturer, was used for amino acid sequence determinations.

Calculation of hydrophobicity

The hydrophobicities (the *Q* values) of the peptides were calculated according to Ney [8] on the basis of the Δf values of Tanford [25].

Sensorial analysis

The bitterness of the hydrolysates ($c = 3\%$ N × 6.25), their hydrophobic peptide fractions ($c = 0.5\%$ N × 6.25) and gel chromatographic fractions was estimated by scoring on a scale from zero to five (0, no bitterness; 1, weak bitter after-taste; 2, weakly bitter; 3, bitter; 4, strongly bitter; 5, extremely bitter) by a panel of three persons.

3. Results and discussion

The elution profiles obtained for five ISSPHs and their HPFs on the Zorbax Bio Series GF-250 column are given in Fig. 1A and B. A high-molecular-mass fraction (fraction I; M_r 30 000–3000), a medium-molecular-mass fraction (fraction II; M_r 3000–1000) and a low-molecular-mass fraction (fraction III; $M_r < 1000$) of ISSPHs were obtained (Table 1). Fraction III is mainly composed of low-molecular-mass peptides and free amino acids. Some of them were retained on the column and eluted with an elution time of more than 12 min (Fig. 1A and B). The high-molecular-mass fraction of ISSPHs and HPFs consists of peptides of M_r 30 000–3000 (Fig. 1A) and 10 000–3000 (Fig. 1B). In peptides with $M_r >$ 10 000 present in the ISSPHs and having a longer chain length, the hydrophobic chains are generally directed towards the interior part of the molecule. Thus they are long enough to allow conformations such as hair-pin loops or clusters. Therefore, these peptides were not extracted from the hydrolysates with 2-butanol. As can be seen from Table 1, ISSPHs and their HPFs contain mainly low-molecular-mass peptides. Their content varies from 55.6 to 82.2% for ISSPHs and from 72.3 to 89.2% for their HPFs. The bitterness of ISSPHs rises as the degree of hydrolysis increases (Table 1). A slight decrease in bitterness was observed at DH 15%. HPFs of all ISSPHs give an extremely bitter taste even at a concentration of 0.5% N × 6.25 (all the hydrolysates were tasted at a concentration of 3% N × 6.25, as described under Experimental). From these results, we can conclude that the bitterness of ISSPHs is mainly due to low-molecular-mass peptides.

The elution profile of HPF-ISSPH-DH 15% obtained on a Sephadex G-25 Fine column (Fig. 2) shows a good correlation with the elution profile of the same sample subjected to GP-HPLC on the Zorbax Bio Series GF-250 column (Fig. 1B). As shown in Fig. 2, bitterness appeared at the end of the high-molecular-mass fraction of HPF (elution volume from 180 to 220

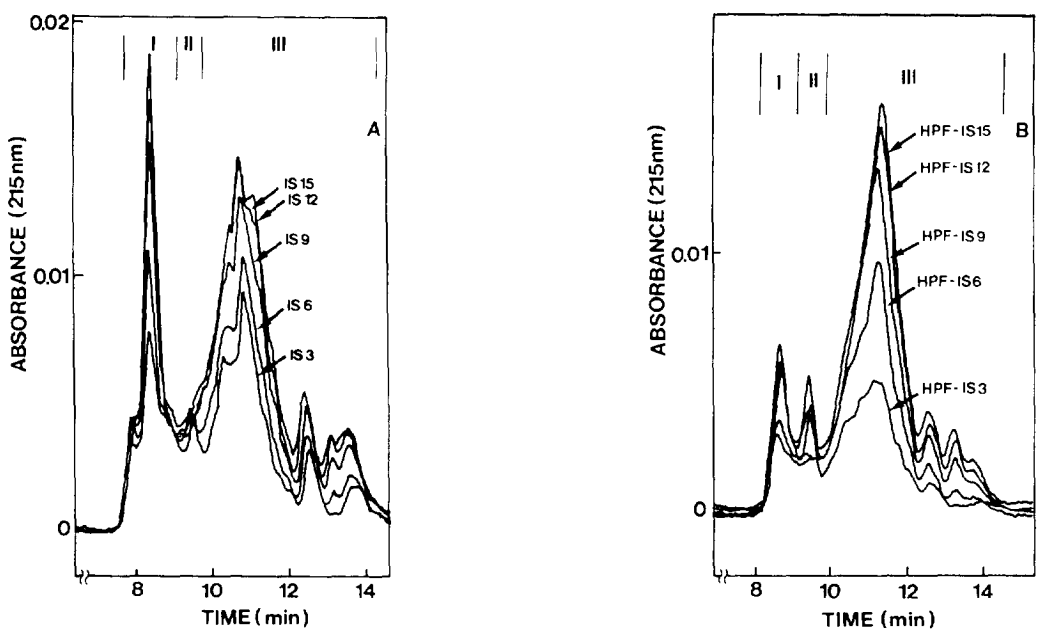

Fig. 1. Elution profiles of (A) five ISSPHs and (B) their HPFs on a Zorbax Bio Series GF-250 column (250 × 9.4 mm I.D.). The molecular mass fractions are designated I, II and III; I = high-molecular-mass fraction; II = medium-molecular-mass fraction; III = low-molecular-mass fraction.

Table 1
Bitterness and molecular mass distribution of ISSPHs and their HPFs obtained by GP-HPLC on a Zorbax Bio Series GF-250 column (250 × 9.4 mm I.D.)

Sample	Bitterness (score)	Area (%)		
		Fraction I: M_r 30 000–3000	Fraction II: M_r 3000–1000	Fraction III: M_r < 1000
ISSPH-DH 3%	0	37.3	7.1	55.6
ISSPH-DH 6%	2	31.5	6.2	62.3
ISSPH-DH 9%	3	23.0	5.8	71.2
ISSPH-DH 12%	3	16.3	5.2	78.5
ISSPH-DH 15%	2	13.9	3.9	82.2
HPF-ISSPH-DH 3%	5	17.2[a]	10.5	72.3
HPF-ISSPH-DH 6%	5	14.2[a]	10.3	75.5
HPF-ISSPH-DH 9%	5	10.7[a]	6.9	82.4
HPF-ISSPH-DH 12%	5	8.3[a]	3.9	87.8
HPF-ISSPH-DH 15%	5	6.7[a]	4.1	89.2

[a] M_r 10 000-3000.

ml), increased gradually in the medium-molecular mass fraction (elution volume from 250 to 290 ml) and reached a maximum intensity in the low-molecular-mass fraction of peptides (elution volume from 290 to 340 ml). The most bitter low-molecular-mass fraction of peptides (M_r < 1000) obtained after GPC on a Sephadex G-25 Fine column was separated into a large number of peptides on the Spherisorb ODS-2 column (Fig. 3) by a gradient of 0.1% (v/v) TFA (A) and 0.1% (v/v) TFA in acetonitrile (B). The eluate from RP-HPLC was divided into eighteen fractions, which were dried and tasted for bitterness. The straight line represents the gradient profile used. Generally, peptides appeared to be separated by RP-HPLC according to their hydrophobicity. Peptides with lower retention times are either small or hydrophobic and less strongly adsorbed on the stationary phase. They tasted slightly bitter in addition to a sour, burning or salty taste (fractions 1–7 and 9). The results of sensorial analysis are in agreement with many statements in the literature that peptides can elicit bitter, sweet, sour, umami, salt, astringent or tickling sensations [26,27]. Expressive bitter tasting fractions of insufficiently separated peptide material (fractions 8, 10, 11–15 and 18) were rechromatographed using a second solvent system consisting of 25 m*M* ammonium acetate (pH 6.0) (A) and 60% (v/v) acetonitrile in 50 m*M* ammonium acetate (pH 6.0) (B) (Figs. 4 and 5). The elution conditions using ammonium acetate in the RP system have been studied previously with casein digests and found to be suitable for peptide separations [20].

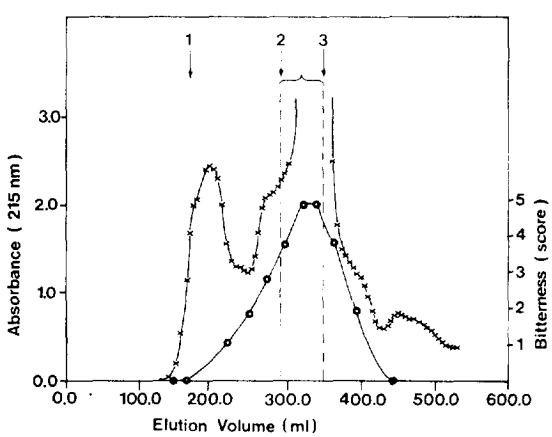

Fig. 2. Chromatogram of the hydrophobic bitter peptide faction from isoelectric soluble soybean protein hydrolysate (HPF-ISSPH-DH 15%) on a column of Sephadex G-25 Fine × = Absorbance at 215 nm; ○ = bitterness. The most bitter low-molecular-mass peptide fraction from 290 to 340 ml (shown with a brace) was rechromatographed by RP-HPLC. The numbers denote elution positions of the following compounds: 1 = cytochrome c; 2 = angiotensin II; 3 = L-leucine.

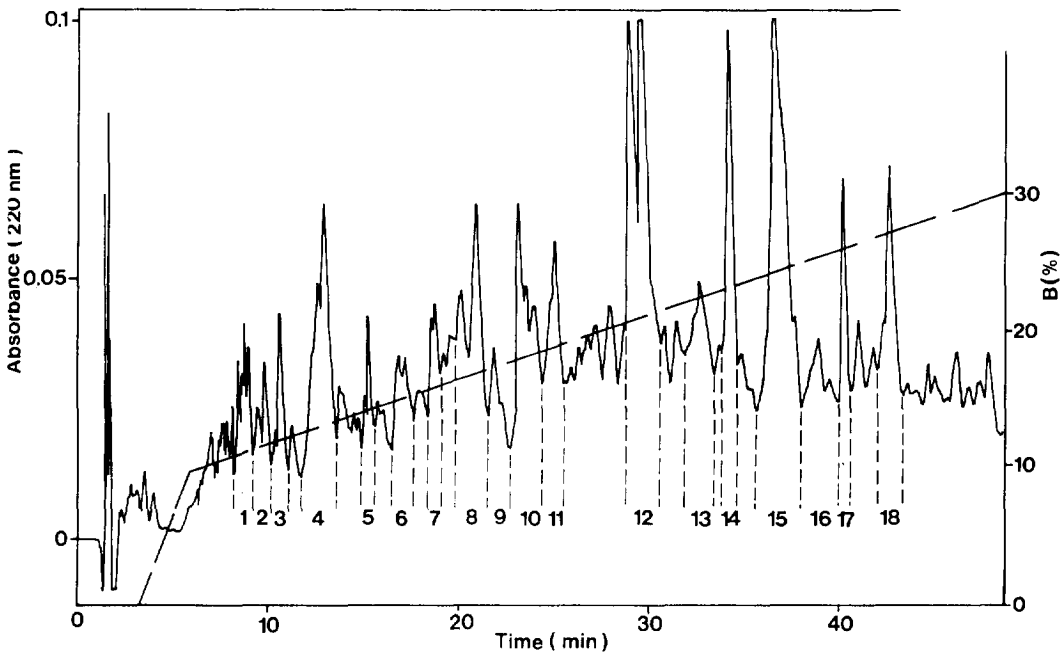

Fig. 3. RP-HPLC of the most bitter low-molecular-mass peptide fraction on a Spherisorb ODS-2 column (250 × 4 mm I.D.) with (A) 0.1% (v/v) TFA and (B) 0.1% (v/v) TFA in acetonitrile. The co-eluted peptide material is divided into fractions 1–18.

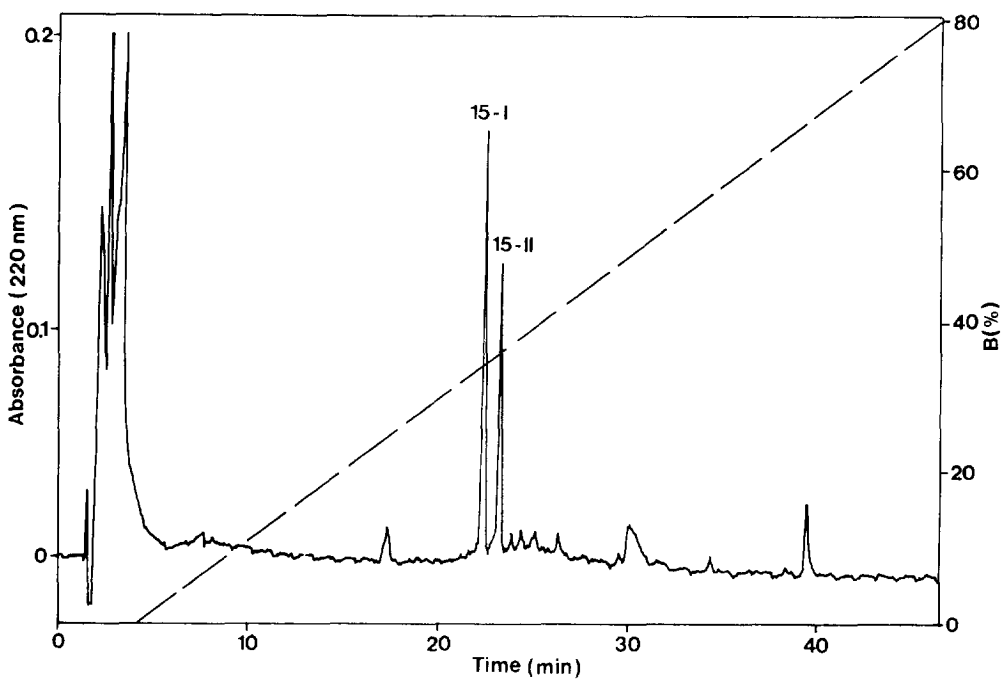

Fig. 4. Elution profile of rechromatographed fraction 15 from Fig. 3, obtained on a Spherisorb ODS-2 column by utilization of a second solvent system (peptides 15-I and 15-II).

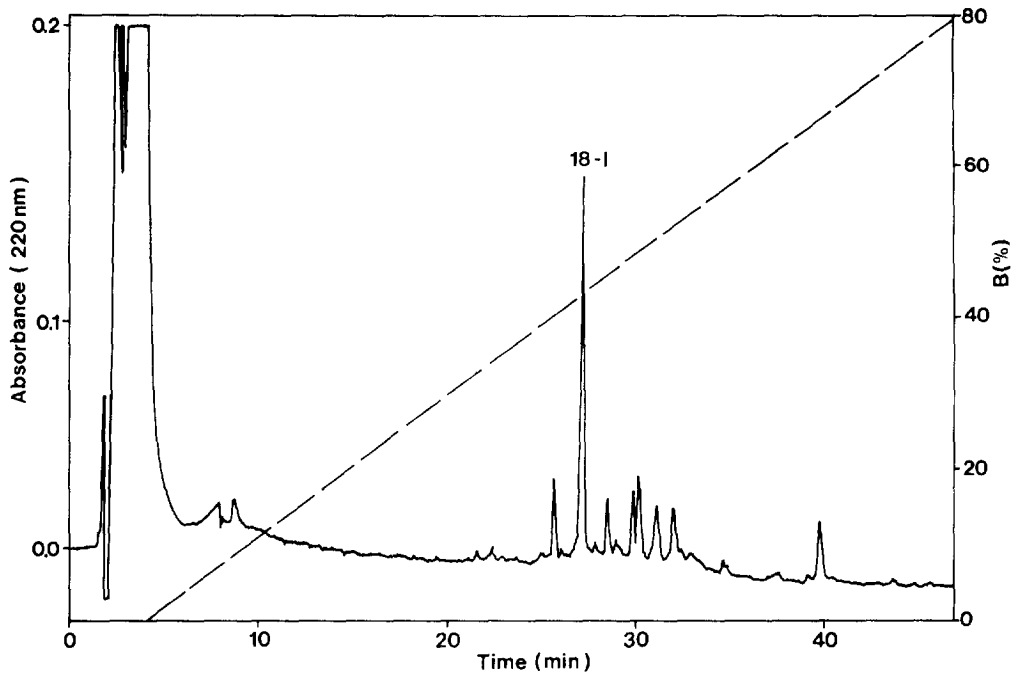

Fig. 5. Elution profile of rechromatographed fraction 18 from Fig. 3, obtained on a Spherisorb ODS-2 column by utilization of a second solvent system (peptide 18-1).

Table 2
Bitter-tasting low-molecular mass peptides isolated from the hydrophobic peptide fraction of isoelectric soluble soybean protein hydrolysate DH 15% (HPF-ISSPH-DH 15%)

RP-HPLC Fraction No.	Peptide	Eluent B (%)[a]	Hydrophobicity, Q (kcal mol^{-1})[b]	Hydrophobic residue	Sequence	M_r
8	8-I	19.65	1.46	2/3	FLS	365.41
	8-II	19.96	1.99	3/4	LLPH	478.57
10	10-I	20.77	1.75	3/4	LVGY	450/52
	10-II	20.54	2.34	3/4	IYIG	464.54
11	11-I	21.37	1.70	3/4	VYDV	494.53
	11-II	21.08	1.90	3/4	SVIY	480.54
12	12-I	22.67	2.23	4/4	VYFV	526.62
	12-II	22.84	2.21	3/4	ISIY	494.56
13	13-I	24.17	2.17	4/4	VVLY	492.60
14	14-I	24.50	2.05	2/3	DIF	392.42
	14-II	24.75	1.77	4/5	GYPVV	533.61
15	15-I	25.75	2.17	4/4	YVVL	492.60
	15-II	26.00	1.60	3/5	SGFTL	509.54
18	18-I	28.64	1.44	3/6	SNLNFL	678.72

[a] Eluent B = 0.1% TFA in acetonitrile.
[b] From Ref. [25].

The isolated peptides, the percentage of eluent B in the mobile phase, hydrophobicity, Q (kcal mol^{-1}), ratio of hydrophobic residues, amino acid sequences and the relative molecular masses of the peptides are given in Table 2. Fourteen bitter-tasting hydrophobic peptides with 3–6 amino acid residues and Q values from 1.44 to 2.34 kcal mol^{-1} were isolated. They are all predominantly composed of hydrophobic amino acids and with leucine, valine or tyrosine at the C-terminal part, which is in good agreement with the specificity of Alcalase, which cleaves predominantly at the carbonyl side of hydrophobic amino acids [22].

4. Conclusion

RP-HPLC has been demonstrated to be a reliable method with a high resolving power for the separation of hydrophobic bitter peptides from soybean protein hydrolysates. Peptides that co-eluate in the first mobile phase were easily separated using a second solvent system on the same RP-HPLC column.

Acknowledgements

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