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The effect of various food parameters on the activity and stability of catalase from *Aspergillus niger* and catalase from bovine liver

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The effects of a number of food relevant parameters on catalase activity and stability were studied. The direct responses of different combinations of the parameters ethanol, pH and ionic strength on bovine liver catalase and *Aspergillus niger* catalase activity were investigated in a full factorial 2^4 statistically designed experiment. Statistically significant effects ($p=0.001$) on both types of catalases were exerted by ethanol (2% ≈ 420 mM), decreased pH (4.5), and high ionic strength ($I=2$). Further, it was found that *A. niger* catalase was comparatively more robust to low pH than bovine liver catalase, but that bovine liver catalase activity was significantly more tolerant to ethanol (2%) than catalase from *A. niger*. The stability of bovine liver catalase and *A. niger* catalase activity to 32 different combinations of the parameters pH, ethanol, ascorbic acid, enzyme concentration, enzyme type, protein concentration, storage temperature and atmosphere in headspace were monitored during 12 days. As expected, the responses to the different treatments varied significantly with time. It was found that ascorbic acid (500 ppm ≈ 2.85 mM), low pH (pH 4.5), and increased storage temperature (25°C) each exerted significantly destabilising effects on catalase activity ($p<0.05$), while addition of ethanol (2%) resulted in a statistically significant stabilising effect ($p=0.001$) that increased during the test period. During storage, ethanol was also found to exert significantly positive two factor interactions with ascorbic acid and temperature, respectively, signifying that addition of ethanol (2%) protected both types of catalases against inhibition by ascorbic acid (500 ppm) and against destabilisation by increased temperature (25°C) during storage. © 1997 Elsevier Science Ltd

INTRODUCTION

Glucose oxidase (EC 1.1.3.4) in conjunction with catalase (EC 1.11.1.6) catalyse oxygen or glucose removal via oxidation of β-D-glucose ($C_6H_{12}O_6$) to gluconic acid ($C_6H_{12}O_7$) in the following *net reaction*:

$$2C_6H_{12}O_6 + O_2 \rightarrow 2C_6H_{12}O_7$$

The glucose oxidase–catalase system has a long history of use in the food industry, primarily in the processing of egg products, where enzymatic removal of glucose prior to spray drying or pasteurisation prevents browning via the Maillard reaction (Scott, 1975; Szalkucki, 1993). Oxygen scavenging by the glucose oxidase–catalase enzyme system has also been known for a long time as a workable antioxidative and antibacterial principle in various food products, including fruit juices (Scott, 1975; Sagi & Mannheim, 1990), fish (Field *et al.*, 1986), shrimp (Kantt *et al.*, 1993), mayonnaise and salad dressings (Scott, 1975; Mistry & Min, 1992), although industrial use is limited.

Glucose oxidase–catalase extracted from *Aspergillus niger* is commercially available and listed as GRAS in the USA and was originally suggested as an antioxidant on the new EC additive list (Toft, 1994) but was eventually not included.

One prerequisite for successful, widespread application is that the enzymes are robust to inactivation in the food micro-environment. In spite of the many reports concerning application of the glucose oxidase–catalase system in foods, there is only little information available regarding the activity and stability responses of the enzymes to parameters such as lipids, ionic strength, ethanol, ascorbic acid, etc.

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Regarding catalase the enzyme from bovine liver is the longest and best studied. Well known inhibitors of the bovine liver catalase include acetate, ascorbate, azide, ethanol, nitrite and various phenols (Zollner, 1993). Ethanol is a noncompetitive inhibitor of catalase with respect to hydrogen peroxide (H_2O_2), but the inhibition of catalase activity is affected by the interaction of inhibitors, and varies with the enzyme type. Davison *et al.* (1986) have thus shown that in the absence of copper the inhibition by ascorbate (2 mM) of bovine liver catalase is prevented and reversed by ethanol (20 mM), but that in the presence of copper (40 μM Cu^{2+}) the protection afforded by ethanol is only effective at much lower ascorbate concentrations, and was shown to protect at [ascorbate] of 0.2 mM. In a study on inhibition of catalase activity in wines it was shown that ethanol concentrations of 0.38% ($\approx$83 mM) and 1.9% ($\approx$413 mM) inhibited *A. niger* catalase activity 49% and 68%, respectively (Temple & Ough, 1975). This inhibition prevents the commercial use of glucose oxidase–catalase as an oxygen scavenger in ethanol containing beverages such as beer and wine (Szalkucki, 1993).

Catalase from *A. niger* is known to be more robust than the bovine liver enzyme to hydrogen peroxide, low pH, elevated temperature and proteolysis (Scott & Hammer, 1960; Wasserman & Hultin, 1981). The reason for the higher stability of the *A. niger* catalase has not been fully elucidated, although it has been shown that deglycosylation of the *A. niger* catalase, i.e. (partial) removal of covalently bound carbohydrate, results in slightly decreased thermal and proteolytic stability (Wasserman & Hultin, 1981). As part of our investigations on the applicability of glucose oxidase–catalase as an antioxidative system in lipid foods we have shown that both glucose oxidase and catalase from *A. niger* remain relatively stable during 24 hours in emulsions containing 1–90% of oil by weight (Meyer, 1993). The purpose of this study was to investigate further the effects of various food relevant factors on the activity and stability of the non-glycosylated bovine and the glycosylated *A. niger* catalase, respectively.

MATERIALS AND METHODS

Materials

A. niger catalase was purchased from Serva Biochemicals (Heidelberg, Germany); bovine liver catalase, bovine serum albumin (BSA) and ascorbic acid were from Sigma Chemical Co. (St Louis, MO, USA).

Methods

Catalase activity was determined by recording the rate of H_2O_2 decay at 240 nm in a double beam spectrophotometer in an assay modified from Aebi (1983): a 0.1 ml sample in 2.90 ml buffered substrate solution with $[H_2O_2]$ = 14 mM.

All results given are averages of duplicate activity determinations.

Individual and interactive effects on catalase activity of factors pH, ionic strength, ethanol, and enzyme type were tested in a full factorial 2^4 design (Table 1): Appropriate combinations were prepared in the cuvette, the enzyme added at $t=0$, and the rate of H_2O_2 decay recorded over 3 min. The levels of the factors investigated were determined from preliminary trials, where the influence of the individual parameters on the activity of each of the two catalases were systematically examined (results not shown).

Effects on catalase stability of factors pH, ethanol, ascorbic acid, enzyme concentration, enzyme type, protein concentration (BSA), storage temperature and atmosphere (air vs N_2) were tested in a fractional 2^8 design (Table 3): Appropriate combinations of parameters were prepared aseptically in 32 different glasses (total vol. 12 ml), which were immediately sealed airtight with rubber septa and aluminium caps. Samples for activity determinations (0.4 ml) were taken aseptically on days 0, 1, 2, 3, 5, 8, and 12.

The PC-programme Modde (Umetri AB, Umeå, Sweden) was used to aid the statistical design of experiments and to fit and analyse data by multiple linear regression.

RESULTS

Effects on catalase activity

As expected, large differences were obtained in the direct activity responses in each of the 16 experiments representing different combinations of the factors ethanol, enzyme type, pH and ionic strength in a full factorial 2^4 statistical design (Table 1). The data obtained show statistically significant negative effects (negative regression coefficients) of ethanol (2% $\approx$ 420 mM) and high ionic strength ($I=2.0$) on both types of enzymes at the 99.9% level ($p=0.001$) and a similar, statistically significant positive effect of increasing pH from 4.5 to 7.0 ($p=0.001$) (Table 2). Significantly positive interactions were found between the following combinations of factors: ethanol*enzyme type and pH*enzyme type at 99.9% ($p=0.001$) and 99% ($p<0.01$) levels of significance, respectively (Table 2). The interaction between ethanol and enzyme type can be interpreted as follows: addition of ethanol (2%) resulted in a relatively larger decrease in *A. niger* catalase activity as compared to the activity drop seen in bovine liver catalase activity [compare the activity decrease between experiment number 5 and 6 with 7 and 8 (Table 1)]. By analogy the interpretation of the two factor interaction between pH and enzyme type is that when the pH was increased from 4.5 to 7.0, the increase obtained in bovine liver catalase activity was comparatively larger than what was obtained for *A. niger* catalase [compare the level of activity increase between experiment number 3 and 7 with 1 and 5 (Table 1)].

Table 1. Catalase activity responses [$\Delta(\ln(Abs_{240\,nm})).s^{-1}$] to ethanol (%), enzyme type, pH and ionic strength (ionic S) in a full factorial 2^4 experiment

Experiment no.	pH	Ethanol %	Enzyme	Ionic S	Activity
1	4.5	0	***A. niger***	0	4.738
2	4.5	2	***A. niger***	0	0.639
3	4.5	0	Bovine	0	1.305
4	4.5	2	Bovine	0	1.158
5	7.0	0	***A. niger***	0	4.703
6	7.0	2	***A. niger***	0	0.949
7	7.0	0	Bovine	0	5.058
8	7.0	2	Bovine	0	4.412
9	4.5	0	***A. niger***	2	2.187
10	4.5	2	***A. niger***	2	0.236
11	4.5	0	Bovine	2	0.000
12	4.5	2	Bovine	2	0.000
13	7.0	0	***A. niger***	2	3.679
14	7.0	2	***A. niger***	2	0.337
15	7.0	0	Bovine	2	2.295
16	7.0	2	Bovine	2	1.949

Table 2. Summary of main effects and statistically significant two factor interactions obtained by multiple linear regression analysis of the activity data shown in Table 1. For significant effects at the 95% confidence level, $p < 0.05$

Factor	Coefficient	*p*	Conf. int. ±
Constant	2.103	0.000	0.338
Ethanol	−0.893	0.001	0.338
Enzyme	−0.081	0.603	0.338
pH	0.820	0.001	0.338
Ionic S	−0.767	0.001	0.338
EtOH*Enz	0.751	0.001	0.338
pH*Enz	0.586	0.004	0.338

Confidence interval (Conf. int. ±): the 95% confidence interval on the regression coefficient value.

Effects on catalase stability

For all the 32 different combinations of the eight parameters tested the activities of bovine liver and *A. niger* catalase were monitored during 12 days. The activities obtained in each of the 32 experiments were calculated as relative activities to the activity obtained at day 0, which was thus set to 100%. For an overview, only the response patterns obtained at days 0, 3 and 12, respectively, are analysed in detail in the following. As expected, the responses to the different treatments varied significantly with time (Table 3). Thus, in some cases (experiment nos 3, 4, 7, 10, 12, 16, 20, 24, 26, and 28), the activities remained close to 100% even after 12 days, while in others (e.g. experiment nos 6, 13, and 29), the catalase activity was almost fully lost after 3 days (Table 3).

Day 3: At day 3 both pH, ethanol, ascorbic acid, and storage temperature were found to exert statistically significant effects on catalase activity (Table 4). Thus, as judged from the signs of coefficients, the catalase stability of both types of enzymes was better at pH 6 as compared to at pH 4.5, addition of 2% ethanol had a positive, stabilising effect, addition of ascorbic acid (500 ppm ≈ 2.85 mM) destabilised the activity, and storage at 5°C was significantly more favorable than storage at 25°C. In addition, the following two factor combinations were found to significantly influence the activity levels at day 3: ethanol*ascorbic acid, ethanol*temperature, and ascorbic acid*temperature (Table 4). The first two interactions manifest a stabilising effect of ethanol, where in the first case, the interaction between ethanol and ascorbic acid, the data indicate that the addition of ethanol (2%) gave a significantly lower decrease in activity by ascorbic acid (500 ppm ≈ 2.85 mM) as compared to the activity drop caused by ascorbic acid when ethanol was not added [compare, for example, the activity differences obtained in experiment nos 18 and 22 with 28 and 32 (Table 3)]. In the second case, the interaction between ethanol and storage temperature, the data signify that the addition of ethanol (2%) significantly lowered the destabilising effect of increasing the storage temperature from 5 to 25°C [compare, for example, the obtained activity differences between experiments number 17 and 25 with 19 and 27 (Table 3)]. Lastly, the significantly negative two factor interaction between ascorbic acid and storage temperature shows that the addition of ascorbic acid (500 ppm ≈ 2.85 mM) significantly enhanced the destabilising effect of increased storage temperature [compare, for example, e.g. the drop in activity obtained between experiment nos 17 and 25 with 21 and 29 (Table 3)].

Table 3. Summary of relative activities (%) obtained at days 0, 3 and 12, in the fractional 2^8 factorial catalase stability experiment. Ascorbic acid (Asc. acid) concentrations are given in ppm. Enzyme concentration (Enz. conc.) and BSA concentrations are in mg/ml. Atmosphere designates type of gas in headspace, and Temp. °C is storage temperature (°C)

Experiment no.	pH	Enzyme	Ethanol %	Asc. acid ppm	Enz. conc.	BSA	Atmosphere	Temp. °C	Day 0	Day 3	Day 12
1	4.5	*A. niger*	0	0	0.125	0.5	nitrogen	5	100	85	72
2	6.0	*A. niger*	0	0	0.125	0.5	air	25	100	102	73
3	4.5	*A. niger*	2	0	0.125	0	air	25	100	97	101
4	6.0	*A. niger*	2	0	0.125	0	nitrogen	5	100	104	105
5	4.5	*A. niger*	0	500	0.125	0	air	5	100	64	16
6	6.0	*A. niger*	0	500	0.125	0	nitrogen	25	100	5	0
7	4.5	*A. niger*	2	500	0.125	0.5	nitrogen	25	100	98	100
8	6.0	*A. niger*	2	500	0.125	0.5	air	5	100	97	96
9	4.5	*A. niger*	0	0	0.625	0	nitrogen	25	100	93	55
10	6.0	*A. niger*	0	0	0.625	0	air	5	100	109	105
11	4.5	*A. niger*	2	0	0.625	0.5	air	5	100	90	86
12	6.0	*A. niger*	2	0	0.625	0.5	nitrogen	25	100	110	109
13	4.5	*A. niger*	0	500	0.625	0.5	air	25	100	5	0
14	6.0	*A. niger*	0	500	0.625	0.5	nitrogen	5	100	98	13
15	4.5	*A. niger*	2	500	0.625	0	nitrogen	5	100	96	86
16	6.0	*A. niger*	2	500	0.625	0	air	25	100	109	103
17	4.5	Bovine	0	0	0.125	0	nitrogen	5	100	92	77
18	6.0	Bovine	0	0	0.125	0	air	25	100	102	86
19	4.5	Bovine	2	0	0.125	0.5	air	25	100	82	66
20	6.0	Bovine	2	0	0.125	0.5	nitrogen	5	100	100	96
21	4.5	Bovine	0	500	0.125	0.5	air	5	100	80	44
22	6.0	Bovine	0	500	0.125	0.5	nitrogen	25	100	14	0
23	4.5	Bovine	2	500	0.125	0	nitrogen	25	100	68	30
24	6.0	Bovine	2	500	0.125	0	air	5	100	113	104
25	4.5	Bovine	0	0	0.625	0.5	nitrogen	25	100	86	35
26	6.0	Bovine	0	0	0.625	0.5	air	5	100	98	92
27	4.5	Bovine	2	0	0.625	0	air	5	100	84	95
28	6.0	Bovine	2	0	0.625	0	nitrogen	25	100	107	101
29	4.5	Bovine	0	500	0.625	0	air	25	100	3	0
30	6.0	Bovine	0	500	0.625	0	nitrogen	5	100	82	55
31	4.5	Bovine	2	500	0.625	0.5	nitrogen	5	—	—	—
32	6.0	Bovine	2	500	0.625	0.5	air	25	100	99	89

Table 4. Summary of main effects and statistically significant interactions obtained by multiple linear regression analysis of the activity response pattern at day 3 (Table 3) for significant effects at the 95% confidence level $p < 0.05$

Factor	Coefficient	p	Conf. int. ±
Constant	83.589	0.001	4.462
pH	6.974	0.004	4.462
Ethanol	13.714	0.001	4.462
Asc. acid	−12.724	0.001	4.462
Temp.	−9.839	0.001	4.462
EtOH*Asc	13.276	0.001	4.462
EtOH*T	8.786	0.001	4.462
Asc*T	−10.901	0.001	4.462

Confidence interval (Conf. int. ±): the 95% confidence interval on the regression coefficient value.

Day 12: Except for the two factor interaction between ascorbic acid*temperature, the same factors as found at day 3 were found to exert statistically significant effects at day 12 (Table 5). As judged from the size of the regression coefficients the effects were of the same magnitude at day 12 as at day 3, except in the case of ethanol, which showed a significantly higher positive effect at day 12 as compared to at day 3 (Table 5).

DISCUSSION AND CONCLUSIONS

The direct activity response data showed, as expected, that addition of 2% ethanol (≈420 mM), a lowering of pH from pH 7.0 to 4.5, and an increase in ionic strength (to $I = 2$) exerted statistically significant negative effects on catalase activity (Tables 1 and 2). All three effects agree well with what have been reported by others (Temple & Ough, 1975; Scott & Hammer, 1960), except that the separate effect of ionic strength has not, to the best of our knowledge, been unequivocally reported earlier. The significant interactions obtained between the parameters ethanol*enzyme type and pH*enzyme type signify that *A. niger* catalase is comparatively more affected by ethanol than bovine liver catalase, and oppositely that bovine liver catalase activity is comparatively more affected by the level of pH than *A. niger* catalase. The results obtained in this factorial 2^4 experiment do not permit any final conclusions to be drawn about the mechanism of inhibition of catalase activity. Hence, it cannot be unequivocally concluded whether the effects obtained are only kinetic or whether the negative and interactive effects on activity are in fact due to changes in the molecular structures of catalase proteins that in turn affect the activities of *A. niger* catalase and bovine liver catalase, respectively. It is well known that the *A. niger* catalase is more robust to lower pH values than bovine liver catalase (Scott & Hammer, 1960), and the more negative effect of ethanol on *A. niger* enzyme also agrees well with the activity responses we recorded in our preliminary, systematic comparisons of dose–activity responses of both catalases to ethanol (results not shown).

It is tempting to interpret the main effect of ethanol to be a consequence of the generally accepted non-competitive inhibition mechanism (Temple & Ough, 1975; Davison *et al.*, 1986) or, alternatively, perhaps as a denaturing effect on catalase.

The results obtained in our fractional 2^8 stability study do not immediately support any of these explanations, however, since it was found, that addition of ethanol stabilised both types of enzymes against activity loss with time, and further, that addition of ethanol protected both types of enzymes against ascorbic acid's inhibitory action during storage and against the significant destabilisation caused by temperature (25°C) (Tables 4 and 5). Although an immediate impression is that the observed stabilising effect of ethanol with time is surprising, our results agree well with observations made by others regarding the mechanism of inhibition of (bovine liver) catalase by ascorbate. Thus, as already touched upon in the introduction, Davison *et al.* (1986)

Table 5. Summary of main effects and statistically significant interactions obtained by multiple linear regression analysis of the activity response pattern at day 12 (Table 3) for significant effects at the 95% confidence level $p < 0.05$

Factor	Coefficient	p	Conf. int ±
Constant	69.824	0.001	4.834
pH	6.864	0.007	4.834
Ethanol	24.636	0.001	4.834
Asc. acid	−14.801	0.001	4.834
Temp.	−6.996	0.006	4.791
EtOH*Asc	14.386	0.001	4.834
EtOH*T	7.067	0.006	4.791

Confidence interval (Conf.int. ±): the 95% confidence interval on the regression coefficient value.

found that, under certain circumstances, the inhibition of catalase activity by ascorbate, could be both prevented and reversed by 20 mM ethanol via reduction of the active enzyme–H_2O_2 complex I (Fe(V)) and the inactive complex II (Fe(IV)), respectively. Our results thus support and expand their conclusions to also be in force at higher concentrations of ethanol, namely at 2% (≈420 mM), and to be valid for both the bovine liver catalase and the more food relevant *A. niger* catalase. Thus, contemplation of the data obtained leads us to conclude that catalase, independent of the type (*A. niger* or bovine liver), apparently is stabilised by ethanol against inhibition by the common activity decay, e.g. inactivation via conversion of the active catalase complex I to the inactive complexes II or III, and against the inhibition caused by ascorbic acid. Our experimental setup does not, however, permit any further conclusions to be drawn regarding the mode and mechanism of interaction between ethanol, ascorbic acid and catalase. The paradox that ethanol influences catalase activity negatively in direct activity measurements, where the activity is monitored during a few minutes, but exerts a statistically significant stabilising effect on catalase activity over longer time periods is probably a result of differences in reaction rates between ethanol and the active and inactive catalase complexes I and II, respectively (Davison *et al.*, 1986). Polyhydric alcohols such as xylitol, sorbitol, and glycerol are known to be able to stabilise a number of enzymes, including stabilisation of the enzyme structure against thermal denaturation (Brumm & Teague, 1989; Combes & Monsan, 1984; Fujita *et al.*, 1982; Ye *et al.*, 1988; Ye & Combes, 1991). A general mechanism of polyol-induced protection has not been established, though, which is why it is difficult to suggest a plausible mechanism for the protective effect of ethanol on catalase activity obtained in our studies. Our results emphasise that the understanding of the detailed mechanism of interaction between ethanol and catalase (with and without ascorbic acid) deserves more research. Likewise, the practical exploitation of the stabilising effect of ethanol in application of the glucose oxidase–catalase system requires further investigations of the phenomenon and applicability trials in real food systems.

It is inconceivable that deliberate addition of ethanol to foods will ever take place with the purpose of stabilising the glucose oxidase–catalase system. However, our observation that 2% ethanol, a concentration relevant in alcoholic beverages like beer, stabilises catalase activity during storage, and that ethanol also stabilises catalase against inhibition by ascorbic acid, may offer new prospects for the applicability of the glucose oxidase–catalase system in alcoholic beverages already containing ethanol (and possibly ascorbic acid).

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