

## Improvement of protein extraction from sunflower meal by hydrolysis with alcalase

By M.M. Yust, J. Pedroche, C. Megías, J. Girón-Calle, M. Alaiz, F. Millán and J. Vioque\*

Instituto de la Grasa, CSIC. Avenida Padre García Tejero 4. 41012-Sevilla, Spain.  
E-mail: [jvioque@cica.es](mailto:jvioque@cica.es)

### RESUMEN

#### Mejora de la extracción proteica de la harina de girasol mediante hidrólisis con alcalasa.

Se ha mejorado la extracción proteica de la harina desengrasada de girasol mediante la adición de la proteasa alcalasa durante la extracción alcalina. Este método ofrece varias ventajas adicionales en comparación con la extracción alcalina tradicional sin alcalasa, que se desarrolla normalmente mediante un proceso de flotación/sedimentación para retirar la fracción lignocelulósica. En comparación a la extracción sin alcalasa, la adición de 0.1% (v/v) de alcalasa mejora los rendimientos de extracción proteica desde un 57.5% a un 87.4%, dando un extracto con un 22% de grado de hidrólisis. Además se obtiene un incremento de hasta 4.5 veces de la solubilidad proteica a bajos pHs, que se correlaciona con el grado de hidrólisis. Los extractos obtenidos con alcalasa tenían un mayor contenido de prolina y glicina, sugiriendo que la proteasa mejora la extracción de las proteínas ricas en prolina y glicina de la pared celular que forma parte de la fracción lignocelulósica. Este extracto proteico puede ser secado directamente sin generación de aguas residuales, y el material resultante rico en fibra podría ser usado para alimentación animal.

**PALABRAS-CLAVE:** Alcalasa - Extracción proteica - Harina de girasol - Hidrolizados proteicos - Proteasa.

### SUMMARY

#### Improvement of protein extraction from sunflower meal by hydrolysis with alcalase.

Extraction of proteins from defatted sunflower meal has been improved by addition of the protease alcalase during alkaline extraction. This method offers several additional advantages as compared to the traditional alkaline extraction without alcalase, which is usually carried out after a sedimentation/flotation step to remove the lignocellulosic fraction. As compared to extraction without alcalase, addition of 0.1% (v/v) alcalase improved the yield of protein extraction from 57.5% to 87.4%, providing an extract that is 22% hydrolyzed. In addition, an increment of up to 4.5 times in protein solubility at low pH values is achieved, which correlates with the degree of hydrolysis. The extracts that were obtained in the presence of alcalase had a higher proline and glycine content, suggesting that the protease improves extraction of proline-rich and glycine-rich cell wall proteins that are part of the lignocellulosic fraction. These protein extracts can be directly dried without generation of wastewater, and the resulting fiber-rich material could be used for animal feeding.

**KEY-WORDS:** Alcalase - Protease - Protein extraction - Protein hydrolysates - Sunflower meal.

### 1. INTRODUCTION

The transformation of by-products of the food industry into new, non-conventional sources of

proteins is of great interest because it represents a potential source of inexpensive protein for feeding livestock and also for human consumption. An additional advantage of using these by-products as a source of protein is that it reduces the environmental problems associated with their disposal. The sunflower (*Helianthus annuus* L.) defatted meal that is left after oil extraction is a typical case of such by-products. Sunflower is one of the more important oilseed crops cultivated in the world, representing the fourth-largest source of edible oil (Lühs & Friedt, 1994). Defatted sunflower meal, which contains about 30% protein (Parrado et al., 1991), has already been used for the preparation of protein isolates (Saeed & Cheryan, 1988) and high added-value protein hydrolysates (Villanueva et al., 1999).

During the industrial extraction of fruit or seed oils, proteins suffer a denaturation process that reduces their solubility. In order to improve solubility, proteins can be hydrolyzed before or during extraction by using proteases. In recent years, the availability of industrial proteases, mainly purified from bacteria and fungi, has enabled large scale production of protein hydrolysates (Vioque et al., 2001). Alcalase is one of these commercial proteases, and has been used to aid in the extraction of protein from chicken bones (Sales et al., 1991), nettle leaves (Dalev et al., 1996) and rice bran (Hanmoungjai et al., 2001). Recently, it has been observed that a limited treatment of *Brassica carinata* defatted meal with alcalase produces a significant increase in the yield of protein extracts (Pedroche et al., 1998). Since treatment with alcalase appears to enhance protein extraction from different sources, the effect of adding alcalase during extraction of defatted sunflower meal protein has now been investigated. Alkaline extraction in the presence of alcalase has been compared with the conventional method for alkaline extraction in the absence of alcalase. This results in several advantages, in addition to allow high yields of protein extraction without having to carry out a preliminary flotation/sedimentation step to remove the lignocellulosic fraction.

## 2. MATERIALS AND METHODS

### 2.1. Materials

Sunflower (*H. annuus* L.) meal was obtained by solvent extraction and was provided by Koipesol (Sevilla, Spain). All chemicals were of analytical grade and were purchased from Sigma Chemical Co. (St. Louis, MO), unless otherwise indicated. Alcalase 2.4 L, a microbial protease from *Bacillus licheniformis* with endopeptidase activity, was provided by Novo Nordisk (Bagsvaerd, Denmark). A main component of this commercial preparation is the serine protease subtilisin A. The specific activity of alcalase 2.4 L is 2.4 Anson Unit per gram.

### 2.2. Protein determination

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

### 2.3. Sedimentation / Flotation fractionation of defatted sunflower meal

A 10 % (w/v) suspension of defatted sunflower meal in water was bubbled with air for 10 minutes to achieve uniform dispersion of the flour. The mixture was allowed to sediment for 15 minutes, so that the suspension was resolved into three phases: a protein fraction that formed a sediment at the bottom of the sedimentation tank; a soluble fraction in the middle, and a lignocellulosic fraction that floated at the top. After the lignocellulosic fraction was mechanically removed, the soluble and protein fractions were separated by vacuum filtration (Bautista et al., 1990).

### 2.4. Protein extraction

Sunflower defatted flour (10 g) was suspended in 0.25% Na<sub>2</sub>SO<sub>3</sub> (100 mL) pH 10. The suspension was extracted by stirring for 1 hour at room temperature in the presence of different concentrations of alcalase. After centrifugation at 8000 g, supernatants were pooled and used for experimentation. An aliquot of the pooled supernatants was taken to dryness to determine extracted matter by gravimetry.

### 2.5. Isoelectric precipitation

For isoelectric precipitation of proteins, the pH of supernatants was adjusted to the isoelectric point of sunflower meal protein (pH 4.3). Precipitates were recovered by centrifugation at 8000 g.

### 2.6. Degree of hydrolysis

The degree of hydrolysis was calculated by determination of free amino groups by reaction with 2,4,6-trinitrobenzenesulfonic acid (Adler-Nissen, 1979). The total number of amino groups was determined in a sample 100% hydrolysed by treatment with 6 N HCl at 110° C for 24 hours.

### 2.7. Amino acid analysis

2 mg of samples were hydrolyzed with 6 N HCl (4 mL) at 110° C for 24 hours in tubes sealed under nitrogen. Amino acids were determined after derivatization with diethyl ethoxymethylenemalonate by high-performance liquid chromatography (HPLC) according to the method of Alaiz et al. (1992), using D,L--aminobutyric acid as internal standard. The HPLC system consisted of a Waters Model 600E multi-system with a Waters Model 484 UV-Vis detector, equipped with a 300 x 3.9 mm i.d. reversed-phase column (Novapack C<sub>18</sub>, 4 m; Waters) that was kept at 18° C during analyses. A binary gradient system formed by mixing (A) 25 mM sodium acetate containing 0.02% sodium azide (pH 6.0) and (B) acetonitrile was used. The solvent was delivered to the column at a flow rate of 0.9 mL/min as follows: time 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). Derivatized amino acids were detected at 280 nm.

### 2.8. Determination of solubility

Protein extracts were assayed for solubility at different pH values that were obtained by addition of 1 N HCl. Solubility was expressed as percentage of total protein that remained soluble after acidification. Protein was determined as nitrogen in the soluble and insoluble fractions obtained by centrifugation at 8000 g for 30 minutes.

## 3. RESULTS AND DISCUSSION

Before alkaline extraction of protein from defatted sunflower meal the lignocellulosic fraction is usually removed using a sedimentation/flotation procedure (Parrado et al., 1991, Villanueva et al., 1999). Fiber is the major component of the low density fraction that remains in the upper phase after sedimentation/flotation (60-70 %), but this upper phase still contains a significant amount of protein (20-25 %) (Villanueva, 1997). This protein is lost as a consequence of carrying out the sedimentation/flotation procedure. In an attempt to increase the yield of protein extraction, assays have been carried out in which the protease alcalase was added during alkaline extraction and the

sedimentation/flotation step was omitted. Alcalase is an inexpensive commercial preparation most active at basic pH, which makes it suitable to be used during alkaline hydrolysis. Several parameters of this new procedure of direct alkaline extraction of the defatted flour using alcalase have been studied in order to assess the possible advantages of using this enzyme.

All the results that are presented here correspond to protein extractions that were carried out at room temperature and pH 10 in medium containing sodium sulphite. Sodium sulphite is a reducing agent that inhibits oxidation of polyphenols (Gheyasuddin, Cater, & Mattil, 1970) and increases yields of protein extraction (Sze-Tao, & Sathe, 2000) due to reduction of disulphide bonds. Table I shows the yields of protein extraction of whole defatted flour (without previous removal of fiber by flotation/sedimentation) in the presence of different concentrations of alcalase. Increasing amounts of alcalase improved the extraction process both in terms of total matter extracted and protein yield. Addition of 0.1% (v/v) alcalase allows for extraction of 87.4% of the original proteins, as compared to 57.5% yield without alcalase. Also, the protein content of this extract, 60.8%, is higher. The improvement in protein extraction correlates with a higher degree of hydrolysis, which goes up to 22% with 0.1% alcalase (Table I).

Removal of the lignocellulosic fraction by sedimentation/flotation before addition of 0.1% alcalase led to a decrease in the yield of protein extraction from 87.4% to 55.5%. Thus, alcalase appears to facilitate extraction of insoluble proteins bound to fiber in the lignocellulosic fraction. Nevertheless, these extracts might not be adequate for preparation of protein isolates. Thus, when proteins were precipitated at their isoelectric point after extraction with 0.01% alcalase or in the absence of the microbial protease, the protein

content of the isolates differed significantly. Protein isolates from extracts obtained without alcalase had an 84.4% protein content, while isolates obtained using 0.01% alcalase only had 68% protein content. This is most likely due to a substantial amount of fiber being associated with and coprecipitating with proteins, as was previously described in the case of protein extracts from olive pomace (Vioque et al., 2000).

Protein extracts obtained using 0.1% alcalase had twice the concentration of proteins of the original flour. This enhances the possible value of these hydrolysates as a source of protein, because the same amount of protein could be provided with half of the material, as compared with extracts obtained without using alcalase. The cost of alcalase is not a limitation because considering how much alcalase costs, extraction/hydrolysis using alcalase is still cheaper than the traditional method. Thus, for the same amount of protein extracted, the cost of alcalase is below that of the amount of flour needed by the traditional methods to compensate for a lower yield. In addition, the cost of the flotation/sedimentation step and the cost of wastewater elimination have to be added to calculate the overall cost of the traditional method, which is not the case with the new method described here.

Solubility is a very important functional property of proteins, since it also determines to certain extent other functional properties such as foaming, emulsion and gelling (Zayas, 1997). Proteases are frequently used for increasing the solubility of proteins, particularly at the isoelectric point of the native proteins (Chobert et al., 1996, Chobert et al., 1988, Turgeon et al., 1992). This increase in solubility is due to the smaller size of peptides and higher hydrophilicity of the resulting hydrolysates, as a consequence of the higher number of free amino and carboxyl groups generated by breaking of peptidic bonds (Mahmoud, 1994). The use of

Table I  
Extracted matter, protein content, yield of protein extraction and degree of sunflower defatted meal using different concentrations of alcalase<sup>a</sup>

	<b>0% Alcalase</b>	<b>0.01% (v/v) Alcalase</b>	<b>0.05% (v/v) Alcalase</b>	<b>0.1% (v/v) Alcalase</b>
<b>Extracted Matter (g)</b>	3.33±0.1	3.32±0.2	4.34±0.4	4.41±0.6
<b>Protein Content (%)</b>	51.7±1.5	53.9±4.2	51.8±3.1	60.8±2.3
<b>Yield of protein extraction (%)<sup>b</sup></b>	57.5±2.7	59.8±3.6	76.0±4.0	87.4±4.9
<b>Degree of Hydrolysis</b>	0.0±0.0	14.7±0.5	16.9±1.8	22.0±2.3

<sup>a</sup> Values represent mean ± SD (n=3).

<sup>b</sup> Based on a sunflower defatted meal protein content of 30%.

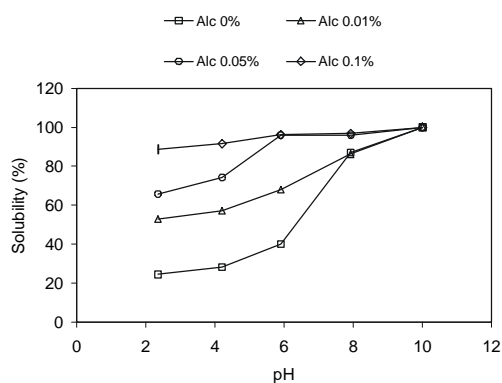


Figure 1  
Solubility in water at different pH values of protein extracts obtained by alkaline extraction in the presence of different concentrations of alcalase.

alcalase for preparation of sunflower protein extracts increased the solubility of the extracts drastically, as shown in figure 1. Thus, addition of 0.1% alcalase to the extraction medium caused an increase in solubility at acidic pH (pH between 2 and 4) from 20 to more than 80%.

The amino acid composition of the protein extracts obtained by treatment with different amounts of alcalase showed small variations that may be due

to differences in how efficiently different proteins are extracted (Table II). An increase in the amino acids glycine and proline, which are abundant in the proteins of the cell wall (Condit & Keller, 1990, Keller et al., 1988, Hong et al., 1990) was observed in the extracts obtained by treatment with alcalase. Thus, it appears that alcalase increases solubilization of proline-rich and glycine-rich proteins that are characteristic of the lignocellulosic fraction. On the other hand, the contents of the sulphur amino acids methionine and cysteine are relatively lower when alcalase is used. This is probably explained by the fact that sulphur amino acids are more abundant in albumins and other water soluble sunflower proteins than in the proteins associated with the lignocellulosic fraction (Baudet, & Mossé, 1977).

An additional advantage of direct alkaline extraction with alcalase as compared to the classic sedimentation/flotation procedure is the elimination of wastewaters which are produced when extracting defatted sunflower meal using traditional alkaline extraction. This represents additional costs of production in order to avoid a negative environmental impact (Borja et al., 2001). Wastewaters are generated by the sedimentation/flotation procedure because vacuum filtration or centrifugation needs to be carried out in order to separate the protein sediment from the soluble fraction, after mechanical removal of the fiber fraction that floats on top (see materials and methods).

Table II  
Amino acid composition (g/100 g protein) of sunflower protein extracts<sup>a</sup>

	0% (v/v) Alcalase	0.01% (v/v) Alcalase	0.05% (v/v) Alcalase	0.1% (v/v) Alcalase
Asp + Asn	8.4 ± 0.1	10.0 ± 0.2	9.6 ± 0.1	8.6 ± 0.2
Glu + Gln	24.3 ± 0.3	24.3 ± 0.4	22.8 ± 0.4	22.7 ± 0.5
Ser	5.5 ± 0.1	5.5 ± 0.1	5.5 ± 0.1	5.7 ± 0.1
His	2.6 ± 0.0	2.5 ± 0.0	2.4 ± 0.0	2.5 ± 0.0
Gly	6.8 ± 0.1	7.6 ± 0.2	7.5 ± 0.2	7.2 ± 0.1
Thr	4.0 ± 0.1	4.2 ± 0.1	4.5 ± 0.1	4.6 ± 0.1
Ala	4.5 ± 0.1	4.7 ± 0.1	5.0 ± 0.1	4.9 ± 0.1
Pro	3.6 ± 0.1	4.3 ± 0.1	4.5 ± 0.1	4.6 ± 0.1
Arg	10.3 ± 0.2	9.7 ± 0.1	9.4 ± 0.1	9.5 ± 0.2
Tyr	2.2 ± 0.0	2.2 ± 0.0	2.4 ± 0.0	2.3 ± 0.0
Val	4.2 ± 0.1	4.3 ± 0.1	4.5 ± 0.1	4.6 ± 0.1
Met	2.5 ± 0.0	0.3 ± 0.0	0.5 ± 0.0	1.3 ± 0.0
Cys	1.9 ± 0.0	1.3 ± 0.0	1.4 ± 0.0	1.6 ± 0.0
Ile	3.8 ± 0.1	3.7 ± 0.1	3.8 ± 0.1	3.7 ± 0.1
Leu	7.1 ± 0.1	7.1 ± 0.1	7.5 ± 0.2	7.4 ± 0.1
Phe	5.2 ± 0.1	5.0 ± 0.1	5.2 ± 0.1	5.0 ± 0.1
Lys	3.5 ± 0.1	3.3 ± 0.1	3.5 ± 0.0	3.8 ± 0.0

<sup>a</sup> Values represent mean ± SD (n=3).

In conclusion, the combined alkaline extraction/hydrolytic process is more efficient for protein extraction than the traditional method (flotation/sedimentation followed by alkaline extraction in the absence of alcalase) because there is no loss of protein into the lignocellulosic fraction. It also provides a final hydrolyzed extract that is more soluble in acidic solutions, and the overall process does not involve generation of polluting wastewaters. This increased solubility may be especially useful in liquid foods, to which hydrolysates can be added to improve their nutritional value. Considering all this, the potential application of this product may not be limited to livestock feeding, but it might also be used for the preparation of foodstuffs for human consumption.

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#### REFERENCES

- Adler-Nissen, J. (1979). Determination of the degree of hydrolysis of food protein hydrolysates by trinitrobenzenesulfonic acid. *J. Agric. Food Chem.*, **27**, 1256-1262.
- Alaiz, M., Navarro, J. L., Girón, J., and Vioque, E. (1992). Amino acid analysis by high-performance liquid chromatography after derivatization with diethyl ethoxymethylenemalonate. *J. Chromatogr.*, **591**, 181-186.
- Baudet, J., and Mossé, J. (1977). Fractionation of sunflower seed proteins. *J. Am. Oil Chem. Soc.*, **54**, 82-86.
- Bautista, J., Parrado, J., and Machado, A. (1990). Composition and fractionation of sunflower meal: use of the lignocellulosic fraction as fermentation source in solid-state fermentation. *Biol. Wastes*, **32**, 225-227.
- Borja, R., González, E., Raposo, F., Millán, F., and Martín, A. (2001). Performance evaluation of a mesophilic anaerobic fluidized-bed reactor treating wastewater derived from the production of proteins from extracted sunflower flour. *Biores. Technol.*, **76**, 45-52.
- Chobert, J.-M., Bertrand-Harb, C., and Nicolas, M.-G. (1988). Solubility and emulsifying properties of caseins and whey proteins modified enzymatically by trypsin. *J. Agric. Food Chem.*, **36**, 883-892.
- Chobert, J.M., Briand, L., Guéguen, J., Popineau, Y., Larré, C., and Haertlé, T. (1996). Recent advances in enzymatic modifications of food proteins for improving their functional properties. *Nahrung*, **40**, 177-182.
- Condit, C., and Keller, B. (1990) Glycine-Rich Cell Wall Proteins Of Dicotyledoneous Plants, in *Recognition And Assembly Of Plants And Animal Extracellular Matrix*, p. 119-135. W.S. Adair and R.R. Mecham (Eds.), Academic Press, New York, NY, USA.
- Dalev, P., Ljubomirova, A. and Ivanov, I. (1996). Enzyme extraction, functional properties and application of leaf protein fractions of nettle. *Biotechnol. Lett.*, **18**, 107-110.
- Gheyasuddin, S., Cater, C. M., and Mattil, K. F. (1970). Preparation of a colorless sunflower protein isolate. *Food Tech.*, **24**, 242-243.
- Hanmoungjai, P., Pyle, D. L., and Niranjana, K. (2001). Enzymatic process for extracting oil and protein from rice bran. *J. Am. Oil Chem. Soc.*, **78**, 817-821.
- Hong, J. C., Nagao, R. T., and Key, J. L. (1990). Characterization of a proline-rich cell wall protein gene family of soybean. A comparative analysis. *J. Biol. Chem.*, **265**, 2470-2475.
- Keller, B., Sauer, N., and Lamb, C. J. (1988). Glycine-rich cell wall proteins in bean: gene structure and association of the protein with the vascular system. *EMBO J.*, **12**, 3625-3633.
- Lühs, W., and Friedt, W. (1994). The Major Oil Crops in *Designer Oil Crops. Breeding, Processing and Biotechnology*, p. 5-58. D. J. Murphy (Ed.), VCH, Weinheim, Germany.
- Mahmoud, M. I. (1994). Physicochemical and functional properties of protein hydrolysates in nutritional products. *Food Technol.*, **48**, 89-95.
- Parrado, J., Bautista, J., and Machado, A. (1991). Production of soluble enzymatic protein hydrolysate from industrially defatted nondehulled sunflower meal. *J. Agric. Food Chem.*, **39**, 447-450.
- Pedroche, J., Vioque, J., Clemente, A., Bautista, J., and Millán, F. Improved protein extraction from *Brassica carinata* with the use of commercial proteases, *Jornada Internacional sobre Proteínas Alimentarias*, Sevilla, 1998.
- Saeed, M., and Cheryan, M. (1988). Sunflower protein concentrates and isolates low in polyphenols and phytate. *J. Food Sci.*, **53**, 1127-1131.
- Sales, A. M., Carvalho, A. C. V., Baldini, V. L. S., Goncalves, J. R., and Beraquet, N. J. (1991). Enzymic protein extraction from bone residues of mechanically separated chicken backs. *Coletanea do Instituto de Tecnologia de Alimentos*, **21**, 233-242.
- Sze-Tao, K. W. C., and Sathe, S. K. (2000). Functional properties and in vitro digestibility of almond (*Prunus dulcis* L.) protein isolate. *Food Chem.*, **69**, 153-160.
- Turgeon, S. L., Gauthier, S. F., and Paquin, P. (1992). Emulsifying property of whey peptide fractions as a function of pH and ionic strength. *J. Food Sci.*, **57**, 601-604.
- Villanueva, A., Vioque, J., Sánchez-Vioque, R., Clemente, A., Pedroche, J., Bautista, J., and Millán, F. (1999). Peptide characteristics of sunflower protein hydrolysates. *J. Am. Oil Chem. Soc.*, **76**, 1455-1460.
- Villanueva, A. (1997) Obtention of peptones with a defined chemical composition from sunflower protein concentrates, Ph.D. Thesis, Universidad de Sevilla.
- Vioque, J., Clemente, A., Pedroche, J., Yust, M. M., and Millán, F. (2001). Obtention and uses of protein hydrolysates. *Grasas y Aceites*, **52**, 132-136.
- Vioque, J., Clemente, A., Sánchez-Vioque, R., Pedroche, J., and Millán, F. (2000). Effect of Alcalase on olive pomace protein extraction. *J. Am. Oil Chem. Soc.*, **77**, 181-185.
- Zayas, J. F. (1997). *Functionality of Proteins in Food*, Springer-Verlag, Berlin, Germany.