ABSTRACT

The proteolytic activity of an industrial preparation of neutral protease from Bacillus subtilis has been tested on caseins and whey proteins of cow's milk. Electrophoretic analysis of κ-casein hydrolysate showed that only electropositive products can be found. Hydrolysis of β-casein generated compounds with low electrophoretic mobility as did hydrolysis of γ-casein. β-Lactoglobulin and immunoglobulins were heavily damaged, whereas α-lactalbumin was slightly hydrolyzed. Because of its wide spectrum of activity and interesting thermophilic properties, this enzymatic preparation can be applied to other fields in the food industry.

INTRODUCTION

The fermentation industry has produced a large number of enzymes used in various industrial applications. In particular, the production of proteases, to replace calf rennet, which is traditionally used to transform milk into cheese (29), is of great interest.

Many tests have been carried out for making use of the proteolytic exocellular system of Bacillus subtilis. Two distinct enzymatic fractions have been isolated, one alkaline and the other neutral. Both are characterized by a wide spectrum of activity on various substrates. Consequently, many developments have been concerned with surface cleaning and preparation of protein hydrolysates (3, 18, 21, 31). Applications have been particularly concerned with milk processings in two distinct ways. The first method consists of using these preparations as substitutes for animal rennet (29). Except for a few studies, referring to Bacillus subtilis proteases as coagulating factors (23, 24, 25, 26), these proteases are claimed by most biochemists and cheese technologists to have too high proteolytic activity and are recommended only when mixed with other clotting proteases (2, 4, 5, 6, 12, 16, 17, 20, 30).

Our own studies dealing with the characterization of Bacillus subtilis neutral protease (27, 28) showed that enzyme sensitivity to pH and temperature was suitable for the cheese making and ripening but that it would be best used at low concentrations.

The second use of these enzymes for the dairy industry is in producing protein hydrolysates to improve the development of manufactured by-products from whey (9, 14, 22, 33). Our study was to characterize the action of a Bacillus subtilis neutral protease preparation on caseins and whey proteins of cow's milk. This protease fits the generally-recognized-as-safe (GRAS) standards, defined by the Joint Expert Committee on Food Additives (13). Its thermophilic character is of significant interest, it is active at ambient temperature, and its optimum activity occurs at about 65°C (7). Another original characteristic was its halophilic behavior (8).
MATERIALS AND METHODS

Enzyme Preparation and Substrates

A commercial dry enzymatic preparation of neutral protease from *Bacillus subtilis* was three times dialyzed (48 h at 4°C) against distilled water to eliminate soluble excipients used for the stabilization of market products, then lyophilized for further storage.

Whole casein obtained by isoelectric precipitation from skim milk was dialyzed as mentioned and lyophilized. Then it was divided into αs (a mix of αs1 and αs2), β-caseins and κ-caseins were precipitated by ion-exchange column chromatography according to the method of Mercier et al. (19). Whey resulting from the isoelectric precipitation of caseins was exhaustively dialyzed against distilled water (72 h at 4°C), then lyophilized. Immunoglobulins and α-lactalbumin were extracted from the whey lyophilitize by ion-exchange column chromatography by the method of Yaguchi et al. (35). β-Lactoglobulin was obtained from the supernatant coming from the treatment of a whey protein solution with trichloroacetic acid (TCA) according to the method of Fox (10).

Electrophoresis

Horizontal electrophoresis was in polyacrylamide-agarose gel according to Uriel (34) as modified by Gripon et al. (11). Twenty-five micrograms of each sample was loaded. The gel was colored by Coomassie blue R 250.

Vertical Electrophoresis in Polyacrylamide Gel

We used the technique of Laemmli and Favre (15) modified as follows: stacking gel (T: 5.2%; C: 3.84%), Tris-HCl .003 M buffer, pH 8.9. Separating gel (T: 9.25%; C: 2.7%), Tris-HCl .018 M buffer, pH 8.9. Running buffer: Tris-HCl .0049 M, pH 8.5. Twenty micrograms of each sample were loaded. The gel was colored by Coomassie blue R 250.

Proteolytic Activity

Substrates were dissolved at concentrations of 1% (wt/vol) in a sodium phosphate .1-M buffer, pH 6.5 for caseins and at pH 8.5 for whey proteins. The enzymatic preparation was added to an enzyme to substrate (E:S) ratio of .37% (wt/vol) for caseins and of .75 or 1.8% (wt/wt) for whey proteins. At given times, proteins were precipitated by adding 5 ml of 24% TCA to 5 ml of the reaction mixture to determine the nonprotein nitrogen (NPN). The precipitate was dialyzed (48 h at 4°C) against distilled water, then lyophilized before further electrophoretic analysis. At the same time, noncasein nitrogen (NCN) was determined according to the method of Aschaffenburg and Drewry (1). Measurements were triplicated; NPN, NCN, and total nitrogen (TN) were measured by the micro-Kjeldahl method.

RESULTS

Casein Hydrolysis

Whole casein hydrolysis, by neutral protease from *Bacillus subtilis*, at 35°C, was optimum at pH 6.5. At 65°C (optimum temperature (7)), pH optimum (pHo) was 8 (Figure 1).

At E:S ratio of .37%, the enzyme hydrolyzed whole casein, generating a fairly substantial amount of NCN, and less NPN (about

![Figure 1. Influence of pH on activity of *Bacillus subtilis* neutral protease preparation. 1) Whole casein, ratio of enzyme to substrate (E:S) = .37%, 35°C, 15 min, 2) whole casein, E:S = .37%, 65°C, 15 min, 3) whey proteins, E:S = .75%, 35°C, 15 min. ΔNPN = Change in nonprotein nitrogen, Nt = total nitrogen.](image-url)
Figure 2. Hydrolysis of caseins by Bacillus subtilis neutral protease preparation. a) Hydrolysis of caseins at 35°C and pH 6.5, rate of enzyme to substrate (E:S) = .37%. 3) β-casein; 4) κ-casein (E:S = .18%); 5) β-casein; 6) αs-casein. b) Hydrolysis of whole casein at 35°C and pH 6.5, E:S = .37%. 1) Nonprotein nitrogen (NPN) % total nitrogen (Nt), 2) noncasein (NCN) % Nt. 50% NCN for 30% NPN after 2-h incubation) (Figure 2b).

κ-Casein hydrolysis was of great importance. We used a proportion of enzyme two times smaller than for the other caseins (Figure 2a). Electrophoresis of the hydrolysate (Figure 3a) showed three electropositive products; the most charged (κBs) was prevalent. β-Casein showed fairly significant hydrolysis. Electrophoretic analysis of the hydrolysate revealed three bands (Figure 3b): βBs1 and βBs2 were electronegative with a small mobility, and βBs3 moved with the migration front.

During αs-casein hydrolysis with Bacillus subtilis protease, NPN production was rather weak (about 8% after 30 min) (Figure 2a), yet electrophoresis showed a fairly quick disappearance of the αs-casein band and of the main hydrolysate as well (αsBs1) (Figure 3c).

Kinetic analysis (Table 1) showed that the protease had an equal affinity for β and αs-casein that was about three times lower than for κ-casein. However, V_max was the highest for this last protein.

Whey Proteins Hydrolysis

Greatest whey protein hydrolysis at 35°C appeared at pH near 8.5 (Figure 1). The degree of hydrolysis was limited, so for sufficient degradation rates, we had to use an E:S ratio five times higher than for caseins. β-Lactoglobulin and immunoglobulins were the most easily hydrolyzed components (Figure 4).

The immunoglobulin band disappeared very quickly (Figure 5a), whereas no related degradation product appeared in the electrophoresis pattern. α-Lactalbumin was hardly hydrolyzed by the protease. No change of the electrophoretic pattern of the hydrolysate was notice-
TABLE 1. Kinetic parameters of the proteolysis of caseins and whey proteins by *Bacillus subtilis* neutral protease preparation.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_m$ $^1$ (mg·ml$^{-1}$)</th>
<th>$V_{max}$ (mg·ml$^{-1}$·min$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole casein$^2$</td>
<td>.43</td>
<td>13.09</td>
</tr>
<tr>
<td>$\kappa$-Casein</td>
<td>1.25</td>
<td>80.97</td>
</tr>
<tr>
<td>$\beta$-Casein</td>
<td>.44</td>
<td>17.17</td>
</tr>
<tr>
<td>$\alpha$-Casein</td>
<td>.35</td>
<td>10.64</td>
</tr>
<tr>
<td>Whole whey proteins$^3$</td>
<td>.84</td>
<td>22.29</td>
</tr>
<tr>
<td>$\beta$-Lactoglobulin</td>
<td>1.04</td>
<td>26.72</td>
</tr>
<tr>
<td>$\alpha$-Lactalbumin</td>
<td>.72</td>
<td>11.08</td>
</tr>
<tr>
<td>Immunoglobulins</td>
<td>2.00</td>
<td>44.46</td>
</tr>
</tbody>
</table>

$^1$ Michaelis constant ($K_m$) and maximum velocity ($V_{max}$) were determined from Lineweaver-Burke plots.

$^2$ For caseins: 35°C, pH = 6.5.

$^3$ For whey proteins: 35°C, pH = 8.5.

... able after 15 min of reaction (Figure 5b). Electrophoresis of $\beta$-lactoglobulin hydrolysate (Figure 5c) showed both genetic variants, as well as products of the hydrolysis, gradually disappeared.

Kinetic studies (Table 1) showed protease had, in general, a low affinity for whey proteins, especially $\beta$-lactoglobulin and immunoglobulins. As far as $V_{max}$ is concerned, we noticed that $\alpha$-lactalbumin was more slowly hydrolyzed than the other two proteins, as confirmed by electrophoretic analysis of the hydrolysates.

**DISCUSSION**

As for most other enzymes, the nature of the substrate was a factor in determining pHo of the enzyme. Moreover, pHo, with casein as a substrate, reached 8 at a reaction temperature of 65°C. This could be explained by heat-induced changes, which modified the protein conformation, and subsequently, the accessibility of the substrate to the enzymatic active site.

Neutral protease from *Bacillus subtilis* produced many casein breakdown products, which showed its low specificity toward caseins. During the first minutes, proteinaceous fractions were produced from $\kappa$-casein, which moved to the cathode under electrophoresis as occurs during hydrolysis with chymosin; but it would be hasty to assert that these products included $\kappa$-paracasein.

$\beta$-Casein hydrolysates, $\beta$s1 and $\beta$s2, had low electrophoretic mobility and might be identical to $\gamma$-caseins. This should corroborate Law and Wigmore's observations (17) on analysis of ripened Cheddar cheese made with a *Bacillus subtilis* neutral protease.

![Figure 4](image-url)
BACILLUS SUBTILIS NEUTRAL PROTEASE

Although production of NPN from \( \alpha_\kappa \)-casein was slower than that from \( \beta \)-casein (23, 24), electrophoresis showed that the \( \alpha_\kappa \)-casein band quickly disappeared as did its hydrolysate. This occurred in spite of their almost equal enzymatic affinity (Table 1). Whey proteins hydrolysis was slower than casein hydrolysis. \( \beta \)-Lactoglobulin and immunoglobulins were proteolyzed more strongly than \( \alpha \)-lactalbumin. Similar results have already been presented (32) with respect to \( \beta \)-lactoglobulin hydrolysis with animal proteases. Within our laboratory, other studies also have shown higher enzymatic sensitivity of \( \beta \)-lactoglobulin than \( \alpha \)-lactalbumin (33).

CONCLUSION

A neutral protease preparation from Bacillus subtilis acted on all casein fractions and on major whey proteins. In spite of its strong proteolytic activity, this protease was more specific, than vegetable proteases on the main milk proteins. This protease was not considered as a coagulating protease in the strictest sense because of the detrimental effects that resulted from excessive proteolysis (29). As we have suggested (27), it can be used at a small concentration or mixed with rennet. However, its activity on all the whey proteins and its strong thermophilic character were of interest for further developments in dairy processings. Biochemical studies are being undertaken to extend the applications of this process to other fields of the food industry.

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REFERENCES

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