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Alcalase Rapeseed Inhibitors: Purification and Partial Characterization

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Extensive rapeseed protein hydrolysate obtained sequentially with Alcalase and Flavourzyme showed inhibitory activity towards Alcalase. Inhibitory activity decreased as the hydrolytic process progressed probably by heat denaturation and/or partial protease degradation.

Alcalase rapeseed inhibitors were purified by gel filtration and subsequent ion exchange chromatography. They are composed of peptides of 8.4 and 6.1 kDa linked by interchain disulphide bonds, as observed by reducing SDS-PAGE, with a native molecular weight of 18 kDa. Aminoacid composition of the inhibitors was characterized by the high proportion of methionine (4.2%) and cysteine (4.6%). Alcalase inhibitors were partially resistant to heat treatment; after heating at 70 °C for 45 minutes more than 50% of the original inhibitory activity remained in the purified protein but after heating at 90 °C for 5 minutes, inhibitory activity decreased very fast to a basal level. The possible relation of these protease inhibitors with the 2S albumin storage proteins is discussed.

Keywords: Alcalase, Rapeseed, Protease inhibitors

INTRODUCTION

Oil crops are important sources of energy, both for human consumption and feeding livestock. They are also sources for many non-edible purposes, providing raw material for a wide range of industrial products. In this sense, oilseeds are becoming of increasing interest as a source of edible proteins.[1] In particular the cultivation of oilseed rape has increased tremendously during the last decades due to rapid progress in rapeseed breeding.[2]

Rapeseed is rich in protein (30%–45%) and hence defatted rapeseed meal may constitute a good source of proteins for humans.[3] Amino acid composition is well balanced in regard to FAO requirements and is rich in sulphur-containing amino acids and lysine, generally below the recomendations of the FAO in legumes and cereals, respectively.[4]

Protein isolates obtained from defatted rapeseed meal can be used for the fortification of foods[5–7] but may also represent an optimal substrate for obtaining protein hydrolysates.[8,9]

Enzymatic modification of proteins of plant and animal origin play an ever increasing role in food and other industries. Hydrolysates which are produced enzymatically are used in

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many areas in the food industry and cosmetics and for this purpose a great variety of different enzymes are used.[10]

The rate of enzymatic hydrolysis will depend on physico-chemical factors, such as temperature or pH and of biochemical elements, such as protease activity or the presence of peptides resistant to hydrolysis or protease inhibitors.

Protease inhibitors have been the target of extensive research in many scientific disciplines. Most of these studies are centred around inhibitors of digestive proteases such as trypsin or chymotrypsin[11] and, in this sense, studies of inhibitors of commercial proteases are limited. In order to study the influence of these inhibitors on the efficiency of enzymatic protein hydrolysis, we have purified and characterized rapeseed inhibitors of alcalase protease.

MATERIAL AND METHODS

Materials

Rapeseed meal (*Brassica campestris* L.), industrially produced by Koipesol (Sevilla, Spain) and obtained by solvent extraction, was used as a protein source. Standards for electrophoresis and gel filtration, Superose 12 HR 10/30 and Mono Q HR 5/5 column were from Amersham Pharmacia (Uppsala, Sweden). Acrylamide, N,N′-methylenebisacrylamide and Coomasie Brilliant Blue G-250 was from Serva (Heidelberg, Germany). Diethylethoxymethylenemalonate was supplied by Fluka (Buchs, Switzerland). Sodium dodecyl sulfate, casein, trichloroacetic acid, trinitrobenzenesulfonic acid (TNBS), D L-α-aminobutyric acid, were purchased from Sigma Co. (St. Louis, MO). All other chemicals were of analytical grade.

The enzymatic complexes used were Alcalase 2.4 L and Flavourzyme 1000 MG 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 was the serine protease subtilisin A. The specific activity of Alcalase 2.4 L was 2.4 Anson Unit (AU) per gram. One AU is the amount of enzyme, which under standard conditions, digests haemoglobin at an initial rate that produces an amount of trichloroacetic acid-soluble product which gives the same colour with Folin's reagent as one milliequivalent of tyrosine released per minute. Flavourzyme 1000 MG is a protease complex of *Aspergillus oryzae* that contains both endo- and exo-protease activities. It has an activity of 1.0 Leucine aminopeptidase unit (LAPU)/g. One LAPU is the amount of enzyme which hydrolyzes 1 µmole of leucine-*p*-nitroanilide per minute.

Rapeseed Protein Hydrolysis

The protein isolate was hydrolyzed batchwise by sequential treatment with Alcalase and Flavourzyme in a pH-stat. Alcalase was added first, and after 60 min Flavourzyme was added to the reactor and incubated for 2 h. The hydrolysis curve was obtained using the following hydrolysis parameters: substrate concentration, 5% (p:v in water); enzyme/substrate ratio, 0.3 AU/g for Alcalase and 50 LAPU/g for Flavourzyme; pH 8 for Alcalase and 7 for Flavourzyme; temperature, 50 °C. The hydrolysis was conducted in a reaction vessel, equipped with a stirrer, thermometer and pH electrode.

The degree of hydrolysis, defined as the percentage of peptide bonds cleaved, was calculated by the determination of free amino groups by reaction with TNBS according to Adler-Nissen.[12]

Purification of Alcalase Inhibitors

Rapeseed protein isolates, obtained as previously described[7] were used for the purification of inhibitors. Protein isolate was loaded on a Superose 12 HR 10/30 gel filtration column with an injection volume of 200 µl. The eluent was 20 mM phosphate, 0.5 M sodium chloride buffer

pH 8.3 at a flow rate of 0.4 ml/min. Fractions where the inhibitory activity was highest were pooled and concentrated. Inhibitors were finally purified by ion-exchange chromatography on a Mono Q HR 5/5 column, which has been previously equilibrated with 0.05 M Tris-HCl, pH 8.0 buffer. After loading the sample, the column was washed with 20 ml of the above mentioned buffer and the proteins were eluted with a 30 ml linear gradient of 0.0–0.5 M NaCl and maintained at 0.5 M NaCl for another 30 ml with a flow rate of 0.5 ml/min. The column effluent was monitored at 280 nm and 1 ml fractions were collected. Column fractions with highest inhibitory activity were pooled and concentrated by lyophilization. This preparation of Alcalase inhibitors was used for all further studies. Protein concentrations were determined by the method of Bradford.[13] Bovine serum albumin was used as standard.

Electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli.[14] Protein samples (2 mg/mL) were mixed 1:1 (v/v) with a solubilization buffer of 10 mM Tris, pH 6.8, 20% glycerol, 2.5% SDS, 0.005% bromophenolblue and 10% β-mercaptoethanol in the case of reducing conditions or without β-mercaptoethanol when using non-reducing conditions. The gel system, containing 0.2% (w:v) SDS consisted of a 22% polyacrylamide resolving gel (pH 8.8) and a 3% stacking gel (pH 6.8). The length of the resolving and stacking gels were 10 and 2 cm, respectively, with a gel thickness of 0.75 mm. Protein bands were stained by immersion of the gels in a 0.05% (w:v) Coomassie brilliant blue G-250 solution, in 45% methanol and 9% acetic acid solution.

Amino Acids Analysis

Samples (10 mg) were hydrolysed with 4 ml of 6 N HCl. The solutions were sealed in hydrolysis tubes under nitrogen and incubated in an oven at 110 °C for 24 h. Amino acids were determined in the acid hydrolysis after derivatisation with diethylethoxymethylenemalonate by reverse phase-high performance liquid chromatography, according to the method of Alaiz *et al.*[15] with D,L-α-aminobutyric acid as internal standard.

Protease Inhibitor Activity

Inhibitory activity was determined by assaying Alcalase activity in the presence and absence of inhibitory extract, with casein as substrate for the protease as described by Batra *et al.*[16] with modifications. Before addition of the substrate, the mixture was incubated for 30 min to allow binding of the inhibitors to the proteases. Casein was then added and the reaction was incubated for 10 min at 37 °C. A blank set was prepared by adding trichloroacetic acid before the addition of the protease solution. A control set was also prepared in the absence of the inhibitor. The reaction was stopped by addition of 1 volume of 5% trichloroacetic acid. The samples were filtered through Whatman No. 1 filter paper and the released tyrosine was determined in an aliquot of the filtrate according to Lowry *et al.*[17] Percentage of inhibition was calculated by comparing the reduction in enzyme activity (tyrosine release) in the presence of the inhibitor with that given by the same enzyme concentration in the absence of the inhibitor. For assay of thermal stability, protease inhibitor samples were heated at 70 °C or 90 °C for the time expressed.

RESULTS AND DISCUSSION

Alcalase Rapeseed Inhibitory Activity during the Hydrolytic Process

Protein isolate used for the generation of hydrolysates was obtained by alkaline extraction and

acid precipitation of proteins as previously described.[7] This protein isolate was hydrolyzed batchwise in a pH-stat using Alcalase for 60 minutes and Flavourzyme for another 120 minutes sequentially. By starting the hydrolysis with Alcalase, predigestion is achieved, increasing the number of N-terminal sites for the action of the exopeptidase Flavourzyme (Figure 1).

Physico-chemical and biochemical factors may affect the protein hydrolysis rate. Among the latter are the presence of protease inhibitors. These compounds are resistant to hydrolysis and may remain in the protein hydrolysate after the hydrolytic process has finished.

Figure 2 shows the residual activity of Alcalase inhibitors, present in protein hydrolysates, after 60 min and 180 min of hydrolysis. After 180 min of hydrolysis, inhibitory activity is lower than at 60 min, probably because of partial degradation of Alcalase inhibitors by heat denaturation, since the hydrolysis is conducted at 50 °C, or/and Flavourzyme activity. When the activity of Alcalase inhibitors was assayed at different times during the process of hydrolysis, the rate of inhibition slightly increased with respect to the original protein isolate in the initial stages (Figure 3). Probably the reduction in the amount of substrate for Alcalase favoured the interaction of inhibitors with the protease.

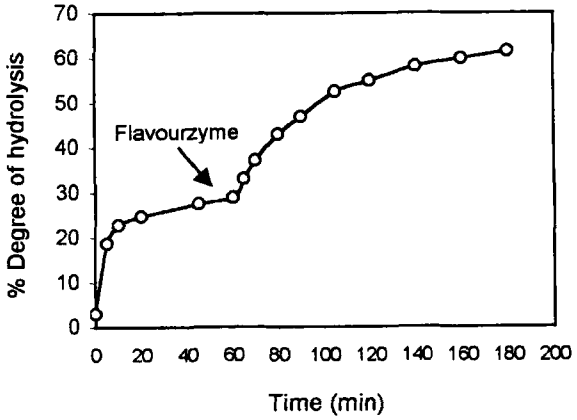

FIGURE 1 Enzymatic hydrolysis of rapeseed protein isolate with Alcalase and Flavourzyme (added after 60 min).

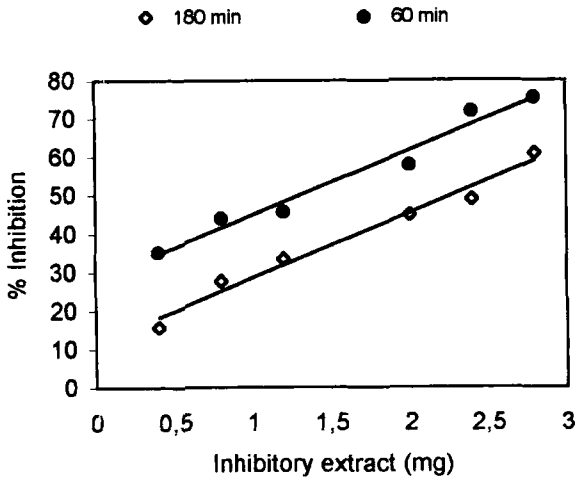

FIGURE 2 Rate of inhibition of Alcalase by rapeseed protein hydrolysates obtained after 60 min and 180 min hydrolysis time.

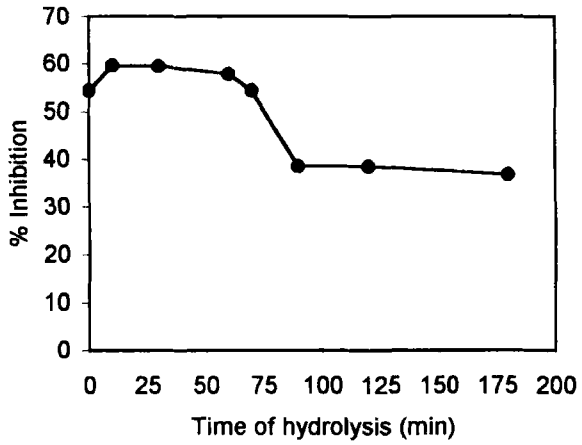

FIGURE 3 Degree of inhibition of Alcalase by hydrolysate extracts obtained at different times.

A gradual decrease in the inhibitory activity was then observed and this was attributed to heat denaturation of inhibitors. Finally, when Flavourzyme was added, the inhibitory activity clearly dropped further probably because of partial degradation of Alcalase inhibitors by Flavourzyme. Thus, at the end of the hydrolytic process the inhibitory activity was about 60% of that observed in the protein isolate.

Purification of Alcalase Rapeseed Inhibitors

Rapeseed protein isolate was used as starting material for the purification of Alcalase inhibitors. Proteins were applied to a Superose 12 HR 10/30 gel filtration column. Figure 4 shows the gel filtration profile of the protein isolate and the inhibitory activity in the different fractions collected. The main inhibitory activity appeared after the maximun of protein absorbance and corresponded to a protein molecular weight of 18 kDa. Fractions where inhibitory activity was highest were pooled (from 14 to 16 ml). These fractions were concentrated and loaded onto a Mono Q HR 5/5 anion exchange column (Figure 5). Under the assay conditions most of the inhibitory activity appeared in proteins not retained by the column and eluting as a single peak in the flow through. SDS-PAGE in reducing conditions (with β-mercaptoethanol) with proteins not retained by the column showed two bands corresponding to 8.4 and 5.1 kDa molecular weights (Figure 6A). Alternatively, SDS-PAGE in non-reducing conditions (without β-mercaptoethanol) (Figure 6B) showed a single band corresponding to 17 kDa molecular weight similar to that deduced from the gel filtration elution volume (18 kDa) (data not shown). Thus, these results suggests that Alcalase rapeseed inhibitors are

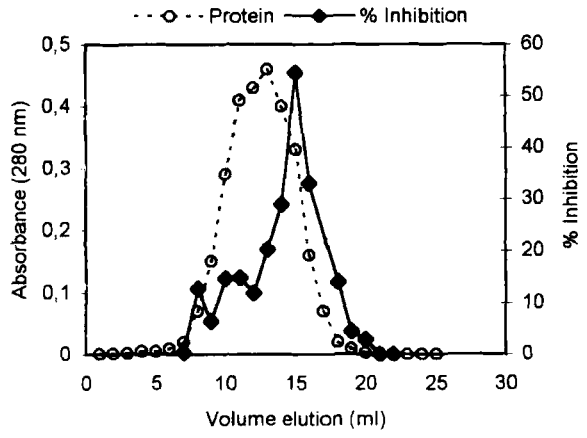

FIGURE 4 Gel filtration elution profile of rapeseed protein isolate and activity of Alcalase inhibitors on a Superose 12 HR 10/30 column.

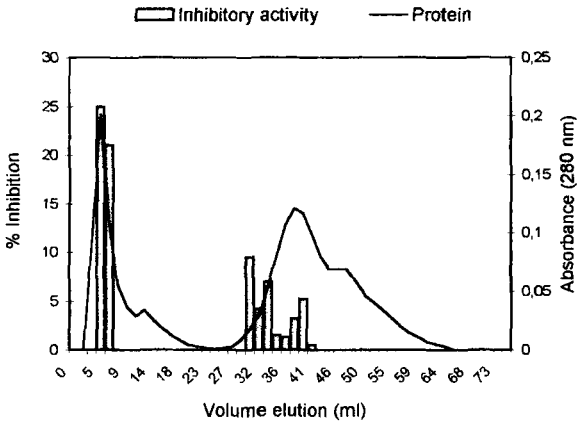

FIGURE 5 Mono Q elution profile of pooled fractions collected from the gel filtration step that contain inhibitory activity.

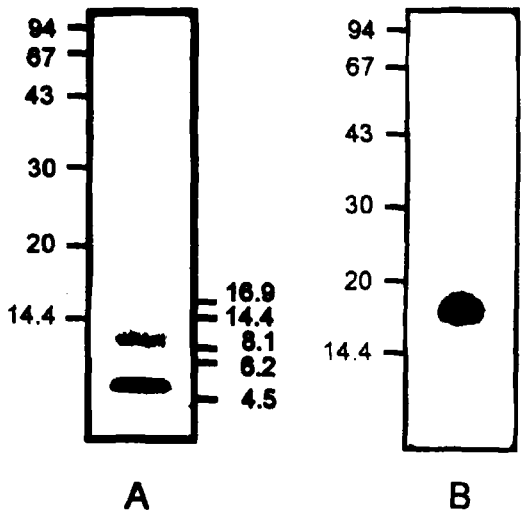

FIGURE 6 (A) SDS-PAGE in reducing conditions (with β-mercaptoethanol) with purified Alcalase inhibitors. Molecular weight markers are indicated on the left and right. (B) SDS-PAGE in non-reducing conditions (without β-mercaptoethanol) with purified Alcalase inhibitors. Molecular weight markers are indicated on the left.

composed of subunits linked by interchain disulphide bonds. Similar low molecular weight protease inhibitors have been purified from other sources such as *B. napus*,[18] chickpea[19] or pea.[20] This type of protease inhibitor possesses a similar structure to that of 2S albumin storage proteins. In fact, sequence similarities have been

established between inhibitors and 2S storage protein in legumes.[21,22] Moreover, inhibitory activity has been described in 2S albumins from several sources such as napin from *B. napus*[23] or chickpea PA1.[24]

Amino Acid Composition

Alcalase rapeseed inhibitors have a high content of sulfur-containing amino acids in regard to the rapeseed protein isolate (Table I). In this sense, the cysteine content of rapeseed inhibitors is more than three times higher than in protein isolates, while the methionine content is more than two times higher. Thus, the total amount of sulfur amino acids in rapeseed inhibitors is 8.8% in contrast to 3.3% in the protein isolate. The high proportion of glutamic acid (28.5%) is also noticeable, although this amino acid is also the main component of the protein isolate, representing 20.6% of the total. It should be noted that a common characteristic of protease inhibitors is their high sulfur amino acid contents and hence the presence of disulfide linkages that appears to protect them against enzymatic proteolysis.[11]

TABLE I Amino acid composition of rapeseed protein isolate and Alcalase inhibitors. Data expressed as g/100 g protein are the mean ± sd of two analyses

	Protein isolate	Alcalase inhibitors
Aspartic acid[a]	10.2 ± 0.2	6.9 ± 0.1
Glutamic acid[b]	20.6 ± 0.3	28.5 ± 0.2
Serine	7.2 ± 0.1	7.0 ± 0.2
Histidine	2.6 ± 0.1	3.6 ± 0.2
Glycine	10.3 ± 0.1	6.5 ± 0.3
Threonine	4.7 ± 0.2	4.0 ± 0.4
Arginine	6.2 ± 0.2	7.3 ± 0.1
Alanine	7.1 ± 0.3	4.2 ± 0.1
Tyrosine	2.1 ± 0.1	1.4 ± 0.1
Valine	5.1 ± 0.1	2.4 ± 0.1
Methionine	2.0 ± 0.2	4.2 ± 0.1
Cysteine	1.3 ± 0.1	4.6 ± 0.1
Isoleucine	3.8 ± 0.2	3.4 ± 0.2
Leucine	8.2 ± 0.2	6.2 ± 0.2
Phenylalanine	4.5 ± 0.1	3.8 ± 0.3
Lysine	4.1 ± 0.1	6.0 ± 0.1

[a]Aspartic acid + asparagine. [b]Glutamic acid + glutamine.

Resistance of Protease Inhibitors to Heat Treatment

A characteristic of a large number of protein enzyme inhibitors is their resistance to denaturation or precipitation by heating.[11] This can be explained as being due to a compact and rigid protein structure and by their relatively high content of disulphide bridges.

Alcalase rapeseed inhibitors were relatively resistant to heat. When rapeseed inhibitors were heated at 70 °C the inhibitory activity decreased with time of treatment; after 45 min at 70 °C more than 50% of the original inhibitory activity remained in the purified protein (Figure 7) but when proteins were heated at 90 °C, inhibitory activity decreased very fast to a basal level around 2% inhibition, even with only 5 minutes treatment (Figure 7). Similar thermal stability has been reported for other serine protease inhibitors, such as the trypsin inhibitors from the legume winged bean.[25] The fact that these protease inhibitors are heat labile at 90 °C favor the use of temperature to heat-inactive proteases at the end of the hydrolytic process, since this treatment will also destroy remaining inhibitory activity that may affect *in vivo* digestibility of the hydrolysate.

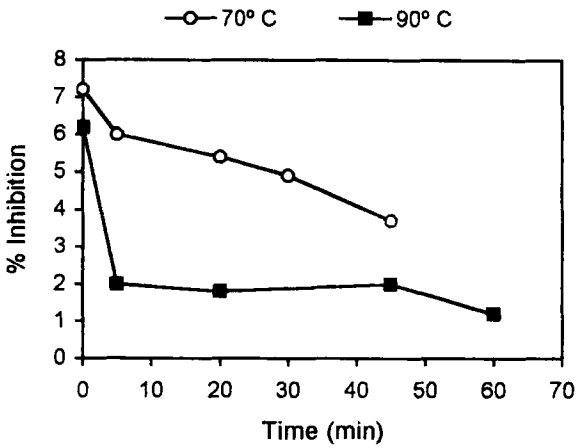

FIGURE 7 Residual activity of Alcalase inhibitors after heat treatment at 70 °C and 90 °C during different times.

In conclusion, Alcalase rapeseed inhibitors are characterized by their low molecular mass, high content of sulfur-containing amino acids and partial resistance to heat denaturation. These characteristics are also observed in other protease inhibitors purified from similar crops. Thus, an inhibitor of subtilisin purified from *B. nigra*[26] with a molecular weight of 15.5 kDa was also thermostable as deduced from the circular dichroism analysis.

Acknowledgements

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