Food Chemistry 47 (1993) 209–212
Analytical Methods Section

Quantitative determination of chymosin activity by thrombelastography

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(Received 24 June 1992; revised version received and accepted 16 September 1992)

An accurate and objective thrombelastographic procedure for the quantitative determination of chymosin in solution and in cheese whey is proposed. Linear relationships between coagulation time and the inverse of chymosin concentration in solution showed determination coefficients (r^2) in the range 0·989–0·997. A satisfactory reproducibility was achieved, with 0·88% as mean coefficient of variation. Determination coefficients for linear relationships between coagulation time and the inverse of chymosin present in whey ranged from 0·995 to 0·998. Reproducibility of chymosin quantification in whey was high, with 0·98% as mean coefficient of variation.

INTRODUCTION

Determination of chymosin and other enzymes used as milk coagulants is a major concern in industrial cheese making and in cheese research (Carlson *et al.*, 1985). Whey concentrates are used as food ingredients. Thus, it is important to know the amount of residual coagulant in those not thermically treated, in order to prevent defects arising in foods during processing or storage.

Assay techniques for chymosin and other coagulants include immunological methods, determination of proteolytic activity and estimation of clotting activity. Immunological methods quantify the concentration of milk coagulants, but not their enzymatic activity (Rothe *et al.*, 1976). Proteolytic activity of milk coagulants may be determined using substrates such as milk, casein, haemoglobin and synthetic peptides (Raymond *et al.*, 1973). Casein-agar diffusion techniques are accurate and sensitive, but assays take up to 48 h to complete (Lawrence & Sanderson, 1969).

Chymosin (EC 3.4.4.3) determinations mainly rely on its clotting activity on milk (Raymond *et al.*, 1973), because of the low proteolytic activity of this enzyme. Clotting point determination (Berridge, 1952) is a laborious and subjective technique based on visual observation of the coagulation point of the milk in tubes placed in a rocking or rotating device. Although the subjectivity of this procedure has been criticized (Kopelman & Cogan, 1976), it continues in use with some modifications for the determination of milk coagulants (Carlson *et al.*, 1985; International Dairy Federation, 1987; Singh & Creamer, 1990).

The increase in viscosity during milk coagulation may be precisely monitored by means of the thrombelastograph, a twisting viscosimeter (Frentz, 1965). The purpose of this investigation was to study the suitability of the thrombelastograph for the quantitative determination of chymosin in solution and in cheese whey.

MATERIALS AND METHODS

Materials

Chymosin used was Maxiren (Gist Brocades NV, Delft, The Netherlands), a commercial preparation of recombinant chymosin obtained from *Kluyveromyces lactis*, with a declared content of 900 μg chymosin/ml. Raw milk was dispensed in 500 ml aliquots, held at −40°C and thawed before use. Coagulation of milk was followed by means of a Hellige thrombelastograph model D (Hellige GmbH, Freiburg im Breissgau, Germany) at 34°C. Phosphate buffered saline (PBS) was adjusted at pH = 6·5. Standard solutions of chymosin were prepared by diluting the enzyme in PBS. Chymosin exposure to light was minimized to avoid inactivation. Wheys used were from cheesemaking trials at the Instituto Nacional de Investigaciones Agrarias.

Determination of chymosin in solution

Chymosin solution (1 ml) was added to 50 ml of milk at 34°C and mixed; 0·35 ml of this mixture was trans-

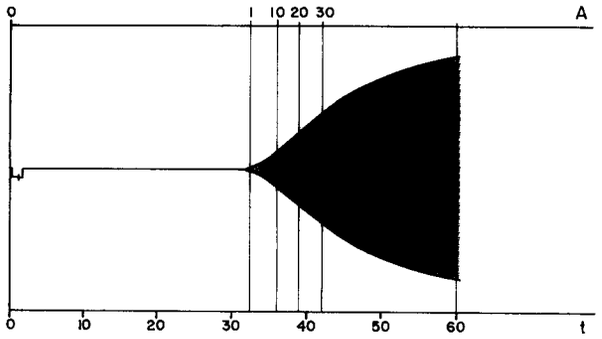

Fig. 1. Thrombelastographic curve showing coagulation times (t, min) for different curve amplitudes (A, mm).

ferred to each of the two vats of the thrombelastograph and the surface covered with vaseline oil. Times needed to reach 1, 10, 20 and 30 mm of amplitude were measured in the resulting graphic, of a diapason form (Fig. 1). The same steps were followed for problem solutions of unknown chymosin concentration and for standard solutions used in calibration curves. The experiment was carried out in triplicate. Regression equations of time on the inverse of chymosin concentration were obtained using the BMDP1R program (Department of Biomathematics, UCLA, Los Angeles, CA).

The reproducibility of this technique was investigated on four series, each consisting of six determinations on the same sample. Coefficients of variations were calculated from data obtained.

Determination of chymosin in whey

Whey (25 ml) at 8°C and 1 ml of PBS were added to 25 ml milk at 60°C and mixed; 0·35 ml of this mixture was transferred to each of the two vats of the thrombelastograph and the surface covered with vaseline oil. Times needed to reach 1, 5, 10 and 15 mm of amplitude were measured in the resulting graphic. For the calibration curve, 25 ml of whey heated to inactivate chymosin and cooled at 8°C and 1 ml of a standard chymosin solution in PBS were added to 25 ml of milk at 60°C. The rest of the steps and the calculation of regression equations were as described above. The experiment was carried out in triplicate.

For the determination of chymosin inactivation temperature 25 ml of whey plus 0·5 ml of a 100 μg chymosin/ml solution were heated at 55, 60, 65 or 70°C for 10 min and thrombelastographic curves obtained as described above were compared. To evaluate the influence of whey protein denaturation on milk coagulation, samples of 25 ml of whey were heated at 65, 70, 75, 80, 85 or 90°C for 10 min and thrombelastographic curves obtained as described above were compared. Influence of acidification in problem wheys due to lactic starter bacteria was investigated by adding 10 UI penicillin/ml and comparing thrombelastographic curves to those obtained for the same whey without penicillin.

The reproducibility of this technique was investigated as described for the determination of chymosin in solution.

Chymosin activity during cheesemaking

The proportion of chymosin found in whey was determined in duplicate cheesemaking trials on a laboratory scale. Chymosin (60, 90, 120 or 150 μg) and lactic starter (5 ml of a coagulated culture in skim milk) were added to milk (500 ml) held at 30°C. Curds were cut into 6–8 mm cubes and scalded at 37°C for 15 min before whey drainage. Chymosin activity in whey was analysed in duplicate as described above. Analysis of variance was performed on chymosin recovery data using the BMDP8V program (Department of Biomathematics, UCLA, Los Angeles, CA).

Thermal inactivation of chymosin in whey at cheesemaking temperatures was investigated in duplicate trials. Chymosin (80 μg) was added to whey (500 ml), previously heated at 70°C for 10 min, and held at 37°C. Whey aliquots (25 ml) were analysed for chymosin activity in duplicate at 30 min intervals as described above. The regression equation of residual chymosin activity on time of incubation was obtained using the BMDP1R program.

RESULTS AND DISCUSSION

Determination of chymosin in solution

The best linear relationship between time (t) needed to reach a certain amplitude (A) and chymosin concentration (RC) was obtained for $t = t_0 + K_1/RC$, where t_0 is a delay time characteristic of the system and K_1 is a constant. Other linear relationships tested were $t = a + b \times RC$ and $RC = a + b/t$. Table 1 shows regression equations of time on the inverse of chymosin concentration for different curve amplitudes. Linear calibration curves were obtained for chymosin concentrations in the range 3–15 μg/ml, which corresponded to coagulation times of 11–41 min for an amplitude of 1 mm.

Higher determination coefficients were recorded for an amplitude of 1 mm than for larger amplitudes, as shown by the data from three trials in Table 2. These results are in agreement with the work by Marshall *et al.* (1982), who concluded that the early stages of curd-firming are affected more by rennet concentration than the later stages.

Determination coefficients obtained in the present

Table 1. Regression equations of time[a] on the inverse of chymosin concentration[b] in solution for different amplitudes[c] of thrombelastographic curves (trial 1)

A	n[d]	Equation
1	10	$t = 3{\cdot}555 + 111{\cdot}782/C$
10	10	$t = 4{\cdot}417 + 123{\cdot}526/C$
20	10	$t = 5{\cdot}270 + 132{\cdot}131/C$
30	10	$t = 6{\cdot}143 + 142{\cdot}239/C$

[a] Time (t) to reach a certain amplitude, in minutes.
[b] Chymosin concentration (C) is expressed in μg/ml.
[c] Amplitude (A) of the thrombelastographic curve, in mm.
[d] Number (n) of determinations.

Table. 2. Determination coefficients (r^2) for the regression equations of chymosin in solution with different amplitudes[a] of thrombelastographic curves

A	*n*[b]	Trial 1	Trial 2	Trial 3
1	10	0·997	0·997	0·989
10	10	0·997	0·987	0·991
20	10	0·997	0·986	0·989
30	10	0·997	0·986	0·990

[a] Amplitude (*A*) of the thrombelastographic curve, in mm.
[b] Number (*n*) of determinations.

work are similar to those published for rennet and chicken pepsin using a rotational type viscosimeter (Kopelman & Cogan, 1976) and for calf rennet using a Formagraph (McMahon & Brown, 1982).

A satisfactory reproducibility was achieved with our technique, as coefficients of variation for an amplitude of 1 mm were as low as 0·48–1·08, with 0·88 as mean value of the four series of determinations. McMahon and Brown (1982) had reported values of 1·41–2·86 and Marshall *et al.* (1982) values of 2·6–3·5. The International Dairy Federation (1987) recommended that the relative difference between two measurements should not be higher than 2%.

Determination of chymosin in whey

A temperature of at least 60°C was necessary to inactivate (in 10 min) chymosin present in whey. When whey was heated at higher temperatures (65–90°C) and added to milk, no significant effect of heating temperature on thrombelastographic curves was observed, excluding the influence of denatured whey proteins on milk coagulation. Although a temperature of 80°C has been suggested to inactivate milk coagulants in whey (Singh & Creamer, 1990), heating at 70°C for 10 min was estimated to be sufficient for the inactivation of chymosin and was used thereafter.

Addition of penicillin to problem wheys had no influence on thrombelastographic curves. It may be concluded that acidification by starter bacteria present in whey had no significant effect on milk coagulation, due to the short time needed to run the assay.

The best equation for recombinant chymosin determination in whey was also $t = t_0 + K_1/RC$. Regression

Table 3. Regression equations of time[a] on the inverse of chymosin concentration[b] in whey for different amplitudes[c] of thrombelastographic curves (trial 1)

A	*n*[d]	Equation
1	10	t = 4·051 + 3·013/C
5	10	t = 5·518 + 3·641/C
10	10	t = 7·780 + 4·221/C
15	10	t = 11·151 + 5·104/C

[a] Time (t) to reach a certain amplitude, in minutes.
[b] Chymosin concentration (*C*) is expressed in μg/ml.
[c] Amplitude (*A*) of the thrombelastographic curve, in mm.
[d] Number (*n*) of determinations.

Table 4. Determination coefficients (r^2) for the regression equations of chymosin in whey with different amplitudes[a] of thrombelastographic curves

A	*n*[b]	Trial 1	Trial 2	Trial 3
1	10	0·995	0·998	0·997
5	10	0·994	0·996	0·994
10	10	0·989	0·994	0·992
15	10	0·979	0·984	0·984

[a] Amplitude (*A*) of the thrombelastographic curve, in mm.
[b] Number (*n*) of determinations.

equations of time on the inverse of chymosin concentration for different curve amplitudes are shown in Table 3. Linear calibration curves were obtained for chymosin concentrations in whey ranging from 0·11 to 0·31 μg/ml. According to data in Table 4, the highest determination coefficients were always found for an amplitude of the thrombelastographic curve of 1 mm.

The reproducibility of the determination of chymosin in whey was satisfactory. Coefficients of variation recorded in the present work for an amplitude of 1 mm ranged from 0·63 to 1·22, with 0·98 as mean value for the four series. Carlson *et al.* (1985) obtained coefficients of variation of 3·63–4·57 when analysing rennet in whey, using a technique based on visual determination of the clotting point.

Chymosin activity during cheesemaking

Percentage recovery of chymosin activity in whey was significantly ($P < 0·001$) influenced by the amount of chymosin added to milk. A higher recovery was obtained for whey from milk to which larger amounts of chymosin had been added (Table 5).

Thermal inactivation of chymosin took place in whey held at 37°C, although it proceeded at a slow rate

Table 5. Chymosin activity[a] found in whey from milk coagulated with different amounts of chymosin

Chymosin added to milk (μg)	Chymosin found in whey (μg)	Recovery in whey (%)
60	43·9	73·1
90	70·8	78·7
120	95·4	79·5
150	116·9	77·9

[a] Average values from duplicate determinations on two 500 ml cheesemaking trials.

Table 6. Thermal inactivation[a] of chymosin in whey at 37°C

Time of incubation (min)	Residual activity (%)
30	95·6
60	94·8
90	93·2
120	92·5
150	91·4
180	90·6

[a] Average values from duplicate determinations on two trials.

(Table 6). The regression of chymosin activity on time of incubation in whey at 37°C was linear ($P < 0{\cdot}001$). Whey incubation at 37°C for 3 h resulted in only a 10% chymosin inactivation.

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