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PII: S0308-8146(97)00037-X

Food Chemistry, Vol. 60, No. 4, pp. 587-592, 1997

0308-8146/97 $17.00+0.00

Survey of enzyme activities in desaponified quinoa *Chenopodium quinoa* Willd.

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(Received 2 August 1996; revised version received 20 December 1966; accepted 20 December 1996)

Quinoa seed, previously desaponified, was assayed for activities of amylase, cellulase, polygalacturonase, invertase, phenolase, alkaline and acid phosphatases, catalase, peroxidase, superoxide dismutase, protease and lipoxygenase. Polygalacturonase, invertase and lipoxygenase activities were not detected. All other enzymes were found to be active in quinoa seed extracts. Protease activity was found with haemoglobin as the substrate, but not with casein. © 1997 Elsevier Science Ltd

INTRODUCTION

Quinoa (*Chenopodium quinoa* Willd.) is a food plant native to the Andean region of S. America. The seed and leaves are used as food. The seed has latterly attracted interest as a novel food in western countries, due to its high nutritional value in terms of protein, lipids, carbohydrates, vitamins, minerals and fibre (Coulter & Lorenz, 1990; Koziol, 1992; Ruales & Nair, 1993). Use of quinoa as a food source is inhibited by the presence of saponins, anti-nutritional compounds with a bitter taste, which can be reduced to acceptable levels by washing and drying the seed or by abrasion of the seed coat (Ridout *et al.*, 1991; Becker & Hanners, 1990).

At present, quinoa consumption is limited to the health food market in the U.K. Potential food applications will require knowledge of the functional properties of the grain and its protein, starch and lipid components. This includes the activities of various enzymes which may limit or enhance its use as a food raw material. For instance, the level of amylase activity in quinoa flour would affect the properties of a wheat flour dough in which it was included. Currently, potential applications of quinoa that have been proposed include an infant weaning food (Ruales & Nair, 1993), non-gluten biscuits for specialist diets (Kuhn *et al.*, 1994) and supplementation of wheat flour (Chauhan *et al.*, 1992).

Quinoa protein content, composition and nutritional value have been well reported, but there are relatively few studies on enzyme activities in quinoa (Lorenz & Nyanzi, 1989; Atwell *et al.*, 1988). This paper seeks to quantify, in desaponified quinoa, the activities of enzymes which are potentially useful or detrimental to food applications.

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MATERIALS AND METHODS

Materials

Quinoa seeds of unspecified variety were from Direct Foods, Barrow-upon-Soar, U.K and were used without further treatment. The quinoa seeds were desaponified in their country of origin (Ecuador), verified by soaking for 24 h in distilled water with no evidence of foam production.

Chemicals were from BDH, Poole, Dorset, U.K. and xanthine oxidase (X4376, 0.4–1.0 units mg^{-1} protein) was from Sigma Chemical Co., Poole, Dorset, U.K.

Enzyme extraction

Seeds were ground in a hammer mill to pass a 0.5 mm mesh. The flour was stirred for 30 min with phosphate buffer (0.1 M, pH 7), unless otherwise stated, in the ratio 1 g:10 ml. The extracting buffer was chosen to optimise activity of the enzyme of interest. Following centrifugation for 10 min at 13 400×*g* the supernatant was filtered through a 0.45 μm filter (type HA, Millipore) to obtain a clear extract. Boiled extract for enzyme assay controls was obtained by heating at 98°C for 5 min then centrifuging as above for 5 min.

Enzyme assays

Carbohydrate hydrolytic activities, namely amylase, cellulase, invertase and polygalacturonase, were followed by spectrophotometric determination of reducing sugar products, by reaction with dinitrosalicylic acid (DNSA). DNSA reagent (Bernfield, 1955) was DNSA 1% w/v, sodium potassium tartrate, 30% w/v in NaOH 0.4 M.

Extract for β-amylase assay was made in 0.1M acetate buffer, pH 5.4, and diluted 1 in 50 before assay. The substrate was soluble starch, 1% w/v, in distilled water. For cellulase, extract was made in 0.1M phosphate buffer, pH7, and the substrate was carboxymethylcellulose (CMC), 1% w/v suspended in distilled water. For invertase, extract was made in 0.1M phosphate buffer, pH 6.0, and the extract diluted 1 in 10. The substrate was sucrose 1% w/v in distilled water. For polygalacturonase assay, the substrates were 0.5% w/v polygalacturonic acid (PGA), 2mM sodium hydroxysulphite in 0.1M acetate buffer, pH 5.0, (Riov, 1975) and, subsequently, pectin (7% methylated) in place of PGA above. For assay of carbohydrate hydrolases extract (1 ml) and substrate (1 ml) were incubated at 37°C. DNSA reagent (2 ml) was added to stop the reaction, the mixture was heated for 5 min in a boiling water bath, distilled water (6 ml) was added and A_{530} read in a Shimadzu UV160A spectrophotometer.

Standard curves were prepared by substituting appropriate reducing sugar solutions (2 ml) for enzyme extract and substrate solution in the assay above: for β-amylase using maltose, for cellulase and invertase using glucose, and for polygalacturonase using galacturonic acid. Activity was expressed as mg of reducing sugar produced per minute per ml of extract ($mg\,min^{-1}ml^{-1}$).

Acid phosphatase and alkaline phosphatase activities were followed by hydrolysis of p-nitrophenylphosphate giving increase in absorbance at 405 nm. The extracting and substrate buffer for acid phosphatase was 0.1M acetate, pH 5.4, and for alkaline phosphatase was 0.1M phosphate, pH 7, or 0.1M carbonate, pH 10. The substrate buffer for acid phosphatase activity was 0.1M acetate, pH 5.4, and alkaline phosphate activity was 0.1M carbonate, pH 10. Substrate concentration was 0.15% w/v. In a 3 ml cuvette, extract (1.5 ml), and substrate solution (1.5 ml) were mixed and the increase in absorbance at 405 nm was followed. Enzyme activity was expressed as μmoles of p-nitrophenoxide produced per min per ml extract, using $\varepsilon = 18.3 \times 10^3 M^{-1}cm^{-1}$.

Protease activity was followed by measurement of protein substrate that was soluble in the presence of trichloroacetic acid (TCA) after incubation with the extract. The extracting buffer was acetate, pH 5, 0.2M. Substrates were casein, haemoglobin or gelatin. Casein was heated in pH 7.6, 0.5M phosphate buffer at 98°C for 10 min, then cooled. Haemoglobin or gelatin were dissolved in pH 5.0, 0.1M acetate buffer. Extract (1 ml), or acetate buffer, was incubated with substrate (2 ml of 1% w/v solution) at 37°C. Reaction was stopped by addition of TCA solution (14% w/v), 2 ml for haemoglobin or gelatin substrates, 3 ml for casein substrate, assay was mixed and stood for 1 h, then filtered. Filtrate (1 ml) was assayed by the Lowry method (Lowry *et al.*, 1951), reading absorbance at 700 nm. The standard curve was produced with tyrosine in 0.6mM HCl. Protease activity was expressed as μg tyrosine produced in assay mixture per min per ml of extract.

Phenolase activity was determined spectrophotometrically by the production of quinone giving an increase in absorbance at 430 nm. The extracting buffer was 0.1M phosphate, pH 6.0. The substrate was 75 mM catechol in distilled water. Extract (0.4 ml), buffer (1.2 ml), and substrate solution (1.4 ml) were mixed in a cuvette and A_{430} followed.

Oxidative enzymes

Catalase activity was followed by a decrease in H_2O_2 concentration, determined by reduction of dichromate giving increase in absorbance at 570 nm (Sinha, 1972). The extract was made with 0.01M phosphate buffer, pH 7. The substrate solution was 0.7% v/v H_2O_2, and colour reagent was 1.25% w/v potassium dichromate in 75% w/v ethanoic acid. Extract (1 ml), 0.1M phosphate buffer, pH 7 (5 ml), and substrate solution (5 ml), were mixed. At intervals, 1 ml of reaction mixture was taken and added to colour reagent (2 ml), heated to 100°C for 10 min and A_{570} read. Catalase activity was expressed as mmol H_2O_2 consumed $min^{-1}ml^{-1}$ extract.

Peroxidase activity was followed by the increase in A_{430} due to formation of purpurogallin from pyrogallol. The extract was made with 0.1M phosphate buffer, pH 7. Extract, 1.5 ml of 1 in 10 dilution, was added to 0.5% v/v H_2O_2 (0.5 ml), and 0.5% w/v pyrogallol (1 ml). Interference from phenolase activity was determined by increase in A_{430} when H_2O_2 was omitted. Peroxidase activity was expressed as $\Delta A_{430} min^{-1}ml^{-1}$ extract.

Superoxide dismutase activity was assayed by the indirect method of McCord and Fridovich (1969), where SOD inhibits the rate of reduction of cytochrome c^{3+} by the superoxide anion, O_2^-.

Extract of quinoa in 0.1M phosphate buffer, pH 7.7, (0.1 ml), was added to 2.8 ml of reagent containing xanthine, 0.76 mg $100\,ml^{-1}$, ferricytochrome c, 12.4 mg $100\,ml^{-1}$ and EDTA 3.4 mg $100\,ml^{-1}$ in 0.05M phosphate buffer pH 7.7. The reaction was initiated by the addition of xanthine oxidase, 0.5% w/v in reagent buffer (0.1 ml), and followed by increase in absorbance at 550 nm. Indirect SOD assays measure the effect of SOD on the steady state concentration of O_2^- which reduces the detector compound. Hence, SOD will cause reduction of ferricytochrome c to be inhibited to some degree. SOD activity is expressed as a percentage of the rate of the reduction when SOD is absent, or '% inhibition'. One unit of SOD is the amount causing 50% inhibition (Beyer & Fridovich, 1987).

Lipoxygenase was assayed by the method of Surrey (1964). Quinoa extract, 1 ml, was added to substrate reagent, 19 ml, consisting of linoleic acid, 0.25% v/v, Tween 20, 0.25% v/v, and NaOH, 0.5 mM, in borate buffer, pH 9 0.0125M. Aliquots of 1 ml were removed at intervals and the reaction stopped with ethanol, 2 ml, 7 ml of 60% ethanol was added and A_{234} determined, against a spectrophotometer blank of substrate only. A control was made using boiled extract.

RESULTS AND DISCUSSION

Amylase

Four separate extracts of quinoa were assayed in duplicate for amylase activity and the results are shown in Fig. 1 and Table 1. The rates of maltose production were linear up to 30 min and show substantial amylase activity. A control made with boiled extract showed no increase in maltose concentration over 30 min. The average activity of the four extracts was 2.29 mg maltose min^{-1} ml^{-1} extract with a standard deviation of 0.64. Variation in amylase activity of different extracts can be accounted for by natural variation in the source material. This assay is considered to measure β-amylase activity only; however, the presence of α-amylase in the extract is likely to increase the measured activity slightly by exposing parts of starch molecules to β-amylase attack. α-amylase activity was not determined separately.

Previously, Lorenz and Nyanzi (1989) found 0.59 mg maltose min^{-1} ml^{-1} extract for unprocessed quinoa seed and 0.29 mg maltose min^{-1} ml^{-1} for washed and heat-treated (80°C) quinoa; these activity rates appeared to decline sharply after 10 min incubation in the assay. The activities found here for amylase are 5 to 10 times greater and, in addition, show linear rates of enzyme activity up to 30 min. Using the same assay method, Marsh *et al.* (1988) found 47 and 61 mg maltose min^{-1} g^{-1} wholemeal flour from ungerminated and germinated wheat flour, respectively, at 25°C and pH 5.8. At a higher temperature (37°C), ungerminated quinoa had half the β-amylase activity of ungerminated wheat.

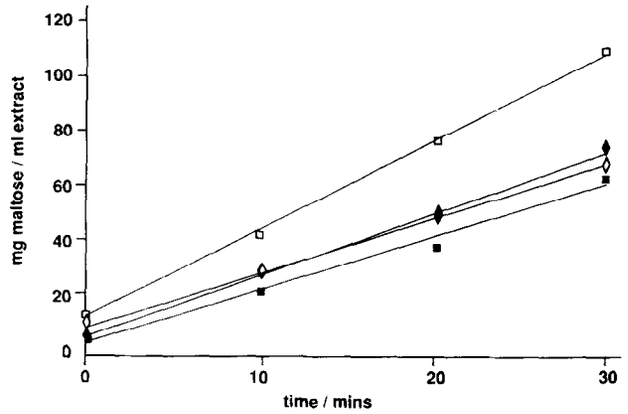

Fig. 1. Amylase activity of four separate extracts of quinoa flour assayed in duplicate. Extract 1(□), extract 2(◆), extract 3(■), extract 4(◇).

Atwell *et al.* (1988) used the Phadebas® method (Barnes & Blakeney, 1974) to determine α-amylase activity in quinoa, and found 1.45 meq g^{-1} dry basis in ungerminated and 6.7 meq gdb^{-1} in germinated seed. It is presumed that by 'meq' the authors mean milli Enzyme Units (EU) where one EU catalyses the hydrolysis of 1 μmol of glycosidic linkage per min at 37°C, as described in the Phadebas® technical literature (Pharmacia, Uppsala, Sweden).

Amylase activity is required to produce fermentable sugars for baking and brewing, and makes starchy weaning foods more calorific. The amylase activity found in quinoa would be useful in these applications.

Invertase

Two separate extracts were assayed in duplicate for invertase and gave results of 0.05 and 0.016 mg reducing sugars min^{-1} ml^{-1} extract over a period of 20 min. Boiled extract as a control gave variable results, one showing a comparable slight increase in reducing sugar over the same time period. Assay of extracts without substrate also showed similar increases in measurable reducing sugar up to 20 min, after which they declined markedly. It was concluded that the changes in reducing sugar concentration did not provide evidence of invertase activity. The extracts had a reducing sugar concentration of 3–4 mg ml^{-1}, and higher concentrations were apparent in boiled extracts, which may have contributed to these results.

Cellulase

Two extracts of quinoa showed initial cellulase activity of 0.012 mg glucose min^{-1} ml^{-1} of extract with a standard deviation of 0.003. The rate of glucose release was linear for the first 20 min, then decreased. Boiled extract showed no activity over the same time period (Fig. 2).

Table 1. Rate of amylase activity of quinoa extracts

	Extract 1	Extract 2	Extract 3	Extract 4
Activity (mg maltose min^{-1} ml^{-1})	3.23	2.18	1.82	1.93
(r^2)	(0.999)	(0.998)	(0.984)	(0.973)

r^2 values refer to regression analysis of mg maltose produced per ml extract versus time (min) plotted for each extract

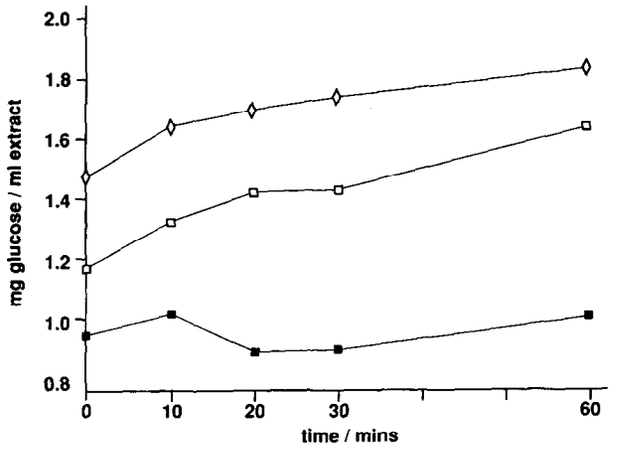

Fig. 2. Cellulose activity in two separate extracts (◇,□) and boiled extract (■).

Lorenz and Nyanzi (1989) measured cellulase activity by viscosity reduction and also found that cellulase activity (confirmed by high glucose and cellobiose concentrations) was greatest in the first 30 min of assay.

The presence of cellulase activity is desirable in cereals used in brewing, where it contributes to viscosity reduction in the worts.

Polygalacturonase

Quinoa extracts caused increases in galacturonic acid concentration over the same time period (22 h), when either polygalacturonic acid or pectin (7% methylated) was used as substrate. The mean activity was 1.7 mg galacturonic acid $22\,h^{-1}$ ml^{-1} extract (sd = 0.7) in the presence of polygalacturonic acid and 1.68 mg $22\,h^{-1}$ ml^{-1} (sd = 0.03) with pectin. However, a control with no substrate gave similar results over the same time period; hence, it was concluded that galacturonic acid production could not be attributed to polygalacturonase activity.

Polygalacturonase is commonly found in pathogenic bacteria and fungi, and in ripening fruit; thus, the presence of measurable levels in dicotyledonous quinoa seeds was not expected.

Phosphatase

Phosphatase activity was determined in duplicate on two extracts. At pH 5.4, the rate of production of p-nitrophenoxide was linear up to 10 min ($r^2 = 0.9997$). The averaged ΔA was $0.03\,min^{-1}$, and activity was 1.09 μmols p-nitrophenoxide min^{-1} ml^{-1} extract with standard error (2×sd) ≤6%.

At pH 10, phosphatase activity was also linear up to 10 min ($r_2 = 0.996$) when the extracting buffer was pH 7. No activity was found when the extracting buffer was pH 10, indicating that the enzyme was not extractable at this pH. The averaged activity of two extracts was 0.70 μmols p-nitrophenoxide min^{-1} ml^{-1} extract, with standard error ≤7.5%. Quinoa seeds demonstrated considerable phosphatase activity at alkaline and acid pH values, suggesting the presence of an alkaline phosphatase and an acid phosphatase. Phosphatases may have a role in phytate breakdown, increasing the availability of iron and other minerals. The phytate content of quinoa has been reported to be between 0.7 g $100\,g^{-1}$ (Ruales & Nair, 1993) and 1.18 g $100\,g^{-1}$ (Koziol, 1992), which is comparable to wholegrain wheat or rye flour, lentils or faba beans (Ruales & Nair, 1993).

Protease

Assays on three quinoa extracts, with casein as substrate, confirmed there was no measurable protease activity with this substrate. With haemoglobin as substrate, the averaged results of four extracts assayed in duplicate gave protease activity of 5.1 μg tyrosine min^{-1} ml^{-1} extract, sd 2.9 (Table 2). Boiled extract showed no protease activity over a 60 min period. A wide range in protease activity was found from different packets of quinoa seed ready-prepared for consumption, as for amylase activity.

Table 2. Effect of substrate on protease activity in quinoa extracts

Time (min)	μg tyrosine ml^{-1} extract				
	Substrate casein				
	Extract 1	Extract 2	Extract 3		
0	130	396	112		
10	130	406	94		
30	143	391	104		
60	155	419	113		
	Substrate haemoglobin				
	Extract 4	Extract 5	Extract 6	Extract 7	Boiled extract
0	220	240	294	151	157
10	308	295	334	166	158
30	362	400	431	206	161
60	489	480	—	294	137

Each extract was assayed in duplicate.

Table 3. Effect of substrate concentration and pH on quinoa phenolase activity

	ΔA_{430} min^{-1} ml^{-1} extract	
Substrate concentration (mM)	pH 6	pH 7
20	0.064 ($n=1$)	—
35	0.09 ± 0.03 ($n=6$)	0.065 ($n=1$)
45	0.11 ± 0.06 ($n=3$)	0.029 ± 0.006 ($n=2$)

n = number of determinations.

The pH optimum for quinoa protease was confirmed to be between 4 and 5.

The average activity found here is 5 times greater than previously reported for mechanically-abraded quinoa (1.05 μg min^{-1} ml^{-1}) and 8 times greater for similarly washed and dried quinoa (0.63 μg min^{-1} ml^{-1}) (Lorenz & Nyanzi, 1989). However, in assays here absorbance was measured at 700 nm, which was found to be the optimum, whereas Lorenz and Nyanzi used 500 nm, which may account for the lower activity found. Protease activity was found here to be 1.6 times greater measured at 700 nm than at 500 nm.

Phenolase

An average of six extracts found ΔA_{430} min^{-1} ml^{-1} extract was 0.09, sd = 0.015, at pH 6 and with catechol concentration of 35 mM in the assay. The rate was linear from 30 to 150 s, where rates were measured, but inactivation was noticeable as the reaction proceeded. This was less apparent at pH 7, where the rate of enzyme activity was slower (see Table 3), but linear for up to 5 min. The rate of enzyme activity was increased by raising substrate concentration in the assay, as shown in Table 3.

Catalase

The averaged activity of two separate extracts (duplicate assays) was 0.06 mmol H_2O_2 consumed min^{-1} ml^{-1} extract (error $\leq$ 2.5%). Thus, there was considerable catalase activity in the grain.

Peroxidase

The substrate used to determine peroxidase activity, pyrogallol, could also be utilised by phenolase. Peroxidase activity was thus determined against a blank containing buffer in place of H_2O_2, showing phenolase activity only, so the rate that was measured was due to peroxidase activity only.

Peroxidase activity from two extracts was found to be 0.42 ΔA min^{-1} ml^{-1} extract, (sd = 0.06). The rate of enzyme activity was linear up to 6 min. Interference from phenolase activity was also determined separately and found to be 0.011 ΔA min^{-1} ml^{-1} extract (sd = 0.005, $n=3$).

Superoxide dismutase

Superoxide dismutase (SOD) is assayed indirectly as neither the disappearance of the substrate nor the appearance of the product can be measured readily.

The relationship between enzyme concentration and per cent inhibition of ferricytochrome c reduction is non-linear, and so a calibration curve was required to establish the relationship between them. Fifty per cent inhibition, or one unit of activity, was given by 30 μl of extract in the assay. SOD activity assayed in three extracts gave 2.9 ± 0.4 units of SOD activity in 100 μl of extract.

Table 4. Summary of Enzyme Activities in Quinoa Extracts

Enzyme	Activity per ml extract (0.1 g fresh weight)
Amylase	6.7 μmoles maltose min^{-1}
Invertase	Not detected
Cellulase	4.0 μmoles glucose h^{-1}
Polygalacturonase	Not detected
Alkaline phosphatase	0.70 μmoles min^{-1}
Acid phosphatase	1.09 μmoles min^{-1}
Protease	5.1 μg Hb min^{-1}
Phenolase	0.09 ΔA_{430} min^{-1}
Catalase	0.06 mmol H_2O_2 consumed min^{-1}
Peroxidase	17.0 μmoles min^{-1}
Superoxide dismutase	29 units
Lipoxygenase	Not detected

Apparent SOD activity can be shown by several components in whole extracts, such as transition metal ions, copper-amino complexes and cytochrome oxidase. Boiled extract in EDTA buffer gave 12% inhibition, which equated to 0.16 units per 100 μl extract. SOD-like activity was greater in boiled extract in non-EDTA buffer, 26% inhibition or 0.4 units $100\,\mu l^{-1}$. This is possibly due to the presence of metal ions such as Cu^{2+}, released from enzymes by boiling, which can show apparent SOD activity (Halliwell, 1984). However, the SOD activity in the unheated extract in EDTA buffer was 18 times greater, indicating the presence of SOD enzyme activity of 274 units g^{-1} fresh weight of quinoa.

Catalase, peroxidase and superoxide dismutase are part of the cellular mechanism in living systems that protects against cell damage by oxygen radicals and related compounds. The effectiveness of this mechanism, post harvest, may have implications in food processing as a natural antioxidant system.

Lipoxygenase

When the ratio of extract to substrate was 1:9 or 1:19, there was no measurable lipoxygenase activity. However, with a ratio of 1:99 there was slight activity of 0.01 ΔA_{234} min^{-1}. Soyabean extract in a ratio of 1:19 showed lipoxygenase activity of 0.09 ΔA_{234} min^{-1}, thus validating the assay method. It was concluded that 0lipoxygenase activity in quinoa was not detectable by this method.

CONCLUSIONS

All enzyme results are summarised in Table 4.

Enzyme assays on crude extracts are prone to interferences from other components of the extracts, but are valid for food investigations as reflecting the chemical complexity of the enzymes' natural environment in food. Quinoa extracts were particularly notable for activities of amylase, protease, phosphatase, phenolase and the oxidative enzymes. No evidence was found for activities of invertase, lipoxygenase or polygalacturonase.

Protease activity was demonstrated on haemoglobin, but not on casein. The levels of activity found for amylase and protease were greater than previously reported (Lorenz & Nyanzi, 1989), which may be due to differences in source or variety of quinoa.

ACKNOWLEDGEMENTS

Mylene Caussette was supported in the U.K. by the ERASMUS scheme of the E.U. We thank Muriel Vanoli for assistance with protease assays.

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