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# Production of *Brassica carinata* Protein Hydrolyzates with a High Fischer's Ratio Using Immobilized Proteases

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*Brassica carinata* protein isolates were hydrolyzed using the digestive enzymes trypsin, chymotrypsin, and carboxypeptidase A in order to obtain hydrolyzates with a high Fischer's ratio. The proteases were immobilized using two glyoxyl-agarose supports of different porosity, 4 and 10% agarose gels, in order to evaluate the effect of substrate diffusion into the support containing the enzyme on the hydrolytic process. Reaction time, substrate concentration, and the enzyme to substrate ratio were optimized in an attempt to increase the Fischer's ratio in the resulting hydrolyzates. Gel filtration chromatography of a hydrolyzate with a degree of hydrolysis of 36% yielded a fraction that represented 31% of the total hydrolyzed proteins and had a Fischer's ratio of 28.3 with a phenylalanine + tyrosine content below 1.5%. This material could be used for preparing special diets when there is a need to increase the supply of branched amino acids and/or reduce the intake of aromatic amino acids.

**KEYWORDS:** ***Brassica carinata*; immobilized enzymes; protein hydrolyzates; Fischer's ratio**

## INTRODUCTION

The development of new or improved products using low-cost agricultural byproducts is of great economical interest. An example of this is the preparation of protein isolates and hydrolyzates using the defatted flours that result from oil extraction in rapeseed, soybean, sunflower, and other oilseed crops. The genus *Brassica* is of great economical and nutritional importance and includes crops that are eaten in different ways as well as used for seed oil extraction. *Brassica* is the second oilseed crop in Europe after sunflower and the fourth worldwide after soybean, palm, and cottonseed (*1*). The defatted *Brassica* meal resulting from oil extraction is rich in protein and may represent a valuable source of protein for human nutrition. However, the presence of certain antinutritional components such as glucosinolates and phytates limits its use so that it is typically used for animal feeding and for preparing organic fertilizer (*2*, *3*).

*B. carinata* (Ethiopian mustard) is related to rapeseed (*B. napus*) and originated from a cross between *B. nigra* and *B. oleracea. B. carinata* seeds are consumed in Ethiopia. Recently, Mediterranean countries such as Spain, Greece, and Italy have been showing interest in the use of this crop for production of biodiesel and solid biomass. In addition, the defatted meal resulting from oil extraction could represent an important source of protein, which would increase the value of *B. carinata* crops. Enzymatic hydrolysis can be used to improve the functional and nutritional properties of oilseed proteins, which are easily denatured during the process of oil extraction (*4*, *5*).

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Extensive protein hydrolyzates are mostly used as nutritional supplements, while tailor-made hydrolyzates with a well-defined composition are used in special medical diets (*6*). In particular, hydrolyzates with a high ratio of branched chain amino acids (BCAA) to aromatic amino acids (AAA), the so-called Fischer's ratio, may be used in the treatment of patients with certain liver diseases such as hepatic encephalopathy (*7*, *8*). This disease is characterized by liver malfunction that results in increased protein catabolism, BCAA deficit, and increased blood AAA concentration. In this situation, high concentrations of AAA can reach the brain, where they can mimic the effects of certain neurotransmitters and/or compete with others, resulting in different brain disorders (*9*). Protein hydrolyzates with a Fischer's ratio higher than 20 and a tyrosine + phenylalanine amount not exceeding 2% of total amino acids may be used in these cases in order to avoid the adverse effect of AAA (*10*). In addition, and attending to its high BCAA and/or low AAA composition, these hydrolyzates could be included in the diet of patients with certain genetic inborn disorders such as phenylketonuria and tyrosinemia (*11*), patients with diabetes (*12*), and athletes looking for increased endurance (*13*).

Protein hydrolyzates with a high Fischer's ratio have been obtained using the corn protein zein (*14*) and casein (*15*, *16*). Hydrolysis of casein using thermolysin and pepsin resulted in a Fischer's ratio of 31.97 (*15*). More recently, our group obtained a casein hydrolyzate with a 48.2 Fischer's ratio using immobilized digestive proteases (*16*).

The production of tailor-made hydrolyzates often requires the use of several proteases. Immobilization facilitates recycling these proteases, which is of great interest because it decreases the cost of the process. Immobilization has some additional

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advantages that result not only in a reduction of costs but also in an improved quality of the final product. These advantages include the possibility of carrying out hydrolysis under unconventional conditions (e.g., in the presence of organic solvents, at high temperature, or in the presence of high urea concentration), a reduction in the production of byproducts due to enzyme autolysis, and no need to inactivate the enzymes by heat or other treatments.

The goal of the present work was to obtain a *B. carinata* protein hydrolyzate with a high Fischer's ratio using immobilized trypsin, chymotrypsin, and carboxypeptidase A in a three-step sequential process. An improvement in the thermal stability of these proteases by immobilization onto glyoxyl-agarose has been observed (*17*). This process would start with a predigestion using trypsin, which would facilitate the subsequent release of peptides with aromatic residues at their C-terminal end by treatment with chymotrypsin and would continue by treatment with carboxypeptidase A in order to release these aromatic residues. Finally, free aromatic amino acids would be eliminated by gel filtration chromatography.

## MATERIALS AND METHODS

**Materials.** *B. carinata* brown seeds were a gift from Koipesol Semillas, S.A. (Sevilla, Spain). Bovine trypsin (E.C. 3.4.21.4), α-chymotrypsin (E.C. 3.4.21.1), and glycidol were purchased from Sigma Chemical Co. (St. Louis, MO). Carboxypeptidase A (E.C. 3.4.17.1) was purchased from Serva (Heidelberg, Germany). Hispanagar S.A. (Madrid, Spain) donated 4 and 10% agarose beads. Organic solvents and all other chemical reagents were of analytical grade.

**Methods.** All experiments were performed at least three times. Data shown correspond to mean values. Experimental error was not higher than 10%.

**Obtention of *B. carinata* Protein Isolates.** *B. carinata* seeds were ground and extracted using hexane in a Soxhlet apparatus. Protein extraction was carried out by suspension of the defatted flour (10% w/v) in 0.25% $Na_2SO_3$ pH 11 and stirring for 1 h. The pellet obtained by centrifugation at 7500*g* for 15 min was extracted twice more by resuspension in half the volume of alkaline solution and centrifugation at 7500*g* for 15 min. The pH of the combined supernatants was adjusted to the isoelectric pH values of 5 and 3.5 and the resulting precipitates were recovered by centrifugation at 7500*g* for 15 min, washed with distilled water, and freeze-dried.

**Activation of Agarose Gels.** The activation of 4 and 10% agarose gels was done according to Guisán (*18*) with slight modifications. The gels were suspended in 1 M NaOH, 0.5 M $NaBH_4$ 2:1 (v/v) (0.7 g swelling agarose is equivalent to 1 mL). These reducing conditions prevent oxidation of the gel. While this mixture was kept on an ice bucket, glycidol was added dropwise in order to reach a 2 M final concentration. The resulting suspension was gently stirred overnight at room temperature. The activated gel was then washed once with abundant distilled water pH 7 and then incubated with distilled water (300 mL) containing 300 μmol of $NaIO_4$/mL gel in order to achieve multipoint attachment. This oxidative reaction was allowed to proceed for 3 h while stirring at room temperature. With use of this procedure, the glyceryl groups obtained in the etherification reaction with glycidol are oxidized by periodate mole to mole. Subsequent measurement of excess $NaIO_4$ by titration with KI is used to determine the number of aldehyde groups that have been produced by the reaction of agarose with glycidol (*19*).

**Enzyme Immobilization.** Immobilization of the enzymes on activated 4 or 10% agarose gels was done according to procedures previously described with slight modifications (*18*, *20*, *21*). The gels (30 mg of enzyme/mL of gel for trypsin and chymotrypsin and 2 mg of enzyme/mL of gel for carboxypeptidase A) were suspended in 0.2 M sodium bicarbonate 1:10 (v/v) and gently stirred at room temperature for 3 h. Derivatives were then reduced by addition of $NaBH_4$ (0.1% w/v). After the mixture was gently stirred for 30 min at room temperature, residual sodium borohydride was eliminated by washing the mixture with abundant distilled water.

**Degree of Hydrolysis.** The degree of hydrolysis was calculated by determination of free amino groups by reaction with TNBS (*22*). The total number of amino groups was determined in a sample completely hydrolyzed by incubation with 6 N HCl at 110 °C for 24 h.

**Protein Determination.** Protein and peptide amounts were determined by elemental analysis as % nitrogen content × 6.25, using a LECO CHNS-932 analyzer (St. Joseph, MI).

**Hydrolysis of Protein Isolates.** Protein isolates, 0.4% (w/v) in 50 mM $NH_4HCO_3$, were taken to pH 8.0 and hydrolyzed at 50 °C in a batch reactor by addition of the immobilized enzymes in the following order: trypsin, chymotrypsin, and carboxypeptidase A. The immobilized enzymes were removed by filtration before addition of the next enzyme. The pH was maintained at 8 throughout the process by addition of $NH_4OH$ 12% (w/v) as required. Aliquots were taken at different times for determination of the degree of hydrolysis (DH).

**Gel Filtration Chromatography (Superose 12 Column).** Lyophilized samples (1% w/v) were dissolved in 0.1 M sodium borate, 0.2 M sodium chloride buffer, pH 8.3. Gel filtration was carried out in an FPLC system equipped with a Superose 12 HR 10/30 column from Amersham Pharmacia LKB Biotechnology (Uppsala, Sweden). Samples (200 μL) were injected at a protein concentration of 1.6 mg/mL. Borate buffer at a flow rate of 0.4 mL/min was used for elution, which was monitored at 280 nm. Blue dextran (2000 kDa) (Pharmacia Biotech.), catalase (240 kDa) (Serva), bovine serum albumin (67 kDa), ovoalbumin (43 kDa), ribonuclease (13.7 kDa), cytochrome C (12.5 kDa) (Pharmacia Biotech.), and bacitracin (1.45 kDa) (Sigma Chemical Co.) were used as molecular weight standards for determination of molecular mass.

**Gel Filtration Chromatography (Biogel P2 Column).** Hydrolyzates were injected into a Biogel P2 (BIORAD, CA) gel filtration column (2 × 55 cm) at a flow rate of 10 mL/h using 50 mM $NH_4HCO_3$. Fractions were collected every 10 min. Cytochrome c (12384 Da), bacitracin (1400 Da), Val$^4$-angiotensin (917 Da), Arg-Lys-Glu-Val-Tyr (693 Da), and Trp-Gly (261 Da) (Sigma Chemical Co.) were used as standards for determination of molecular mass.

**Amino Acid Analysis.** Samples were hydrolyzed by incubation in 6 N HCl at 110 °C for 24 h in tubes sealed under nitrogen. Amino acids were determined in the acid hydrolyzate by high-performance liquid chromatography (HPLC), according to the method of Alaiz et al. (*23*) after derivatization with diethyl ethoxymethylenemalonate, using D,L-α-aminobutyric acid as internal standard, and a 300 × 3.9 mm i.d. reversed-phase column (Novapack $C_{18}$, 4 μm; Waters). A binary gradient system with the solvents (A) 25 mM sodium acetate, 0.02% sodium azide (pH 6.0) and (B) acetonitrile was used. The elution gradient, at a flow rate of 0.9 mL/min, was as follows: 0.0−3.0 min, linear gradient from A/B (91/9) to A/B (86/14); 3.0−13.0 min, elution with A/B (86/14); 13.0−30.0 min, linear gradient from A/B (86/14) to A/B (69/31); 30.0−35.0 min, elution with A/B (69/31). The column was maintained at 18 °C.

**Electrophoresis.** Tricine-sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed according to the method described by Schaggerr and von Jagow (*24*) with slight modifications. The acrylamide solutions that were used for preparation of the gels were defined by the letters T (total percentage concentration of acrylamide and bisacrylamide) and C (percentage concentration of the cross-linker relative to T) according to Hjerten (*25*). Gels consisting of 20% T, 6% C and 4% T, 3% C were used for separation and stacking, respectively. The length of the separating and stacking gels were 6 and 2 cm, respectively, with a gel thickness of 1 mm. Electrophoresis was performed at a constant voltage of 60 V for stacking and 120 V for separation. The gels were fixed in 20% methanol, 8% acetic acid for 15 min before they were stained in 0.25% coomassie brilliant blue G in 45% methanol, 10% acetic acid for 24 h. Destaining of the gels was performed in 10% acetic acid.

## RESULTS

**Hydrolysis of *B. carinata* Protein Isolate.** Hydrolysis of the *B. carinata* protein isolate was based on the procedure previ-

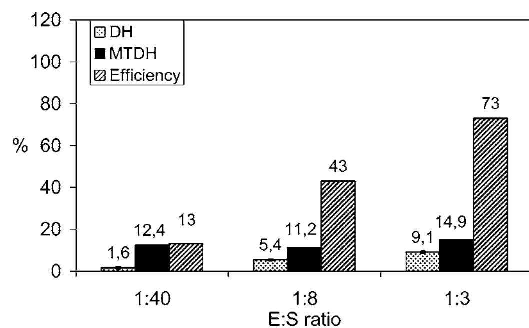


**Figure 1.** Effect of the E:S ratio on the hydrolysis by immobilized trypsin. Hydrolysis was carried out for 24 h at three different E:S ratios. Efficiency (hatched bars), maximum theoretical degree of hydrolysis (MTDH, solid bars), and experimental degree of hydrolysis (DH, dotted bars) are shown. Efficiency was calculated as (DH/MTDH) × 100.

ously described for hydrolysis of casein using trypsin, chymotrypsin, and carboxypeptidase A immobilized on 10% glyoxyl-agarose supports (*16*). A first digestion using immobilized trypsin was carried out in order to improve the exposure of aromatic residues that otherwise remain buried inside proteins and to allow the generation of shorter peptides that would readily interact with chymotrypsin and carboxypeptidase A. The preferred targets of trypsin are the peptidic bonds Arg-Xaa and Lys-Xaa, which are different than the target sites for chymotrypsin, namely, Xaa-Phe, Xaa-Tyr, Xaa-Trp, and Xaa-Leu. Carboxypeptidase A releases amino acids from the carboxylic end of peptides, having more affinity for tyrosine, phenylalanine, leucine, and tryptophan residues.

Hydrolysis with immobilized trypsin was carried out using a substrate concentration of 2% (w/v), an enzyme to substrate ratio of 1:40, a pH of 8, and a temperature of 50 °C. Hydrolysis of *B. carinata* proteins following this protocol provided a yield that was low as compared with that obtained in the hydrolysis of casein (*16*). Thus, while treatment of casein for 2 h hydrolyzed 73% of the peptidic bonds that were susceptible to hydrolysis by trypsin, treatment of the *B. carinata* isolate for 24 h yielded a degree of hydrolysis of 1.6%, which is equivalent to hydrolysis of only 13% of the susceptible peptidic bonds (**Figure 1**). Decreasing the substrate concentration to 0.2% and changing the enzyme to a substrate ratio to 1:8 and 1:3 resulted in degrees of hydrolysis of 5.4 and 9.1%, respectively. Because these degrees of hydrolysis were still rather low, additional changes were introduced into the procedure in order to test conditions that could improve hydrolysis of the *B. carinata* protein isolate by trypsin, chymotrypsin, and carboxypeptidase A. These included using a substrate concentration of 0.4% and immobilization of the enzymes on 4% glyoxyl-agarose, which has a higher porous size than the 10% glyoxyl-agarose used before. In addition, purification of globulins and albumins before hydrolysis was carried out in order to determine whether there is any component in these fractions that might interfere with the activity of the proteases.

Results of these additional experiments for immobilized trypsin, chymotrypsin, and carboxypeptidase A are shown in **Figures 2**, **3** and **4**, respectively. In **Figures 2** and **3**, as in **Figure 1**, the maximum theoretical degree of hydrolysis (MTDH) is given by the number of amino acid residues that are a target for trypsin (**Figures 1** and **2**) or chymotrypsin (**Figure 3**). In **Figure 4**, corresponding to carboxypeptidase A, MTDH is calculated as twice the degree of hydrolysis that was obtained after hydrolysis using trypsin and chymotrypsin, since a theoretical removal of all the residues at the carboxylic end would result in a twofold increase of the degree of hydrolysis. We have

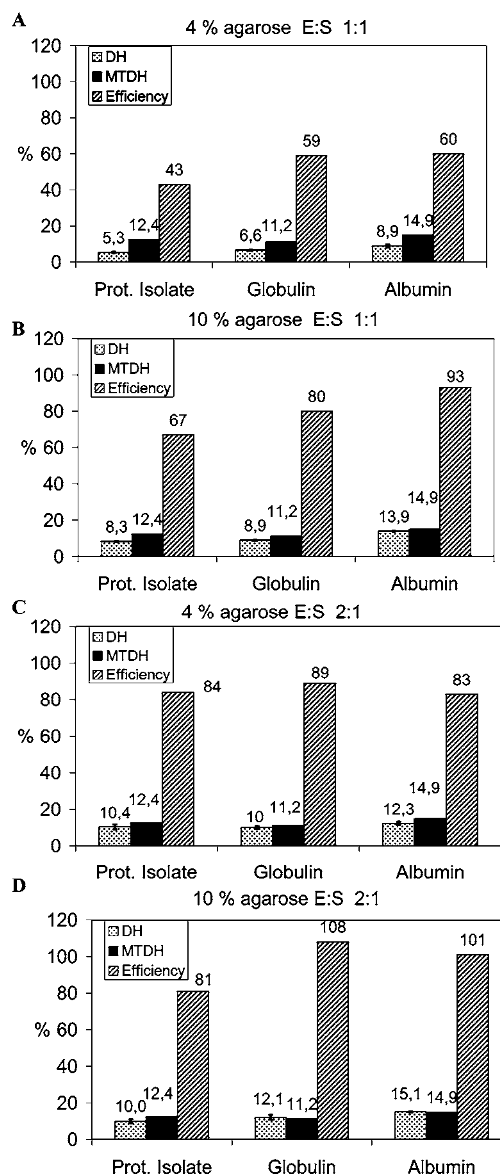


**Figure 2.** Effect of the E:S ratio and type of agarose on the hydrolysis by immobilized trypsin. Hydrolysis was carried out for 3 h using trypsin previously immobilized using 4% (**A** and **C**) or 10% (**B** and **D**) agarose and an E:S ratio of 1:1 (**A** and **B**) or 2:1 (**C** and **D**). Efficiency (hatched bars), maximum theoretical degree of hydrolysis (MTDH, solid bars), and experimental degree of hydrolysis (DH, dotted bars) are shown. Efficiency was calculated as (DH/MTDH) × 100.

previously reported that although carboxypeptidase A has higher affinity for tyrosine, phenylalanine, leucine, and tryptophan residues, other residues are also released from the carboxylic end if hydrolysis is allowed to proceed long enough (*21*).

In general, no important differences exist between the efficiency of the enzymes immobilized on either 4% or 10% glyoxyl-agarose, suggesting that diffusion of the protein into the porous matrix of the agarose support containing the immobilized enzymes is not the main limiting factor (**Figures 2** and **3**, **panels A** and **C** vs **panels B** and **D**, and **Figure 4**, **panel A** vs **panel B**). Previous studies showed that the thermal stability of these enzymes immobilized on 10% agarose was 3−6 times higher than the thermal stability of the same enzymes immobilized on 4% agarose (*17*). Considering all this, it was concluded that 10% glyoxyl-agarose is the best choice for immobilization of trypsin, chymotrypsin, and carboxypeptidase A.

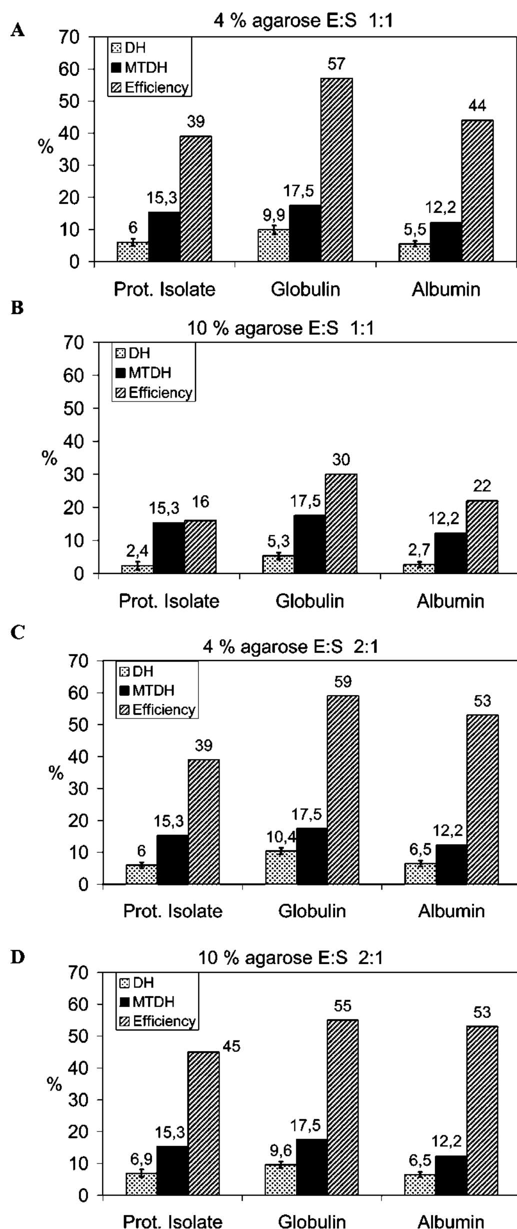


**Figure 3.** Effect of the E:S ratio and type of agarose on the hydrolysis by chymotrypsin. Hydrolysis was carried out for 3 h using chymotrypsin previously immobilized using 4% (**A** and **C**) or 10% (**B** and **D**) agarose and an E:S ratio of 1:1 (**A** and **B**) or 2:1 (**C** and **D**). Efficiency (hatched bars), maximum theoretical degree of hydrolysis (MTDH, solid bars), and experimental degree of hydrolysis (DH, dotted bars) are shown. Efficiency was calculated as (DH − DHo) × 100/MTDH, where the initial DH values after hydrolysis with trypsin (DHo) for the protein isolate, globulins, and albumin were 10.4, 10.0, and 12.3%, respectively.

Unlike the size of the pores in the support (4% vs 10% glyoxyl-agarose), the enzyme to substrate ratio had a significant effect on the hydrolysis process. Thus, high enzyme to substrate ratios were needed in order to obtain effective hydrolysis in a relatively short period of time such as 3 h (**Figures 2** and **3**, **panels A** and **B** vs **panels C** and **D**). Isolation of the globulin and albumin fractions also had an effect on hydrolysis since both fractions were hydrolyzed slightly better than the original isolate containing both fractions (**Figures 2−4**). Interestingly, the efficiency of the hydrolysis of globulins with carboxypeptidase A was higher than 100% (**Figure 4**), indicating that this enzyme was releasing more amino acids than those initially exposed by trypsin and chymotrypsin in the carboxy-terminal end of peptides.

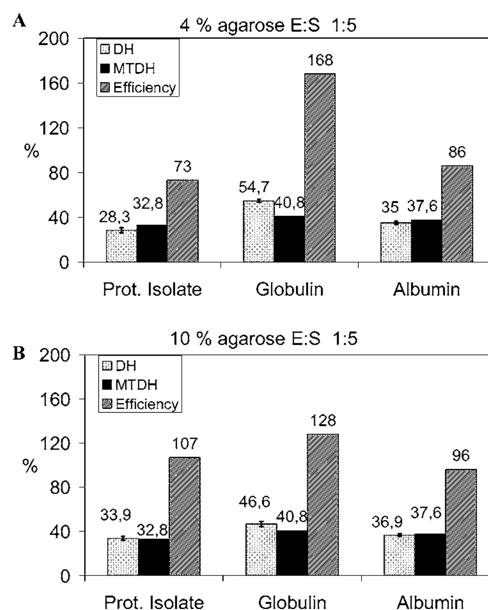


**Figure 4.** Effect of the E:S ratio and type of agarose on the hydrolysis by carboxypeptidase A. Hydrolysis was carried out for 3 h using carboxypeptidase A previously immobilized using 4% (**A**) or 10% (**B**) agarose and an E:S ratio of 1:5 (**A** and **B**). Efficiency (hatched bars), maximum theoretical degree of hydrolysis (MTDH, solid bars), and experimental degree of hydrolysis (DH, dotted bars) are shown. Efficiency was calculated as (DH/MTDH) × 100 where MTDH is twice the DH obtained after hydrolysis using trypsin and chymotrypsin (16.4, 20.4, and 18.8% for protein isolate, globulin, and albumin, respectively).

**Table 1.** Optimized Procedure for Hydrolysis of *B.* Carinata Seed Proteins

| E:S (w/w) | enzyme (time) | DH[a] | MTDH | efficiency[d] |
|---|---|---|---|---|
| 2:1 | TP (2 h) | 9.4 ± 1.1 | 12.4[b] | 76.0 |
| ((2:1 | QT (2 h[e]) | 6.9 | 15.3[b] | 45.0)) |
| 2:1 | TP (2 h) + QT (2 h) | 16.3 ± 1.2 | 27.7[b] | 59.0 |
| 1:5 | TP (2 h) + QT (2 h) + CPA (2 h) | 32.4 ± 1.4 | 32.6[c] | 99.0 |

[a] Degree of hydrolysis as determined by reaction of free amino groups with TNBS.[22] Data are given as percentage and represent the mean ± standard deviation of triplicate experiments. [b] Maximum theoretical degree of hydrolysis as calculated from the amino acid composition of the *B. carinata* protein isolate and considering the theoretical target sites for each enzyme. [c] Maximum theoretical degree of hydrolysis calculated as twice the DH obtained after hydrolysis using trypsin and chymotrypsin. [d] Calculated as (DH/MTDH) × 100. [e] Calculated from *B. carinata* digestion with trypsin plus chymotrypsin minus trypsin digestion.

Based on the experiments described above, **Table 1** shows the main parameters for the optimized hydrolysis of *B. carinata* seed proteins using trypsin, chymotrypsin, and carboxypeptidase A. The *B. carinata* protein isolate was chosen as the substrate for this hydrolytic process because there was no large advantage in using the purified globulin or albumin fractions. The proteases were immobilized onto 10% modified agarose gels and the substrate concentration was 0.4% (w/v). Hydrolysis with each enzyme was allowed to proceed for 2 h and the enzyme to substrate ratios were 2:1 for trypsin and chymotrypsin and 1:5 for carboxypeptidase A. Gel filtration and SDS-PAGE analyses of the protein isolate, the two intermediate hydrolyzates, and the final hydrolyzate that were produced are shown in **Figures 5** and **6**. The *B. carinata* isolate included peptides as small as 2.5 kDa and proteins as big as 300 kDa including globulins (cruciferin) (**Figure 5**). Fractions lower than 30 kDa were

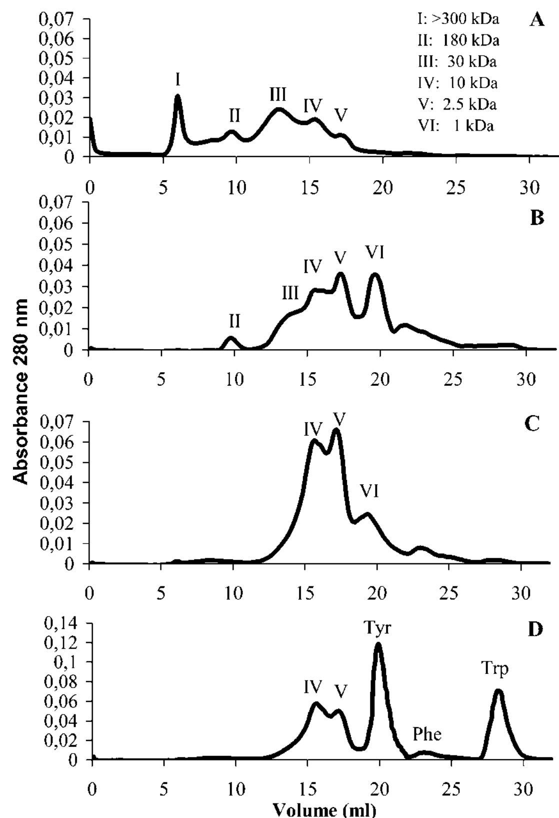


**Figure 5.** Gel filtration chromatography of the *B. carinata* protein isolate and hydrolyzates. The following samples were subjected to gel filtration chromatography using a Superose 12 column: protein isolate (**A**), trypsin hydrolyzate (**B**), trypsin + chymotrypsin hydrolyzate (**C**), and trypsin + chymotrypsin + carboxypeptidase A hydrolyzate (**D**).

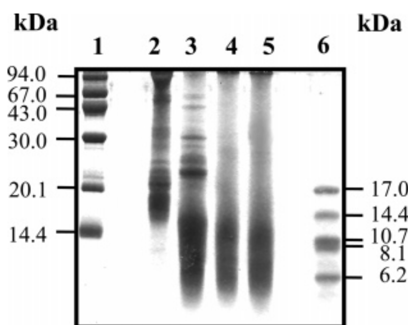


**Figure 6.** SDS-PAGE of the *B. carinata* protein isolate and hydrolyzates. The original protein isolate (lane 2) and the hydrolyzates resulting from hydrolysis using trypsin (lane 3), trypsin + chymotrypsin (lane 4), and trypsin + chymotrypsin + carboxypeptidase A (lane 5) were run together with molecular weight standards (lanes 1 and 6).

identified by SDS-PAGE as the α and *β* subunits of the globulins which showed as bands between 30 and 20 kDa (**Figure 6**). Treatment with trypsin resulted in the almost complete disappearance of the larger proteins from the electrophoretic and chromatographic profiles. This observation further supports the view that diffusion of proteins into the support containing the immobilized enzyme was not a decisive limiting factor for the hydrolysis of *B. carinata* protein.

Following treatment with trypsin, incubation with chymotrypsin caused the hydrolysis of 45% of the target peptidic bonds: Xaa-Phe, Xaa-Tyr, Xaa-Trp, and Xaa-Leu. The efficiency of the hydrolysis using first trypsin and then chymotrypsin was 60%. Incubation with chymotrypsin completed the hydrolysis of the remaining large proteins, producing a large fraction of peptides with molecular weight lower than 10 kDa (**Figure 5C**). SDS-PAGE shows that the remaining α and *β* subunits completely disappear at this stage, and that a smear of low molecular weight peptides are produced (**Figure 6**).

Carboxypeptidase A releases aromatic amino acids from the carboxylic end of proteins. Treatment of the trypsin-chymotrypsin hydrolyzate with this enzyme approximately doubled the degree of hydrolysis (**Table 1**). SDS-PAGE analysis and gel filtration chromatography did not reveal significant differences between these two hydrolyzates, except for the presence of peaks corresponding to free tyrosine, phenylalanine, and tryptophan in the gel filtration profile (**Figures 5** and **6**).

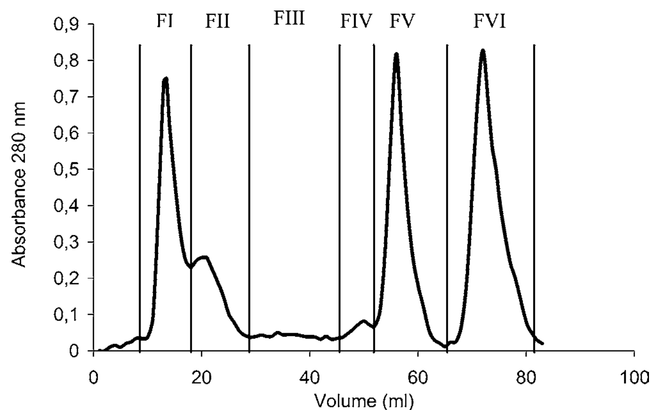


**Figure 7.** Separation of peptidic fractions by gel filtration chromatography. The *B. carinata* final protein hydrolyzate obtained using trypsin, chymotrypsin, and carboxypeptidase A was applied to a Biogel P2 column.

**Table 2.** Fractions Obtained by Gel Filtration Chromatography of the *B. carinata* Protein Hydrolyzate[a]

| | molecular masses (kDa) | yield (%) |
|---|---|---|
| F I | >1.8 | 52.0 |
| F II | 1.8–1.4 | 13.0 |
| F III | 1.4–0.5 | 31.0 |
| F IV–F VI | free amino acids | 4.0 |

[a] Protein was hydrolyzed using trypsin, chymotrypsin, and carboxypeptidase A and injected into a Biogel P2 column. Fractions were collected and combined according to the elution profile and molecular weight.

**Purification of a Peptidic Fraction with a High Fischer's Ratio.** With the goal of obtaining peptide fractions with a high Fischer's ratio, hydrolyzates prepared following the optimized procedure described above were loaded on a Biogel P2 gel filtration column (**Figure 7**). Biogel P2 offers a separation range lower (1.8−0.1 kDa) than that provided by Superose 12 (300−1 kDa), which was used for characterization of the hydrolyzates as described before (**Figure 5**). The eluate was divided into six fractions according to molecular weight as shown in **Table 2**. The largest fraction, fraction I, represented more than 50% of the total proteins eluted from the column and is made up of proteins and peptides with molecular weights ranging from 10000 to 1800 Da that are not retained by the column. Fraction II contained peptides between 1800 and 1400 Da and represented 13% of the total proteins eluted. Fraction III represented 31% of the total proteins and included peptides between 1400 and 500 Da. Fractions IV, V, and VI corresponded to peptides below 500 Da and free amino acids, mainly free phenylalanine, tyrosine, and tryptophan, respectively, and represented 4% of the total proteins.

**Table 3.** Amino Acid Composition of the Fractions Described in Table 2[a]

| | F I | F II | F III | F IV | F V | F VI | hydrolyzate |
|---|---|---|---|---|---|---|---|
| Asp[b] | 13.8 ± 1.8 | 8.6 ± 0.9 | 4.2 ± 1.7 | 1.3 ± 0.3 | 0.4 ± 0.2 | 0.4 ± 0.1 | 10.4 ± 1.0 |
| Glu[c] | 26.0 ± 2.0 | 21.2 ± 0.9 | 12.8 ± 1.6 | 2.4 ± 1.1 | 0.7 ± 0.1 | 0.6 ± 0.0 | 19.5 ± 1.2 |
| Ser | 6.2 ± 0.2 | 6.1 ± 0.6 | 2.7 ± 0.8 | 0.7 ± 0.5 | 0.0 ± 0.0 | 0.4 ± 0.1 | 4.9 ± 0.3 |
| Hys | 2.0 ± 0.3 | 3.0 ± 0.3 | 3.4 ± 0.6 | 4.1 ± 0.4 | 0.5 ± 0.1 | 0.0 ± 0.0 | 2.7 ± 0.2 |
| Gly | 7.5 ± 0.3 | 6.6 ± 0.1 | 3.6 ± 0.1 | 1.5 ± 1.3 | 0.4 ± 0.1 | 1.5 ± 0.2 | 5.6 ± 0.4 |
| Trr | 6.6 ± 0.6 | 5.9 ± 0.9 | 4.5 ± 0.7 | 0.5 ± 0.3 | 0.2 ± 0.1 | 1.5 ± 0.1 | 4.5 ± 0.3 |
| Arg | 4.9 ± 0.1 | 10.3 ± 3.7 | 13.4 ± 1.1 | 3.5 ± 2.1 | 0.4 ± 0.1 | 2.2 ± 0.2 | 8.8 ± 0.6 |
| Ala | 4.9 ± 0.1 | 6.3 ± 0.6 | 3.7 ± 0.3 | 1.3 ± 0.6 | 0.4 ± 0.1 | 0.5 ± 0.1 | 5.2 ± 0.5 |
| Pro | 5.1 ± 1.6 | 5.7 ± 1.3 | 3.6 ± 0.9 | 1.8 ± 0.3 | 0.1 ± 0.0 | 0.1 ± 0.0 | 6.4 ± 1.1 |
| Tyr | 0.4 ± 0.2 | 1.2 ± 0.7 | 0.3 ± 0.2 | 5.6 ± 1.6 | 88.2 ± 6.3 | 1.5 ± 0.3 | 2.4 ± 0.6 |
| Val | 5.7 ± 0.6 | 5.7 ± 0.5 | 7.0 ± 2.0 | 1.5 ± 0.2 | 0.1 ± 0.0 | 0.9 ± 0.2 | 6.0 ± 1.3 |
| Met | 0.4 ± 0.1 | 1.9 ± 0.5 | 1.3 ± 0.3 | 0.3 ± 0.2 | 0.3 ± 0.2 | 0.4 ± 0.3 | 1.5 ± 0.3 |
| Cys | 2.8 ± 0.3 | 1.1 ± 0.5 | 0.5 ± 0.2 | 0.1 ± 0.2 | 0.0 ± 0.0 | 0.1 ± 0.0 | 2.0 ± 0.3 |
| Ile | 3.4 ± 0.1 | 4.0 ± 0.5 | 5.7 ± 0.2 | 3.3 ± 0.7 | 0.3 ± 0.0 | 1.0 ± 0.2 | 4.7 ± 0.4 |
| Leu | 6.6 ± 0.5 | 6.4 ± 0.3 | 26.7 ± 5.2 | 7.0 ± 2.2 | 0.5 ± 0.1 | 0.8 ± 0.3 | 7.7 ± 0.5 |
| Phe | 1.0 ± 0.2 | 2.4 ± 0.4 | 1.2 ± 0.5 | 62.0 ± 5.2 | 1.5 ± 0.2 | 0.6 ± 0.2 | 4.1 ± 0.7 |
| Lys | 2.8 ± 0.8 | 4.1 ± 0.9 | 5.4 ± 1.1 | 0.7 ± 0.3 | 0.2 ± 0.0 | 0.7 ± 0.5 | 3.5 ± 0.4 |
| Trp | 0.0 ± 0.0 | 0.2 ± 0.1 | 0.1 ± 0.0 | 2.6 ± 1.3 | 6.1 ± 1.6 | 86.9 ± 4.4 | 0.8 ± 0.1 |
| F.R. | 11.7 ± 2.6 | 4.7 ± 1.1 | 28.3 ± 8.2 | 0.2 ± 0.0 | 0.01 ± 0.00 | 1.2 ± 0.1 | 2.8 ± 0.1 |

[a] F.R.: Fischer's ratio = valine + isoleucine + leucine/tyrosine + phenylalanine. [b] Asp = Asp + Asn. [c] Glu = Glu + Gln. Data are expressed in % and represent the average ± standard deviation of three independent experiments.

The amino acid composition of the six fractions that were pooled from the Biogel P2 column eluate is shown in **Table 3**. In fractions I−III, the content in aromatic amino acids were reduced considerably so that the Fischer's ratios were higher than those in the original protein hydrolyzate. Some amino acids were present in large amounts in some fractions as compared with the amino acid composition of the original hydrolyzate. These are the residues that are at the target sites for trypsin and α-chymotrypsin, including arginine and leucine in fraction III, phenylalanine in fraction IV, tyrosine in fraction V, and tryptophan in fraction VI. An analysis of free amino acids in these fractions showed that the amino acids phenylalanine, tyrosine, and tryptophan in fractions IV, V, and VI, respectively, were free amino acids (not shown), while fraction III contained both small peptides and free amino acids (45% arginine, 35% leucine, 30% isoleucine, 20% valine, 10% lysine, and 10% phenylalanine). This reflects hydrolysis of the residues in the carboxylic end of peptides by carboxypeptidase A after hydrolysis with trypsin and chymotrypsin.

## DISCUSSION

The hydrolysis of *B. carinata* protein using immobilized trypsin, chymotrypsin, and carboxypeptidase A has proven to be difficult and certainly not as straightforward as hydrolysis of other proteins such as casein (*16*). This difficulty might be explained by several factors, including diffusion problems, the intrinsic resistance of globulins to proteolysis due to its compact and globular structure (*26*) even after thermal treatment (*27*), and the presence of protease inhibitors that may reduce enzymatic activity (*28*, *29*). Considering the porous nature of the agarose support, diffusion problems might be caused by the size of the *B. carinata* protein aggregates (*30*), which can be as large as 300 kDa (**Figure 5**). Nevertheless, the lack of effect of using a support with larger pore size and the almost complete disappearance of the larger proteins after treatment with trypsin support the view that diffusion of the substrate protein into the matrix containing the immobilized enzymes is not the main limiting factor. In any case, an optimization study has been carried out so that good yields of hydrolysis have been obtained by using a higher enzyme to substrate ratio.

The final goal of our research was to produce a protein hydrolyzate with a high Fischer's ratio. One of the fractions obtained by gel filtration chromatography of the *B. carinata* hydrolyzate (fraction III in **Figure 7**), representing one-third of the original protein present in the original hydrolyzate, meets the requirements to be included in the diet of patients with hepatic encephalopathies. Thus, this fraction has a Fischer's ratio of 28.3 and a content in aromatic amino acids (phenylalanine and tyrosine) lower than 2% of the total amino acids. Considering these parameters, this fraction could also be used in the treatment of other metabolic disorders such as phenylketonuria and tyrosinemia. The interest in nutritional formulas based on protein hydrolyzates of plant origin has increased in recent years. These hydrolyzates are well absorbed and can be customized in order to meet specific nutritional needs (*31*−*33*).

The procedure here described facilitates the production of a food supplement of great interest for specialized nutrition and has the additional advantage of using an inexpensive material as the source of protein. The use of immobilized enzymes reduces the cost as compared with the use of soluble enzymes because immobilization permits recycling of the enzymes and increases its stability. This product could be easily scaled up to allow production of the high Fisher's ratio hydrolyzate in large quantities. Additional studies on several biological activities that are present in *B. carinata* protein hydrolyzates are now underway.

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