Effect of Alcalase™ on Olive Pomace Protein Extraction

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ABSTRACT: The effect of Alcalase™ treatment on olive pomace protein extraction has been studied. Alcalase improves protein extraction from 5 to 30% of total protein. This improvement is not accompained by an increase in degree of protein hydrolysis, probably because protease activity is inhibited by secondary metabolites and the substrate is higly denatured and resistant to hydrolysis. The increase in protein extraction is attributed to fiber solubilization as a result of secondary activities of Alcalase. Protein extracts with a high content of soluble fiber have improved functional properties, with respect to olive pomace, such as water and oil absorption. Emulsifying and foaming activities were inappreciable. The products obtained represent a suitable source of soluble fiber and protein and may contribute to the improvement of the economic status of olive pomace by-product.

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KEY WORDS: Alcalase, fiber solubility, olive pomace, protein solubility.

Olive oil is a product of great economic importance in countries such as Spain, Italy, Greece, and Turkey. Olive pomace is a by-product of oil extraction. After residual oil (5–8%) is extracted, pomace, with a caloriphic power of 3,500 kcal/kg, is usually employed as fuel (1).

Recently, interest in plant by-products has been promoted by the use of their components and for the reduction of environmental problems associated with by-product elimination. For example, defatted sunflower and rapeseed meals have been used for the extraction of proteins and for obtaining protein isolates (2–4) or even high added-value protein hydrolysates (5–7). In this sense, olive pomace, favored by the high amount produced and low price, could represent a cheap source of dietary fiber or proteins for use in the food industry. In fact, it has been suggested that after pitting and increasing protein content to 15–18%, pomace could be used for animal feeding (1).

In a previous paper we studied the chemical composition of olive pomace (8). This by-product is characterized by the high-fiber content, around 70%, and low-protein amount, about 6%. In addition, the yield of protein extraction is low, around 6.9% of total proteins, mainly because these proteins are highly denatured and/or associated with the fiber and other components such as tannins (9,10). These results represent a drawback for the utilization of olive pomace as source of protein. Recently, we have observed that a limited treatment of *Brassica carinata* defatted meal with the protease Alcalase™ produces a significative increase in protein extraction (11). Similarly, to improve protein extraction in olive pomace, we treated this by-product with Alcalase. The results obtained are discussed and the characterization of the products are described.

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MATERIAL AND METHODS.

Raw material. Extracted olive pomace was purchased from the experimental plant of Instituto de la Grasa (Seville, Spain). Olive pomace was defatted with hexane for 12 h in a Soxhlet extractor, lyophilized, and stored in screw-capped bottles under nitrogen until use. Trinitrobenzenesulfonic acid (TNBS) was purchased from Sigma Chemical Co. (St. Louis, MO). Acrylamide and bisacrylamide were from Serva (Heidelberg, Germany). All other chemicals were of analytical grade.

Proteolytic enzyme. The enzymatic complex used was Alcalase 2.4 L (Novo Nordisk, Bagsvaerd, Denmark). Alcalase 2.4 L is a microbial protease of *Bacillus licheniformis* with endopeptidase activity. A main component of the commercial preparation is the serine protease subtilisin A. The specific activity of Alcalase 2.4 L is 2.4 Anson unit (AU) per gram. One AU is the amount of enzyme, which under standard conditions, digests hemoglobin at an initial rate that produces an amount of trichloroacetic acid-soluble product that gives the same color with the Folin reagent as 1 mequiv of tyrosine released per min.

Incubation of olive pomace with Alcalase. Olive pomace (10 g in 100 mL water) was incubated with Alcalase (0–100 mg) for 1 h at 50°C and pH 8. The extraction was conducted in a reaction vessel, equipped with a stirrer, thermometer, and pH electrode. After incubation, the enzyme was inactivated by heating at 80°C for 15 min. Solubilized proteins were recovered by centrifuging at 4,000 × *g* for 30 min. The supernatant was lyophilized. Extraction without Alcalase was carried out in the same way but omitting the enzyme from the procedure.

Precipitation of proteins. Extracted proteins in the supernatant (above) were precipitated by adjusting the pH to the isoelectric point (pH 2) with 0.5 N HCl and stirring at room temperature for 1 h. The samples were centrifuged at 4,000 × *g* for 30 min and the pellet was dried.

Fiber determination. Total, soluble, and insoluble fibers were determined according to the enzymatic–gravimetric method (12).

Total nitrogen determination. Nitrogen was determined by the micro-Kjeldahl method (13).

Determination of free amino groups. The number of free amino groups was determined by reaction with TNBS according to Adler-Nissen (14).

Gel-filtration chromatography. Gel filtration was carried out in a fast-protein liquid chromatography system equipped with a Superose 12 HR 10/30 column from Amersham Pharmacia (Uppsala, Sweden). Injection volume was 200 µL. The eluent was 20 mM phosphate buffer, 0.5 M sodium chloride buffer pH 8.3 at a flow rate of 0.4 mL/min. The molecular weights were determined with a calibration curve made with blue dextran 2000 (2,000 kDa), catalase (240 kDa), α-amylase (200 kDa), bovine serum albumin (67 kDa), ovoalbumin (43 kDa), carbonic anhydrase (29 kDa), and ribonuclease A (13.7 kDa) as molecular weight standards.

Electrophoresis. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli (15). The gel system, containing 0.2% (wt/vol) SDS, consisted of a 15% polyacrylamide resolving gel (pH 8.8) and a 3% stacking gel (pH 6.8). The lengths of the resolving and stacking gels were 10 and 2 cm, respectively, with a gel thickness of 0.75 mm. Electrophoresis was performed at a constant current of 25 mA. Protein bands were stained by immersion of the gels in a 0.05% (wt/vol) Coomassie brilliant blue G-250 solution, in 45% methanol and 9% acetic acid. Molecular weights were determined with the low molecular weight standard kit from Amersham Pharmacia.

Determination of functional properties. Water and oil absorption was determined by the method of Carcea-Bencini (16) with modifications. Protein (1 g) was stirred with 10 mL distilled water (pH 7) or corn oil and centrifuged at 2,200 × *g* for 30 min. The volume of the supernatant was measured. The water- or oil-holding capacity is expressed as the number of grams of water or oil held by 100 g of protein. Foam capacity and foam stability were measured by the method of Nath and Narasinga-Rao (17). Emulsifying activity was determined according to Naczk *et al.* (18).

RESULTS AND DISCUSSION

Extraction of olive pomace proteins. In an attempt to increase protein-extraction yield from olive pomace, a by-product highly denatured as a result of olive oil and olive pomace oil extraction, we have treated this material with the protease complex Alcalase.

Results show that the proportion of proteins extracted from olive pomace increased with the quantity of Alcalase used. The concentration of enzyme at 20 mg/100 mL (Fig. 1) was enough to reach a maximum of protein solubilization. Similarly, the amount of protein extracted increased with the time of enzymatic treatment, but after 1 h, there was no further increase in protein extraction (Fig. 2).

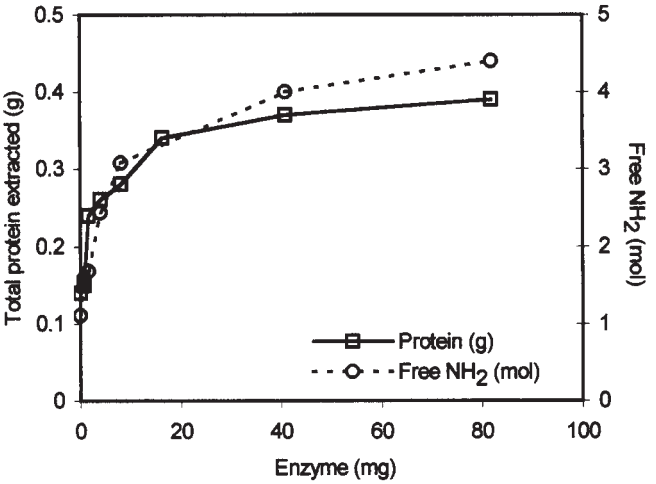

FIG. 1. Alcalase dependence of olive pomace protein extraction. Enzyme concentration was 0–100 mg/100 mL of reaction mixture. Olive pomace was incubated with Alcalase for 1 h at 50°C and pH 8.

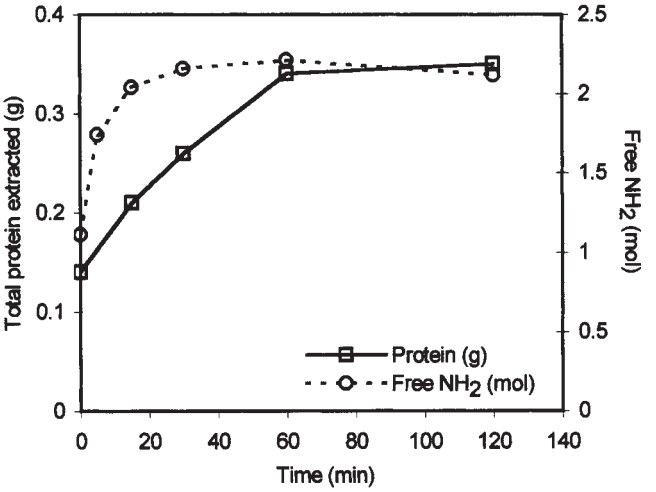

FIG. 2. Time course of olive pomace protein extraction with Alcalase, given as total protein extracted vs. time. Olive pomace was incubated with Alcalase (E/S: 5 mg/g) at 50°C and pH 8. E/S, enzyme/substrate.

Protein extraction depended also on the temperature. Thus, at 50°C without enzyme, the proportion of protein extracted was two times higher than that extracted at room temperature (Fig. 3). Temperature was also crucial on the yield of protein extraction with Alcalase. At 50°C, the optimal temperature for Alcalase, 30% of the proteins in olive pomace were extracted, six times more than without Alcalase at room temperature. Although Alcalase treatment solubilized 30% of the proteins from olive pomace, this extract still possessed a low-protein content (12.8%) (Table 1). The extract obtained without Alcalase had a lower protein content (2.8%), even below that observed in the original olive pomace. This suggests the presence of other compounds, such as fiber, strongly bound to proteins and also solubilized with them. Proteins extracted with Alcalase were precipitated at the isoelectric point of

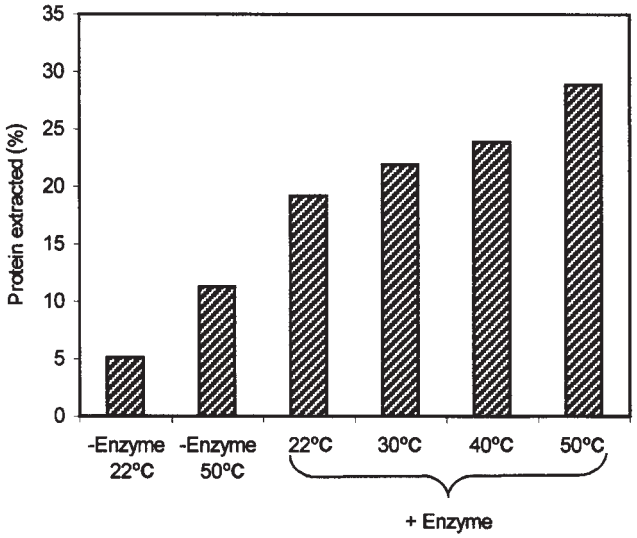

FIG. 3. Temperature dependence of olive pomace protein extraction. Olive pomace was incubated with Alcalase (E/S: 5 mg/g) for 1 h and pH 8. See Figure 2 for abbreviation.

olive pomace proteins (pH 2). The pellet obtained after centrifugation also had a low protein content (8.2%), supporting the hypothesis that other components, such as fiber, bound to proteins.

Characterization of the extracted proteins. Proteins extracted after Alcalase treatment are supposed to be partially hydrolyzed, but the ratio between the number of free amino groups and the amount of protein extracted was similar, independent of the proportion of enzyme used or time of incubation (Figs. 1,2). If there is protein hydrolysis, a net increase in the number of free amino groups would be expected as peptides of smaller size are generated.

To confirm these results, we have characterized the proteins extracted with and without Alcalase treatment. Gel filtration profile of each protein extract was very similar, with a main peak corresponding to a molecular weight of 30 kDa (Fig. 4). SDS-PAGE protein profiles of the protein extracts also were alike with a smear of proteins centered around 20 kDa (data not shown). The low resolution of SDS-PAGE was probably due to the presence of fiber interacting with the proteins. Another indication of the similar nature of both protein extracts was the solubility around the isoelectric point. One of the main consequences of protein hydrolysis is the increase in solubility around the isoelectric point, but in this case, both protein extracts show similar solubility at this pH, with 33.7% for the extract without Alcalase vs. 34.8% for the one obtained with the enzyme. Thus, all physicochemical data suggest that the incubation with Alcalase probably does not hydrolyze olive pomace proteins. In this sense, it has been reported that secondary metabolites, such as glucosinolates or phenols, are capable of reacting with protein side chains, changing physicochemical properties such as hydrophobicity, isoelectric point, and molecular weight of proteins, resulting in an alteration of protease activity and also substrate digestibility (19). In the case of olive pomace, in addition to these interactions, proteins are probably denatured to a large extent as a result of the process of olive oil and olive pomace oil extractions. All of these factors may result in the absence of protein hydrolysis.

If olive pomace proteins are not hydrolyzed, then the increase in solubility could be attributed to other factors. In this sense, Alcalase is a commercial protease, and although the protease subtilisin A is the main component, there are other peptides present in the Alcalase complex, as observed by SDS-PAGE (data not shown). In this sense, Alcalase may have other secondary activities, such as polysaccharide degradation that may have an influence on the solubility of fiber and favor the solubilization of proteins associated to this fiber.

Fiber and protein composition of olive pomace and protein extracts. Olive pomace and protein extracts are characterized by high fiber content, although fiber contents of protein extracts and precipitated proteins are below that observed in the original olive pomace meal. But the main difference in fiber composition between protein extracts and the original olive pomace is the increase in soluble fiber in the first with respect to olive pomace (Table 1). Thus, while soluble fiber is

TABLE 1
Fiber and Protein Composition of Olive Pomace and Protein Extracts[a]

	Olive pomace flour	Extraction without Acalase	Extraction with Alcalase	Precipitated proteins from Alcalase extract
Total fiber[b]	74.1 ± 6.2	42.5 ± 1.3	36.2 ± 2.2	38.4 ± 6.2
Soluble fiber[c]	10.2 ± 0.9	97.1 ± 0.5	98.3 ± 0.2	16.4 ± 0.9
Insoluble fiber[c]	89.8 ± 0.9	2.9 ± 0.5	1.7 ± 0.2	83.6 ± 0.9
Total protein[b]	6.0 ± 1.3	2.8 ± 0.2	12.8 ± 2.1	8.2 ± 1.4
Free protein[d]	31.0 ± 5.4	51.4 ± 2.7	46.5 ± 2.4	43.3 ± 3.7
Protein bounded to fiber[d]	69.0 ± 5.4	48.6 ± 2.7	53.5 ± 2.4	56.7 ± 3.7
Protein bounded to soluble fiber[e]	29.4 ± 1.9	99.1 ± 1.3	98.3 ± 2.5	15.0 ± 3.5
Protein bounded to insoluble fiber[e]	70.6 ± 1.9	0.9 ± 1.3	1.7 ± 2.5	85.0 ± 3.5

[a]Olive pomace (10 g/100 mL water) was extracted with Alcalase (50 mg) or without enzyme for 1 h at 50°C and pH 8.
[b]As percentage with respect to 100 g of dry matter.
[c]As percentage with respect to total fiber.
[d]As percentage with respect to total protein
[e]As percentage with respect to protein bound to fiber. $n = 3$.

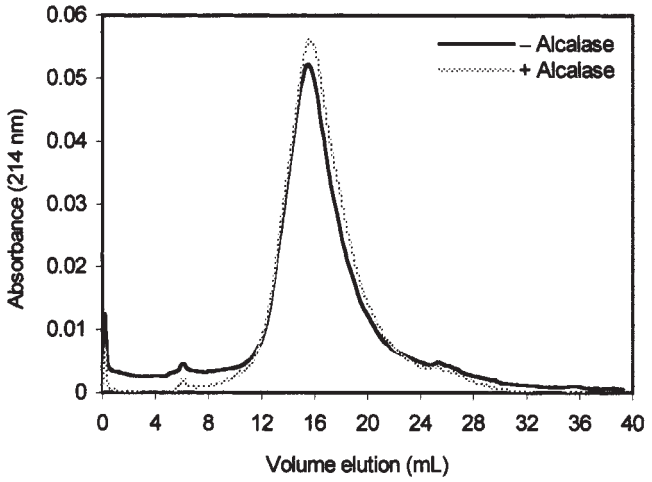

FIG. 4. Gel filtration protein profile of proteins extracted with (+) or without (–) Alcalase.

the minor fraction in olive pomace (10.2%), after protein extraction, almost all the fiber in these extracts is soluble (97.1–98.3%). In turn, precipitated proteins show a predominance of insoluble fiber. Precipitated proteins are characterized by their low solubility, and this is extended in the case of olive pomace to the fiber, giving another indication of the close relationship between protein and fiber in olive pomace. The protein extract obtained after Alcalase treatment has the highest protein content, 12.8%. In contrast, extracts obtained without Alcalase treatment have a protein content even below the original olive pomace.

In both protein extracts, an increase in free protein with respect to olive pomace proteins is observed. Thus, it seems that the process of protein extraction favors solubilization of fiber and, as a consequence, liberalization of proteins that were probably interacting with this fiber in the original olive pomace meal. These results suggest the use of cell-wall degradative enzymes better improve protein recovery in materials such as olive pomace with a high amount of fiber.

As observed in Table 1, the relative fiber and protein composition of extracts without and with Alcalase treatment is similar. The significant difference is in the protein content and yield of protein extraction that is improved when Alcalase is first used.

Functional properties. In exploring and developing new sources of additives or foods, functional properties are a main factor in determining their potential utilization.

Functional properties are related to the chemical composition of the material studied. This is apparent in the case of olive pomace and protein extracts. Thus, water absorption values were low in the olive pomace meal and precipitated proteins, in accordance with their low content in soluble fiber (Table 2). On the other hand, both protein extracts show a very high water absorption rate, *ca*. 700%, probably due to the high soluble-fiber content.

The differences in fat absorption were not that drastic, although both extracts showed higher fat absorption than the olive pomace flour. Precipitated proteins have a similar fat absorption to that observed in both extracts. This high oil absorption may also be related to fiber, but in addition to the nature of proteins present in these materials. Oleosins, the protein components of plant oil bodies, predominate in this protein fraction. Oleosins are small (15–25 kDa) amphipathic proteins that form a monolayer completely encircling the surface of storage oil bodies (20). Thus, in olive pomace extracts, the increase in protein content and percentage of free proteins, mainly oleosins, may enhance oil absorption.

TABLE 2
Functional Properties of Olive Pomace Flour and Protein Extracts

	Water absorption[a]	Fat absorption[a]
Olive pomace flour	128.3 ± 6.5	212.2 ± 52.8
Extraction without Alcalase	699.3 ± 1.0	515.9 ± 52.7
Extraction with Alcalase	683.7 ± 1.6	427.3 ± 70.8
Precipitated proteins	153.1 ± 23.8	507.3 ± 74.8

[a]Expressed as g/100 g of dry matter, $n = 3$.

No emulsion or foam was obtained, probably because of the low protein content of the material studied.

Thus, olive pomace protein extracts could be used as additives in food systems where a high water or oil absorption is required.

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