The lipolytic properties of Candida mycoderma and Debaryomyces kloeckeri isolated from limburger cheese and some properties of the lipases produced by these yeasts

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Lipolytic microorganisms frequently play important roles on the development of pungent odour and flavour in certain types of cheeses.

During the course of investigation on the production of volatile flavour substances by yeasts isolated from limburger cheese, viz. Candida mycoderma and Debaryomyces kloeckeri, it was suggested that these yeasts had lipolytic properties, since each of these strains produced remarkably large amount of n-butyric acid in whole milk media after relatively short time of incubation5).

Although considerable literatures3,7,11,13,17) have been published on the lipases produced by moulds and bacteria isolated from various cheeses, little is known of the lipolytic properties of yeasts subsisting in cheeses16,18).

In the present paper, production of lipase by C. mycoderma and D. kloeckeri isolated from limburger cheese and some properties of the lipases produced were investigated.

**Experimental**

1. Strain used: C. mycoderma and D. kloeckeri, which had been previously isolated from limburger cheese by authors5) were used throughout this work. These strains were incubated at 30°C and transferred fortnightly into Koji-extract-agar medium2).

2. Preparation of lipase solution: Each strain of C. mycoderma and D. kloeckeri was cultivated in the medium (Medium-LP) consisting of 2.0% of soluble starch, 2.0% of peptone, 0.5% of potassium phosphate, dibasic, 0.1% of urea, 0.1% of magnesium sulfate and 0.5% of calcium carbonate, pH being 6.8.

After 48 hr’s incubation at 30°C cells were removed by centrifugation. The supernatant was used as lipase solution, followed by dialyzing against cold running water overnight.

3. Standard conditions for lipase assay: To a mixture consisting of 0.2 ml of tributyrin and 2 ml of M/15 acetate buffer solution (pH 4.5) was added 10 ml of the lipase solution. The reaction mixture was then incubated at 21°C with a constant reciprocal agitation (140 reciprocations per minute). After 3 hr’s incubation, 30 ml of 2:1 mixture of alcohol and ethyl ether was added to stop reaction.

The mixture was titrated with N/100 alcoholic caustic potash, using phenolphthalein as an indicator.

Lipase activity was calculated and expressed in acid degrees, which were defined as ml of N/100 alcoholic caustic potash required to neutralize liberated fatty acids under the condition described above.

4. Estimation of lipolysis of milk fat: Ten ml of the lipase solution was added to a mixture consisting of 2 ml of M/15 acetate buffer solution (pH 4.5) and 0.2 ml of milk fat which was prepared according to the description of NAKANISHI and NAKAE[20]. A control was the same composition except that 10 ml of distilled water was used instead of 10 ml of the lipase solution.

They were incubated at 21°C for 3 hr. with shaking, then lipids in the reaction mixture were extracted three times with 30 ml portions of 1:1 mixture of methanol and chloroform. After removing the solvent from the extracted mixture, fractionation of the lipids was quantitatively carried out by thin-layer chromatography on 20 by 20 cm chromatoplate coated with thin-layer (250 μ) of Wako Gel B-O and dried in an oven at 120°C for 30 minutes. The plate was spotted with 20 μl of the lipids and developed with petroleum ether–ethyl ether–acetic acid (82:18:1 v/v) [14].

The spots were detected by spraying 2',7'-dichlorofluorescein and viewing it under ultraviolet light.

For the determination of composition of fatty acids liberated from milk fat, free fatty acids fraction was obtained by elution from the thin layer plate.

Fatty acid methyl esters prepared by trans methylation with 0.5% sulfuric acid–methanol were analyzed by gas liquid chromatography using twin column and hydrogen flame ionization chromatograph (Shimazu GC-IC).

Three mm-ID×187 cm stainless steel columns were packed with 20% diethylene glycol succinate polyester on 60–80 mesh Celite. The columns were conditioned at 190°C. Carrier gas was nitrogen at flow rate of 35 ml per minute.

The proportion of each fatty acid methyl ester was determined by triangulation.

5. Sephadex column chromatography of the lipase solution: After hydration in M/15 acetate buffer, pH 4.5 for 72 hr., Sephadex G–100 was packed to a height of 45 cm in a glass column (2.5 by 50 cm).

The column was equilibrated with the same buffer. A flow rate of 60 ml per hour was maintained by an adjusted screw cock. Ten ml of each lipase solution was applied into the column and eluted with the same buffer for about 10 hr. at room temperature. Ten ml of aliquot samples were collected in a fraction collector, and absorbance at 280 μμ and lipase activity were measured.

Results and Discussion

1. Lipolytic activities of C. mycoderma and D. kloeckeri

Lipolytic activity was calculated and expressed on the basis of gram dry weight of cells. In Fig. 1 was represented changes of the lipolytic activities of C. mycoderma and D. kloeckeri during incubation. As will be seen from Fig. 1, apparent lipolytic activities are observed in both yeasts, that is, the lipolytic activity of either C. mycoderma or D. kloeckeri clearly increased soon after starting of incubation and reached the maximum on the 8th day of incubation or on the 4th day, respectively.
Lipase production by yeasts from limburger cheese

Lipase solutions of each strain of *C. mycoderma* and *D. kloeckeri* were prepared from the Media-LP incubated for 0, 2, 4, 6, 8, 10 and 12 days in the same manner as described in this text, then activity assay of lipase was estimated under standard assay condition.

Fig. 1. Production of lipase by *C. mycoderma* and *D. kloeckeri* during incubation.

Each strain of *C. mycoderma* and *D. kloeckeri* was incubated at 30°C for 2 days in the Media-LP of which pH were adjusted to various values as indicated with M/15 acetate buffer (pH 4.0~6.5) and McIlvaine buffer (pH 7.0 and 7.5) solutions. Each lipase solution was prepared by the method described in this text and the enzyme activity was assayed under standard assay conditions.

Activity was measured by reducing value method.

Fig. 2. Effect of pH on the production of lipase by *C. mycoderma* and *D. kloeckeri*.

Fig. 2 shows the effect of pH of the growing medium on the lipolytic activities of *C. mycoderma* and *D. kloeckeri*. Each of these yeasts is found to have relatively high lipolytic activities in wide pH ranges from 4.0 to 8.0, and the optimum pH for the production of lipase is observed to be near 5.5 in *D. kloeckeri* and near 7.0 in *C. mycoderma.*
In an earlier paper\textsuperscript{6}, it was reported that the pH of limburger cheese after the manufacture is about 5.0, and the pH rose gradually and reached 5.5 on 25th day after the manufacture and 7.0 on 35th day.

From these results, therefore, it can be suggested that \textit{C. mycoderma} and \textit{D. kloeckeri} would produce lipase optimally in relatively later stage of limburger cheese ripening.

2. Lipolysis of milk fat by \textit{C. mycoderma} and \textit{D. kloeckeri}

As shown in the thin-layer chromatogram of Fig. 3, five spots were obtained from each of the test samples. Spots 1, 2, 3, 4 and 5 in Fig. 3 were identified as phospholipids, free fatty acids, cholesterol, diglyceride and triglyceride, respectively.

![Thin-layer chromatogram of milk fat treated with the lipases of \textit{C. mycoderma} and \textit{D. kloeckeri}](image)

A: Milk fat treated with the lipase of \textit{C. mycoderma}
B: Milk fat treated with the lipase of \textit{D. kloeckeri}
C: Control

Spots
A-1\textsuperscript{)} Phospholipids A-4\textsuperscript{)} Diglyceride
B-1\textsuperscript{)} Free fatty acids B-4\textsuperscript{)} Triglyceride
C-1\textsuperscript{)} Cholesterol C-4\textsuperscript{)}

Fig. 3. Thin-layer chromatogram of milk fat treated with the lipases of \textit{C. mycoderma} and \textit{D. kloeckeri}

<table>
<thead>
<tr>
<th>Fatty acids</th>
<th>\textit{C. mycoderma} (%)</th>
<th>\textit{D. kloeckeri} (%)</th>
<th>Control (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Butyric</td>
<td>0.47</td>
<td>0.38</td>
<td>0.31</td>
</tr>
<tr>
<td>Caproic</td>
<td>0.94</td>
<td>0.76</td>
<td>1.48</td>
</tr>
<tr>
<td>Caprylic</td>
<td>6.82</td>
<td>3.80</td>
<td>1.58</td>
</tr>
<tr>
<td>Capric</td>
<td>4.94</td>
<td>5.52</td>
<td>0.84</td>
</tr>
<tr>
<td>Lauric</td>
<td>9.17</td>
<td>5.90</td>
<td>2.11</td>
</tr>
<tr>
<td>Myristic</td>
<td>21.41</td>
<td>11.04</td>
<td>11.22</td>
</tr>
<tr>
<td>Palmitic</td>
<td>22.58</td>
<td>19.04</td>
<td>34.21</td>
</tr>
<tr>
<td>Palmitoleic</td>
<td>28.08</td>
<td>5.14</td>
<td>2.86</td>
</tr>
<tr>
<td>Stearic</td>
<td>5.64</td>
<td>22.47</td>
<td>16.73</td>
</tr>
<tr>
<td>Oleic</td>
<td>---</td>
<td>25.90</td>
<td>28.60</td>
</tr>
</tbody>
</table>

\textsuperscript{---: undetected}
Lipase production by yeasts from limburger cheese

In contrast to the control, remarkable liberations of diglyceride and free fatty acids are observed in both test samples. These results may be taken to indicate that both strains of C. mycoderma and D. kloeckeri hydrolyzed milk fat.

The fatty acid composition of each free fatty acids fraction appeared in Fig. 3 was given in Table 1. From this table it is noted that the percentages of lauric, myristic, palmitoleic, stearic and oleic acids were extremely differed between the test samples, although the differences observed in the percentages of butyric, caproic, caprylic, capric and palmitic acids from sample to sample were not so striking.

As may be seen in most of microbial lipases\(^1\), these differences were considered to be perhaps caused by the differences of the positional and fatty acid specificities of lipase toward milk fat triglycerides between C. mycoderma and D. kloeckeri.

3. Effect of pH and temperature on the lipase activities of C. mycoderma and D. kloeckeri

Lipase activities of C. mycoderma and D. kloeckeri under several conditions of pH are shown in Fig. 4.

Both lipases of C. mycoderma and D. kloeckeri were most active at pH 4.5 and reduced their activities when the reaction was moved from the optimum pH to the alkaline side. It can be assumed that activities of these lipases in limburger cheese were considerably limited, because pH of limburger cheese is always above 5.0 throughout the ripening period, viz. the pH of limburger cheese after the manufacture is near 5.0 and the pH gradually rose and reached 7.6 at the end of 40 days\(^6\).

Effect of temperature on the lipase activities of C. mycoderma and D. kloeckeri is shown in Fig. 5, from which it is noted that the optimum temperature of the lipase of C. mycoderma and that of D. kloeckeri are 35°C and 30°C, respectively.

These results are consistent with the general observation that microbial lipases are most active within the temperature range 30°C to 40°C.\(^9\)

The enzyme assay was carried out under standard assay conditions except at various values of pH as indicated.

The buffers used were: m/15 acetate buffer, pH 4.0 to 6.5 and McIlvaine buffer, pH 7.0 to 8.0.

Fig. 4. Effect of pH on the activities of lipases produced by C. mycoderma and D. kloeckeri
The enzyme activity was assayed under standard assay conditions except at various temperature as indicated.

Activity was measured by reducing value method.

Fig. 5. Effect of temperature on the activities of lipases produced by *C. mycoderma* and *D. kloeckeri*

Fig. 6. Gel filtration of the lipase solution of *C. mycoderma* on Sephadex G-100

Fig. 7. Gel filtration of the lipase solution of *D. kloeckeri* on Sephadex G-100
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4. Gel filtration of lipase solution on Sephadex G-100

The elution pattern of the lipase solution of C. mycoderma and that of D. kloeckeri by gel filtration on Sephadex G-100 column were shown in Fig. 6 and Fig. 7, respectively.

Two main peaks (peak I and II) having lipase activity are seen from the elution pattern of the lipase solution of C. mycoderma (Fig. 6) and three main peaks (peak III, IV and V) from that of D. kloeckeri (Fig. 7).

Gelotte reported that when more than one peak of activity was obtained by Sephadex gel filtration, only the final peak was considered to contain lipase in unit molecular form and that the other peaks were assumed to consist of lipases either in a polymerized form or associated with inactive high molecular weight material4).

If this was the case, the possibility would be considered that the lipase of C. mycoderma and that of D. kloeckeri were contained in peak II and peak V in unit molecular form, respectively.

For estimating the ranges of molecular weights of these lipases, skim milk was chromatographed on the same Sephadex G-100 column according to the description of Morr et al.10). From the elution pattern of skim milk, the ranges of molecular weight values of the lipase of C. mycoderma (peak II) and that of D. kloeckeri (peak V) were estimated to be 30,000~38,000 and 12,000~21,000, respectively.

Acknowledgements

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Summary

In the present study the lipolytic properties of Candida mycoderma and Debaryomyces kloeckeri which were isolated from limburger cheese and the some properties of the lipases produced by these yeasts were investigated.

Results obtained were as follows.

(1) Both C. mycoderma and D. kloeckeri produced lipase; the optimum pH for the production of lipase by C. mycoderma or by D. kloeckeri was observed to be near 5.5 or near 7.0, respectively.

From these facts it was suggested that these yeasts would produce lipase optimally in limburger cheese in relatively later stage of ripening.

(2) Thin-layer chromatographic analysis showed that these lipases apparently hydrolyzed milk fat. Percentages of myristic, palmitic, palmitoleic, stearic and oleic acids in fatty acids liberated from milk fat by C. mycoderma were extremely differed from those in fatty acide liberated from milk fat by D. kloeckeri.

(3) Both lipases of C. mycoderma and D. kloeckeri were most active at pH 4.5 and reduced their activities when the reaction was moved from the optimum pH to the alkaline side.

The optimum temperature for the activity of the lipase of C. mycoderma and that of D. kloeckeri were 35°C and 30°C, respectively.

(4) By gel filtration of the lipase solutions of C. mycoderma and D. kloeckeri on Sephadex G-100,
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the ranges of the approximate molecular weights of lipase of C. mycoderma and these of D. kloeckeri were estimated to be 30,000~38,000 and 12,000~21,000, respectively.

References

Lipase production by yeasts from limburger cheese

リンブルガーチーズより分離した酵母のリパーゼの生産性と産出リパーゼの特徴

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リンブルガーチーズに棲息する酵母のリパーゼの生産性と産出したリパーゼの諸性質を知る目的でリンブルガーチーズから分離した2種の酵母、Candida mycoderma ならびに Debaryomyces kloeckeri について実験を行なった。得られた結果は次のとおり要約される。

（1）両酵母とも明らかにリパーゼの生産性を有していることが認められ、C. mycoderma は pH 7.0, D. kloeckeri は pH 5.5 においてそれぞれリパーゼの産出至適 pH を有していた。このことから、リンブルガーチーズにおいてこれら酵母によりリパーゼの産出される時期は主に発酵後20日以降と推定された。

（2）それら酵母により産出されたリパーゼは乳脂肪を分解し、各種脂肪酸を遊離させるが、遊離した脂肪酸のうち、ミリスチン酸、パルミト酸、ステアリン酸およびオレイン酸の遊離状態に両リパーゼ間の著しい相違を認めることが出来た。

（3）両リパーゼとも pH 4.5 においてリパーゼ活性が最も高く、かつ C. mycoderma の生産するリパーゼは 35℃ 付近で、また D. kloeckeri の生産するリパーゼは 30℃ 付近でそれぞれ高いリパーゼ活性を有していた。

（4）Sephadex G-100 によりそれらリパーゼを酵別し、おおまかな分子量を推定すると C. mycoderma の生産するリパーゼの分子量は約 30,000～38,000, D. kloeckeri のそれは約 12,000～21,000 と推定された。