

# CHARACTERIZATION OF COLBY CHEESE MADE WITH FISH ENZYMES

JOAQUIM F. PONTE TAVARES, ARTHUR HILL, RICK YADA & ELIZABETH GULLET

## ARQUIPÉLAGO



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Queijo tipo "Colby" foi fabricado, em quatro ensaios, com extractos de pepsina extraídas de estômagos de atum e de bacalhau, usando-se como testemunho um extracto de estômago de vitelo ("rennet" comercial). Os extractos enzimáticos foram padronizados, de modo a que os volumes usados conduzissem aos mesmos tipos de coagulação do leite, usando-se para o efeito, como substrato de Ca, uma solução de "Berridge" a pH 6.3. Os tempos de coagulação foram cerca de duas vezes superiores aos de extractos de vitelo. As texturas e os rendimentos em queijo, obtidos por acção dos três extractos enzimáticos não diferiram significativamente. Os queijos foram analisados depois de estarem um mês numa câmara de cura a 10°C. As análises sensoriais de todas as amostras de queijo revelaram o desenvolvimento de um gosto amargo. Microfotografias das microestruturas das coalhadas e dos queijos com 1 mês de cura, tiradas com um microscópio electrónico, não revelaram diferenças significativas entre as amostras analisadas. Pode concluir-se que o grau de pureza das pepsinas de atum e bacalhau podem produzir queijo tipo "Colby" aceitável que não requiere mais de três meses de cura, e que como fundido, as enzimas usados são inactivados pelas temperaturas elevadas a que são submetidos. Purificações posteriores dos extractos enzimáticos poderão melhorar a especificidade das enzimas e, embora a custos mais elevados, poderão reduzir a lentidão da proteólise.

Joaquim F. Ponte Tavares, Universidade dos Açores, Rua da Mãe de Deus, PT-9500 Ponta Delgada, Açores, Portugal. - Arthur Hill, Rick Yada & Elizabeth Gullet, Department of Food Sciences, University of Guelph, Guelph, Ontario N1G 2W1, Canada.

## INTRODUCTION

Traditionally the abomasum of suckling calves has been used as the source of rennet for production of Colby type cheese. This enzyme is of special value in cheese-making because it converts milk casein into curd in such a way as to give high yields and desirable proteolysis in the aged cheese. Most proteases are inferior to rennet for cheese-making because of their broader specificity

for protein substrates. Excessive proteolysis during curdling and subsequent steps in the cheese-making operation lead to defects in the product. The high cost of commercial rennet has sparked interest in the possible use of rennet substitutes. Foremost among such possible substitutes are the proteases from the stomach of fishes. Some gastric proteases from marine organisms are relatively unstable at temperatures above 30° C. It follows that it may be possible to rennet milk and

subsequently heat denature the coagulating enzyme during subsequent steps in the cheese-making process. According to BREWER et al. (1984) it is possible to prepare a satisfactory cheddar cheese with Atlantic cod pepsin as a rennet agent. When the conventional cheddar cheese process is employed, there is somewhat more loss of fat and protein to the whey fraction, indicating that additional protein degradation occurs during formation and the early stage of cheddaring. The high molecular activity of fish pepsins at low reaction temperatures also occurs when milks are treated to initiate the clotting process. Cold renneting of milk with a catalyst having a low temperature coefficient for the enzyme can be accomplished with lower enzyme concentration.

TAVARES & RAND (1980) published the first study on the milk clotting properties and successful application of fish pepsins to cheese manufacture. They found that tuna gastric enzymes may, in fact, be closer to calf rennet for cheese manufacture than other animal pepsins. These findings were substantiated by BREWER et al. (1984) using isolated cod pepsin.

In 1982, MACCABE & RAND reported the isolation and purification of zymogen for proteolytic enzymes from the gut portion of flounder. Mozzarella type cheese was made and only the flavour was judged slightly inferior to the standard. When tested in a cooked product the flavour, texture and overall acceptability were higher.

These investigations clearly demonstrated that the gastric and pancreatic fish proteases from many species of fish could be potential sources of milk coagulating enzymes.

In the present study Colby-type cheese was made using enzymes extracted from the stomachs of cod (*Gadus morhua*) and bigeye tuna (*Thunnus obesus*), in addition to commercial rennet. The cheeses obtained by these three methods were subjected to sensory analysis and electron microscopy.

## MATERIAL

**Reagents and instruments.** Low heat and nonfat dried milk powder were purchased from New Dundee Creamery, New Dundee, Ontario, Canada. Commercial rennet was provided by Dairy and Food Laboratories (Waukesha, Wisconsin).

Meat grinder, Waring blender, Polytron homogeniser, Beckman L8-70 ultracentrifuge unit, Namer Viscometer (Namer-Co. Edison, N. J.), IBM compatible computer via an analog-to-digital converter, Scanning electron microscope, and equipment of the cheese pilot plant of the University of Guelph were used. Reagent grade chemicals and distilled water were used throughout.

## METHODS

**Batch extraction and activation.** Atlantic cod (*Gadus morhua*) and bigeye tuna (*Thunnus obesus*) were caught from fisheries zone 3 km off the coast of Atlantic Canada and from deep North Atlantic Ocean in proximity to Azores Islands, respectively. They were obtained from Canada Institute Fisheries Technology and from University of the Azores (Department of Oceanography and Fisheries), respectively. The stomachs received frozen in dry ice, were thawed, split cleaned and briefly rinsed 3 times in tap water. The tissue chopped into small pieces using a sharp knife was mixed with equal weight of distilled water (DW) containing 10% NaCl of total weight (tissue and DW). On the following day the mixture was transformed into a slurry by means of a meat grinder and a blender. Sometimes only the polytron homogenizer was used to obtaining the enzyme extract. The slurry was ultracentrifuged in a Beckman L8-70 M ultracentrifuge at 35,000 x g for 60 min. The supernatant obtained from ultracentrifugation, designated as crude pepsinogen, was employed for cheese making after activation of the zymogen. Pepsinogen extracts were stored at -20° C and were activated just prior to use. Activation was accomplished by adding 0.1 N HCl to pH 4.0, holding at room temperature for 60 min and re-adjusting the pH to 5.0 with 0.1 NaOH.

**Enzyme assay.** Pepsin activity was assayed by the measurement of coagulation time and curd firmness of milk using a Namer Viscometer. Fresh low heat skim milk powder (97% total solids) at pH 6.7-6.8 was reconstituted to 9%, 11%, 12%, 13%, and 15% T.S. and stored overnight. The milk, plain or with added milk fat, homogenized or not, was warmed and held at the coagulation temperature of 32° C for 1 hr prior to the addition of enzyme extract.

Single strength commercial rennet was used in concentration of 0.4 ml/l of milk and compared with cod and tuna stomach protease extracts in concentrations of 2.5 ml for the cod enzyme and of 20 ml, 30 ml, 40 ml, and 50 ml, for the tuna enzyme per liter of milk, respectively. The coagulation temperature of 32°C was chosen to assess the suitability of Nameter Viscometer for determining the effects of enzyme concentration on the rate of milk aggregation.

**Cheese manufacture.** Colby cheese was produced using the tuna and cod pepsin preparations, and with calf rennet as a control, in four trials. The enzymes were standardized to the same milk-clotting strength on Berridge as substrate of Ca at pH 6.3; the setting times were a little more than twice as long as calf. Cheeses were stored under 10°C and analysed one month after production. Sensory analysis of the products was done.

**Sensory analysis.** Samples of Colby cheese made with rennet and enzymes from cod and tuna were evaluated by 100 consumers in the sensory evaluation facilities at the University of Guelph. Of the 100 judges, 60 were female and 40 male with a large proportion (79%) between the age of 18 and 25; 19% were 26 to 45 years of age. Ninety percent of judges consumed mild cheddar cheese types. Four replications were evaluated, 25 consumers evaluated each rep. Cheeses were presented at room temperature in covered 30 ml sample cups which had been coded with 3-digit codes. Judges were instructed to use the unsalted crackers and water provided, to clear their palates between samples. Preference for flavour, texture, and overall preferences were evaluated using the 9-pt hedonic scale (9 corresponded to very good). In addition, acceptability was assessed as definitely accept, perhaps accept, perhaps reject, and definitely reject. Data were collected on the computerized sensory data collection system of Compusense, Guelph, Ont. Canada. Presentation of samples was randomized among judges. Participants were screened for preference for cheddar type cheese. Hedonic data were analysed by ANOVA and by Tukeys Honestly Different Test, using SAS (version 1.0). Judge effect was included in the error term as this was an untrained effective panel of judges.

**Scanning electron microscopy.** Also observed was the fine structure of milk curds (Fig. 1) and cheese samples (Colby type) (Fig. 2) made by different milk coagulating enzymes by using the scanning electron microscope (Hitachi S-570). The procedure for sample preparation was that of SAIO (1981) with some modifications. Small pieces (< 2 mm cube) were fixed at room temperature with 2% glutaraldehyde in phosphate buffer (0.07 M  $\text{Na}_2\text{HPO}_4 + 0.07 \text{ M } \text{KH}_2\text{PO}_4$ -pH 5.8) for 90 min. After five washes in 0.1 M phosphate buffer (pH 5.8) at 10 min intervals, samples were postfixed in 1% osmium tetroxide in the same buffer for 90 min at room temperature. The dehydration was done using a 10% incremental ethanol (50, 60, 70, 80, 90, and 95%) series, leaving samples at each concentration for 15 min followed by three rinses with 100% ethanol. The samples were then rinsed three times with chloroform. Critical point drying (CPD) was conducted using  $\text{CO}_2$ .

For freeze drying, the samples were dehydrated using the ethanol series, frozen in liquid nitrogen and transferred to a Polaron E 5300 freeze drier and dried for 24 hr.

All of the samples were mounted on stubs and sputter coated with 20-30 nm of gold palladium (60: 40) using Anatech Hummer VII Sputter coater. The observations were made at 10 KV.

## RESULTS AND DISCUSSION

**Evaluation of Colby Cheese.** Sensory analysis. Consumer acceptability of Colby cheese made with rennet, cod enzymes and tuna enzymes was determined.

ANOVA showed that treatment effect was significant for preferences for flavour, texture, and overall preference (Table 1). A significant rep effect was obtained for flavour and overall preference. Significant treatment x rep interactions were obtained for flavour, texture, and overall preference. These significant effects indicated that there was a high degree of variability within the treatments. Flavour scores for rep 2 were significantly lower than for the other replications (Table 2). Examination of the interactions determined this result from a lower preference for rennet cheese flavour for this rep. Tukeys Honestly Different Test did not reveal any significant difference between replication means for overall preference, indicat-

ing that the difference here probably resulted from a difference in variability around the mean. Examination of the treatment x rep interactions showed the cheeses made with tuna enzymes were more variable among reps. Although all the cheeses varied, this differed for cheese and rep. Rep 3 for tuna appeared to be quite different from the other reps and was more preferred.

Mean scores and SD for treatment effects are shown in Table 3. Cheese made with tuna enzymes was less preferred ( $P<0.05$ ) for flavour and overall preference than that made by rennet or cod enzymes. Cheeses made from cod and tuna enzymes were preferred less for texture ( $P<0.05$ ) than that made with rennet. SD obtained are large indicating a spread in the ratings. Examination of the frequency for scores obtained for each cheese showed a very high proportion in the dislike range for all the cheese (Table 4). This was particularly evident for flavour, suggesting the cheeses had not sufficiently ripened when the test was conducted at one month of age. The number of ratings obtained for preference for tuna enzyme cheese compared to the cod enzyme cheese for texture and overall preference probably reflects the high rat-

Table 1  
ANOVA for Colby cheese made with rennet, cod and tuna enzymes.

Source	df	SS	MS	F value	Pr>F
Flavour					
Treat.	2	38.887	19.443	4.21	0.0157
Rep.	3	36.947	12.316	2.67	0.0479
Treatxrep	6	86.793	14.466	3.13	0.0054
Error	288	1328.960	4.614		
Texture					
Treat.	2	95.387	47.693	11.61	0.0001
Rep.	3	17.213	5.738	1.40	0.2438
Treatxrep	6	109.547	18.258	4.45	0.0003
Error	288	1182.640	4.106		
Overall Preference					
Treat.	2	101.360	50.680	11.60	0.0001
Rep.	3	37.947	12.649	2.90	0.0355
Treatxrep	6	103.013	17.169	3.93	0.0009
Error	288	1257.760	4.367		

df =degrees of freedom (of treatments-1)  
MS=mean squared  
SS =sum of squares  
F = Frequency value. Very discriminatory between samples.  
Pr>F. Anything below 0.05 is significant.

Table 2  
Mean scores<sup>1</sup> for replications of Colby cheeses made by rennet, cod and tuna enzymes.

Rep.	Flavour		Texture		Overall preference	
	M	SD	M	SD	M	SD
1	4.67ab <sup>2</sup>	2.26	5.31	2.13	5.08	2.27
2	4.37b	1.87	4.80	2.02	4.56	1.94
3	5.31a	2.35	5.44	2.16	5.42	2.22
4	4.56ab	2.35	5.23	2.34	4.61	2.43

<sup>1</sup> - n=75

<sup>2</sup> - means within a column not followed by the same letter are significantly different ( $P<0.05$ ).

M =mean scores;

SD=standard deviation.

ings obtained for this cheese in rep 3.

Examination of the accept/reject data (Table 5) supports the evidence that the cheese was not liked. Only 23% would definitely accept the rennet, and this was reduced to 14 % and to 15% for the cod and tuna fish enzymes, respectively. These data were supported by comments of judges, and it became difficult to get people to participate as negative feed-back occurred. The cheese made with the tuna enzyme developed beads of moisture on the surface when it was cut indicating a poor ability to hold water.

**Microstructure.** Scanning electron micrographs of milk curd and of Colby-type cheese samples. Most cheeses are made from whole milk, the coagulation of which is achieved by a combined action of starter lactic acid bacteria and a proteolytic coagulant such as rennet.

Scanning electron microscopy (SEM) is better suited for studies of more advanced stages of

Table 3  
Mean scores<sup>1</sup> for hedonic ratings of Colby cheeses made with rennet, cod and tuna enzymes.

Treat.	Flavour		Texture		Overall preference	
	M	SD	M	SD	M	SD
Rennet	5.15a <sup>2</sup>	2.16	5.90a	1.94	5.60a	1.97
Cod	4.76ab	2.09	5.16b	2.02	4.98a	2.03
Tuna	4.27b	2.37	4.52b	2.31	4.18b	2.46

<sup>1</sup> - n=100

<sup>2</sup> - means within a column not followed by the same letter are significantly different ( $P<0.05$ ).

M =mean scores;

SD=standard deviation.

Table 4

Percent of judgements in low preference and high preference categories.

	Flavour Preference		Texture Preference		Overall Preference	
	low %	high %	low %	high %	low %	high %
Rennet	51	15	23	25	37	18
Cod	63	8	26	9	58	9
Tuna	64	7	64	13	67	12

cheese-making than the initial stages of milk coagulation, as it is considerably easier to examine the more compact structures produced after the removal of excessive water in the form of whey. Substantial changes take place in the ratios of the remaining substances forming the curd. Most of the lactose and water are removed with whey and the curd is composed mainly of casein (=24%), fat (=34%), and salts (=5.5%). There is approximately 35% water in Cheddar cheese.

Casein micelles in milk aggregate during cheesemaking and fat globules are entrapped in the coagulum. To study the microstructure of curd by SEM it is necessary to remove the fat unless a cold stage is used.

In freshly cheddared curd, for example, the macrostructure of stretched granules is reflected at the microscale by the development of protein fibres which may be observed by SEM. Following the removal of the tensile force, however, the nature of the fibrous microstructure is rapidly changed as the protein fibres in the matrix contract, whereas the parallel orientation of the curd granule junctions is preserved. In addition to protein, fat as the other major component of cheese was found to play an important role in cheese processes. This indicates that the exclusive use of

SEM in the examination of cheese is of limited value. Nevertheless, SEM was used alone in studying the effect of calf rennet and substitutes such as bovine pepsin (EINO *et al.* 1976) and porcine pepsin on the microstructure of cheddar cheese. The traditional calf rennet cheese was softer but was found to be more compact during the first 6 months of ripening than the bovine pepsin cheese.

The microstructure of curd granule junctions in cheese was studied by KAALAB (1977). The junctions, which appeared as dark veins under a light microscope, were found by SEM to contain considerably less fat and, hence, more protein than the interior portions of the curd granules. This was evident from the high number of empty cavities in the interior areas of the curd granules following the extraction of fat in contrast to very few cavities found in the curd granule junctions. Consequently, the areas rich in cavities scattered light and appeared lighter than the compact junctions. The differences in the microstructure of the junctions and the interior portions of the granules should be taken into consideration when cheeses are subjected to electron microscopy.

According to what was described above, casein micelles, whey proteins, fat globules, and lactose as the major constituents of milk are capable of undergoing a great variety of changes, particularly under the effects of proteolytic enzymes and/or lactic bacteria, leading to various dairy products. The microstructure of such dairy products is controlled, to a great extent, by the manufacturing processes. The microstructure determines some properties of the product, for example viscosity, syneresis, firmness and texture.

Based on this knowledge, the scanning electron micrographs of the one month cheeses were studied. No major differences between tuna and cod pepsin cheese compared to the control were revealed (Figs. 1 and 2). It would appear that this level of purity of tuna and cod pepsin might be capable of producing acceptable Colby cheese for applications that do not require aging of more than three months and that use heat to inactivate the enzyme, such as processed cheese spread. Further purification may improve the specificity of the enzyme and reduce delayed proteolysis, but would increase, concomitantly its cost.

Table 5

Percent definitely accept and of definitely reject assessments.

Treatment	Definitely accept %	Definitely reject %
Rennet	23	16
Cod	14	23
Tuna	15	49

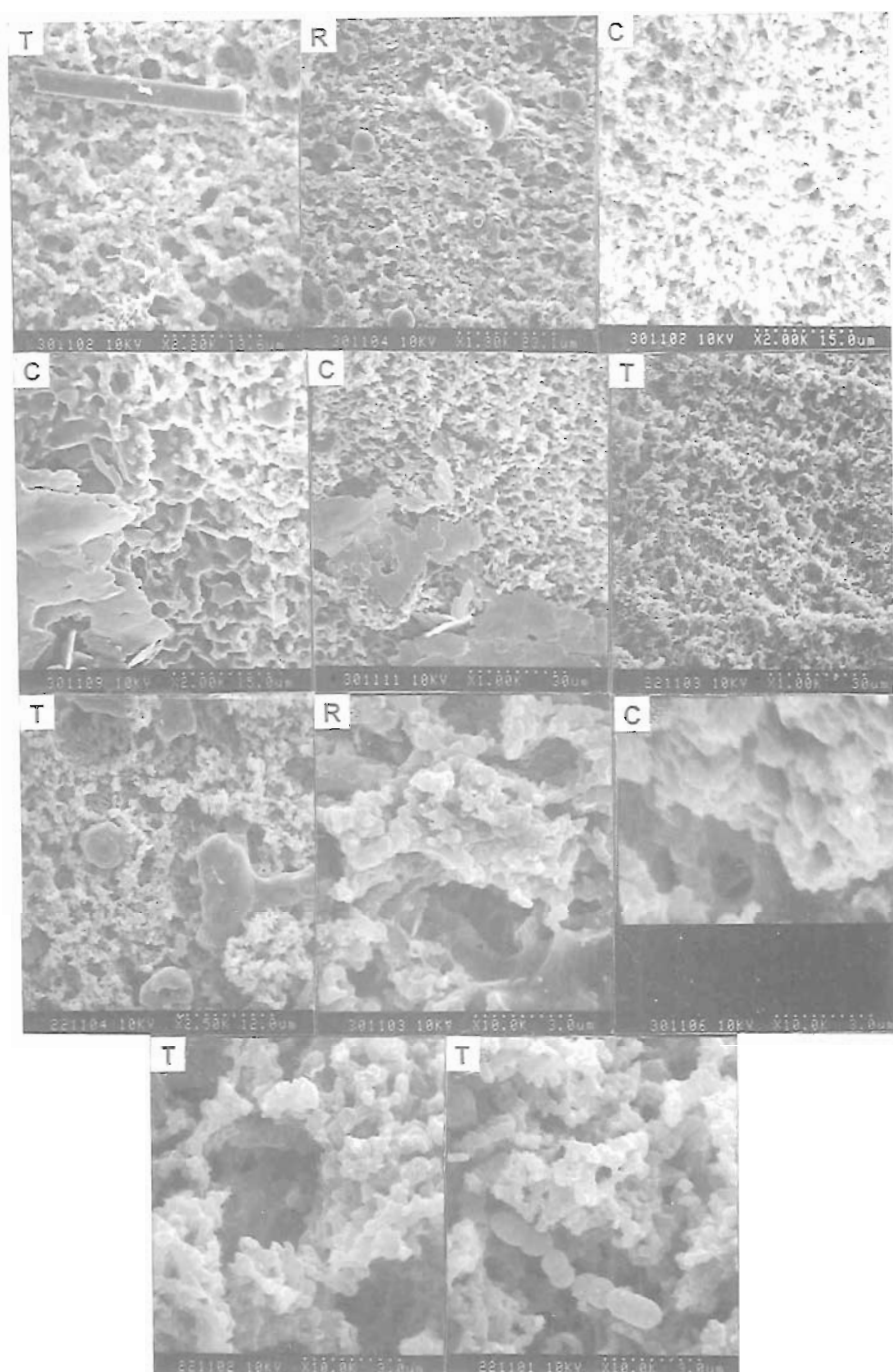


Fig. 1. Scanning Electron Microscopy (SEM). Images of freeze dried milk curds made with Commercial Rennet (R) as control, Cod Stomach Enzyme (C), and Tuna Stomach Enzyme (T). Images Nos. 301102, 301104, 301108, 301109, 301111, 221103, and 221104 were obtained at amplification between \* 1.00 K, and \*2.50 K. Images Nos. 301103, 301106, 221101, 221102 were obtained at amplification \*10.0 K.



## CONCLUSIONS

1. The process of curd formation using proteases from the stomach of cod and tuna showed no difference when compared to the conventional method.

2. The structure of the curds was studied with the electron microscope, and no significant differences were detected between those made with commercial rennet, and those made with the substitutes (cod and tuna stomach enzymes).

3. The cheeses were submitted to a taste panel,

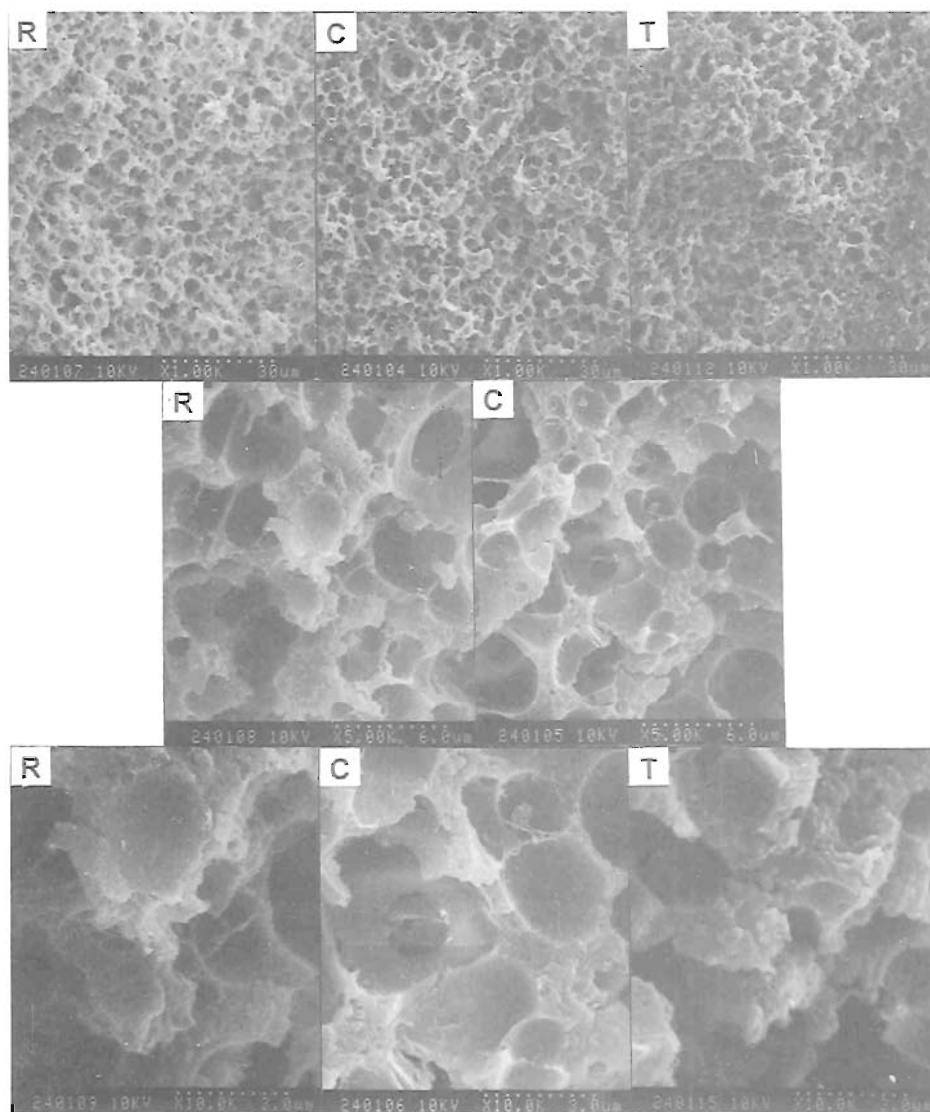


Fig. 2. Scanning Electron Microscopy (SEM). Images of freeze dried cheese samples (Colby type) made with Commercial Rennet (R), as control, Tuna Stomach Protease (T), and Cod Stomach Protease (C). Images Nos. 240107 (R), 240104 (C), and 240112 (T) were obtained at amplification \*1.00 K. Images Nos. 240108, and 240105 were obtained at amplification \*5.00. Images Nos. 240109, 240106, and 240115 were obtained at amplification \*10.0 K. No substantial differences were noted among the samples of cheeses made with the above mentioned three enzymes.

and no significant organoleptic or textural differences were reported.

4. None of the cheeses were of acceptable commercial quality, probably because they were not sufficiently ripened at time of testing.

5. The cheeses made with cod enzymes were less acceptable than that made with commercial rennet, and this is not likely to be corrected by further ripening.

6. The cheeses were highly variable between replications.

7. It appears that the use of these fish enzymes, as rennet substitutes, is technologically feasible.

8. Further studies to characterize the efficacy of tuna and cod pepsins in cheese processing are necessary to better evaluate their industrial potential.

#### ACKNOWLEDGEMENT

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#### REFERENCES

- BREWER, P., N. HELBING & N.F. HAARD 1984. Atlantic cod pepsin. Characterization and use as a rennet substitute. Canadian Institute. *Journal of Food Science. Technology* 17: 38-43.
- EINO, M.F., D.A. BRIGGS, D.M. IRVINE & D.W. STANLEY 1976. A comparison of microstructure of Cheddar cheese curd manufactured with calf rennet bovine pepsin, and porcine pepsin. *Journal of Dairy Research* 43: 113-115.
- KAALAB, M. 1977. Milk gel structure. VI. Cheese texture and microstructure. *Milchwissenschaft* 32 (7): 449-458.
- MACCABE, V. & A.G. RAND Jr. 1982. Fish Stomach Enzymes for Cheese Manufacture. *Sea Grant Final Report*. University of Rhode Island, U.S.A.
- TAVARES, J.F.P. & A.G. RAND Jr. 1980. Abstract: Recovery of milk coagulating enzymes from tuna waste. *25th Atlantic Fisheries Technological Conference*. St. Johns. Newfoundland.
- SAIO, K. 1981. Microstructure of traditional Japanese soybean foods. *Studies of Microstructure*: 275-282.

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