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Purification and characterization of chymosin and pepsin from kid

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The objective of this work was to study the characteristics of the gastric aspartic proteinases chymosin and pepsin which are constituents of the kid rennet. The two enzymes were extracted from abomasal tissue of one kid from a local indigenous breed, separated from each other by DEAE-cellulose chromatography and then were purified by gel filtration and anion-exchange chromatography. The molecular weights of the purified kid chymosin and pepsin as determined by gel filtration were 36 kDa and 40 kDa respectively. The isoelectric point of kid chymosin was as multiple forms of 3–6 zones at pH 4·6–5·1, while that of kid pepsin was at pH $\leqslant$3·0. Kid pepsin contained 0·37 molecules phosphorous per molecule and was totally inhibited by 5 μM pepstatin A, being more sensitive than kid chymosin. Both enzymes were almost equally as proteolytic as calf chymosin on total casein at pH 5·6. Kid pepsin activity was more pH and temperature dependent than kid chymosin activity. In comparison with the calf chymosin temperature sensitivity, the order of increased sensitivity was: calf chymosin <kid chymosin <kid pepsin.

Keywords: Milk clotting enzymes, kid chymosin, kid pepsin.

Among the different kinds of milk clotting enzymes, the gastric aspartic proteinases, chymosin (EC 3.4.23.4) and pepsin A (EC 3.4.23.1) are the most commonly used. These are secreted as prochymosin and pepsinogen in the forth stomach of ruminants and their ratio depends on age and feeding regimes of the animals. The younger the animal, fed with milk, the more prochymosin is secreted (Andren, 1992). Calf rennet that contains high percentage of chymosin is considered to be the best but, nowadays, as older cattle are slaughtered, bovine rennets that contain high percentage of pepsin are widely used in cheesemaking. In addition, liquid rennets or rennet pastes from small ruminants are used in the manufacture of special Italian, Spanish or Greek cheeses (Guinee & Wilkinson, 1992). Bovine, lamb and kid rennets have been characterized by Barzaghi & Rampilli (1996) and Jordan et al. (1999). However, for better understanding of rennet properties, characterization of chymosins and pepsins individually is needed.

Calf chymosin, bovine pepsin and their zymogens have been extensively studied in terms of molecular, physicochemical and technological characteristics (Chow & Kassell, 1968; Foltmann et al. 1977, 1979; Uchiyama et al. 1981; Martin & Corre, 1984). Lamb chymosin and sheep pepsin have been also characterized (Fox et al. 1977; Baudys et al. 1988; Pungercar et al. 1990; Rogelj et al. 2001), while there is limited information on characteristics of kid chymosin or goat pepsin (Amourache & Vijayalakshmi, 1984; Suzuki et al. 1999).

The aim of this work was to purify kid chymosin and pepsin using different procedure than those described in the previous studies, to study some of their physicochemical and technological properties and finally to have information that may be useful in cheesemaking.

Materials and methods

Extraction of enzymes

The abomasum of a kid 25 days old from a local indigenous Greek breed, which had been fed with milk and grass, was excised immediately after slaughter and the tissue was separated, washed, salted and air dried. The dried tissue was cut manually to small pieces, homogenized in a STOMACHER LAB BLENDER 400 (Seward medical, London, UK) with 150 ml NaCl solution (60 g/l) containing

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boric acid (20 g/l) and then extracted overnight at 4 °C under continuous stirring. The crude extract was taken after centrifugation at 6000 ***g*** for 30 min and the tissue was re-extracted with 150 ml of the above solution. The crude extracts from the two successive extractions were combined and activated with 1 M-HCl at pH 2·0 for 30 min. The pH was finally adjusted to 5·5 with 1 M-NaOH.

Isolation of chymosin and pepsin

The two enzymes were initially separated from each other by anion-exchange chromatography according to the International Dairy Federation (IDF) method (1997a). The crude extract was dialysed against 2 × 3 l distilled water for 8 h using a dialysis membrane (Sigma Chemical Co., St Louis, MO 63118, USA) with molecular weight cut-off 12 kDa and was subsequently applied to a Pharmacia C10/10 column (Amersham Pharmacia Biotech, Uppsala, Sweden) packed up to 5 cm height with resin Fractogel EMD DEAE 650 M (Merck, Darmstadt Germany) and equilibrated with 25 mM-piperazine/HCl buffer, pH 5·3 (buffer A). Stepwise elution was performed initially with 51 ml 0·25 M- and after with 51 ml 0·5 M-NaCl in solution A at a flow rate 1·33 ml/min with the aid of a peristaltic pump P-1 (Pharmacia, Uppsala, Sweden). Fractions of 3 ml were collected and the absorbance was measured at 280 nm. The active fractions of chymosin peak as well as those of pepsin A peak were separately pooled and dialyzed against 2 × 5 l distilled water for 8 h and lyophilised.

Purification of chymosin

The lyophilised fraction of kid chymosin was dissolved (at a level of 14 mg/ml) in 50 mM-sodium citrate buffer – containing 0·2 M-NaCl and 0·2 g NaN_3/l, pH 5·6, filtered through 0·45 μm filter (Millex-HV, Millipore Corporation, Bedford, USA) and using the same buffer was subjected to gel filtration chromatography on a Sephacryl S200 26/60 (Pharmacia) column. The column was attached to an advanced protein purification system (WATERS, 34 Marple Street, Milford, MA 01757, USA) consisted of an helium degasser, one pump to mix four solvents (WATERS 600 Controller), a Rheodyne injector (model 7125, Rheodyne Inc., Cotati, California, USA) and a UV detector (WATERS 486). The data acquisition and processing were performed by the MILLENIUM v. 2.15 software (1994, WATERS Corp.). The flow rate was 1 ml/min, absorbance was measured at 280 nm and 3 ml fractions were collected. The active fractions were pooled, dialysed against 3 × 5 l distilled water for 12 h and lyophilised.

For further purification, the lyophilisate was dissolved in 6 ml buffer A (see above) at a level 0·5 mg/ml and applied to a high resolution column MonoQ HR 5/5 (Pharmacia) equilibrated with the same buffer and connected to the WATERS system. The elution was performed at a flow rate 1 ml/min and the gradient was 100% buffer A for 5 min, 0–30% buffer B (1 M-NaCl in solution A) over 40 min, 30–100% buffer B over 10 min.

Purification of pepsin

The lyophilised pepsin fraction from the DEAE–anion exchange chromatography was dissolved at a level 5 mg/ml in 6 ml 20 mM-sodium phosphate buffer containing 0·2 M-NaCl and 0·2 g NaN_3/l, pH 6·3 and subjected to gel filtration chromatography as described previously for the kid chymosin. The active fractions were pooled, dialyzed and lyophilised. Finally, the lyophilisate was dissolved in solution A and was further purified on MonoQ HR 5/5 column. Elution was carried out with 0–40% buffer B (1 M-NaCl in solution A) over 15 min and 40–100% buffer B over 60 min at a flow rate 1 ml/min.

Enzyme assay

The crude extract and the eluted fractions at each purification step were monitored for their milk clotting activity (MCA) according to the REMCAT method (IDF, 1997b). One unit was equal with 1 IMCU.

Protein determination

The protein contents of the crude extract and fractions were determined by the method described by Lowry et al. (1951).

SDS- and Urea-polyacrylamide gel electrophoresis

SDS-PAGE (12% T and 0·1% SDS in the separation gel and 2% SDS and 5% β-mercaptoethanol in the sample buffer) was carried out according to Laemmli (1970) in order to check the purification steps of each enzyme. It was performed on a mini electrophoresis unit (mini VE Hoefer, Pharmacia). Proteins were fixed with 15% TCA, stained with Coomassie blue R250 (Serva, Heidelberg) 2·5% in methanol : acetic acid : water (5 : 1 : 4) and de-stained with methanol : acetic acid : water (4 : 1 : 5) solution. The molecular weight marker 70L (Sigma) containing substances with *Mr* 14·2–66 kDa was also used. Urea-PAGE was carried out according to Andrews (1983) on the above electrophoresis unit. After electrophoresis, the gels were fixed, stained and de-stained as above.

Molecular weight and isoelectric point determination

The molecular weights of the purified enzymes were estimated by gel filtration on a Superdex 75 HR 10/30 (Pharmacia) column attached to WATERS system. Aliquots (100 μl) of the purified chymosin (0·02 mg/ml) and pepsin (0·12 mg/ml) were individually applied to the column, the elution was carried out with buffer A containing 0·2 M-NaCl and 0·2 g NaN_3/l and the absorbance was measured at 280 nm. The column calibration was achieved using the following proteins: Aprotinin (6·5 kDa),

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ribonuclease A (13·7 kDa), chymotrypsinogen A (25 kDa), ovalbumin (43 kDa) and bovine serum albumin (67 kDa). For comparison, the molecular weights of calf chymosin and bovine pepsin fractions isolated from calf rennet (Ha-La, Hansen) according to the IDF method (1997a) were also determined.

The pI of the purified enzymes was determined by isoelectric focusing (IEF) on a 0·2 mm polyacrylamide gel with pH range 2–7 containing 10% ampholytes as follows: Servalyte 2–4 (Serva) and Ampholine 2·2–5, 4–6·5 and 5–7 (Pharmacia) at a ratio 1 : 1 : 2 : 0·75 respectively. The analysis was performed on a LKB 2117 multiphor II apparatus (Pharmacia) with a power supplier LKB 2197 and refrigerator system DESAGA FRIGOSTAT (DESAGA, Heidelberg), under the conditions described by the Commission Regulation (1996). IEF markers with pI 3·6, 4·6 and 5·1 (Sigma) were also used.

Determination of organic phosphorous content of pepsin

Lyophilised purified pepsin (5 mg) was dissolved in 1 ml 0·1 M-sodium acetate buffer, pH 5·7 and dialyzed at first against 6 × 1 l of the same buffer for 24 h and then against 4 × 1 l bi-distilled water for 10 h, in order to remove any inorganic phosphate. The dialysed pepsin was lyophilised and then analysed according to the IDF method (1990).

Inhibition by pepstatin

Solutions 0·01, 0·1, 1·0, 10, 50 and 100 μM-Pepstatin A (Sigma) in ethanol : acetic acid 90 : 10, pH 3·0 were mixed with equal volume of 1 μM-purified enzyme solution and incubated for 5 min in duplicate. The MCA was measured on the modified Berridge substrate, pH 6·5 at 35 °C (IDF, 1997b).

Relative proteolytic activity

The relative proteolytic activity of the purified enzymes as well as that of commercial calf chymosin (Sigma-Aldrich chemie, Gmbh P.O. 1120, 89552 Steinheim, Germany) was measured on bovine, ovine and caprine whole acid casein (20 mg/ml) in 0·1 M-sodium phosphate (0·3 NaN_3 g/l) buffer, pH 5·6. Each substrate was treated with each enzyme at a level of 0·01 U/ml for 24 h at 32 °C. After incubation, the substrate was boiled for 2 min to inactivate the enzyme. To the enzyme-substrate system an equal volume of 24% TCA was added and after 30 min the mixture was filtered through a Whatmann qualitative filter paper. Two trials were carried out and the amount of 12% TCA-soluble nitrogen in the filtrates was estimated by the method of Lowry et al. (1951).

Effect of substrate pH and temperature on MCA

The effect of substrate pH and temperature on MCA, expressed as milk clotting time, was measured according to IDF (1997b). Milk substrates with pH values ranging from 6·0 to 6·8 at 35 °C and milk substrates with pH 6·6 at temperatures ranging from 30 to 60 °C were used. In addition, the effect of substrate pH on the temperature inactivation of the MCA of the purified enzymes was studied. For comparison, commercial calf chymosin (Sigma) was also used. The three enzymes were standardized to equal coagulation times at pH 6·6 and 35 °C. The experiment was carried out in duplicate.

Results and discussion

Purification of enzymes

The first chromatographic purification step, based on the reference method of IDF (1997a) for the determination of the enzymatic contents of a rennet, is shown in Fig. 1a. According to this method, chymosin is eluted first at low salt concentration followed by pepsin at higher salt concentration. Thus, in this study, the first peak eluted with 0·25 M-NaCl (fractions 1–9) corresponded to chymosin, while the second, eluted with 0·5 M-NaCl (fractions 20–26), corresponded to pepsin. The chymosin peak also contained substances such as inert proteins and pigments that eluted together with chymosin fractions but had much lower MW (Fig. 1c, lane 3). The co-elution of rennet pigments with the chymosin fraction has been also reported by Martin et al. (1982). By using this method of chromatography, chymosin was not purified (purification factor 1, Table 1), but it was simply separated from pepsin. The inert materials were eliminated by gel filtration on Sephacryl S200 column and in this way kid chymosin was purified 13 folds. Finally, chymosin was eluted as one main peak from MonoQ column with 0·25 M-NaCl (Fig. 1b). However, as the peak was not absolutely symmetrical two main active fractions (32–35 min and 36–38 min) were initially pooled and applied to SDS-PAGE (Fig. 1c, lanes 5 & 6). It was shown that the two fractions concerned were rather the same substance and thus they were finally pooled together as one peak. The asymmetry of the peak was possibly due to the existence of isoforms taking also into consideration the results of isoelectric focusing below. Chymosin from other ruminants (calf, buffalo) appears to be heterogeneous due mainly to genetic polymorphism (Foltmann, 1970; Donelly et al. 1986; Mohanty et al. 2003). On the contrary, Baudys et al. (1988) have found only one peak of chymosin from Mongolian lamb by FPLC. The purification of kid chymosin achieved by MonoQ column was 28-fold with specific activity 170 units/mg and a yield 4% (Table 1).

With regard to kid pepsin purification, DEAE cellulose chromatography resulted in a 5-fold purification of this enzyme with specific activity 33 units/mg (Table 1). Since the method used in this purification step permits the estimation of enzyme content, it was estimated that pepsin activity content of the abomasal tissue used was 72% of the total activity. Gel filtration chromatography increased the purification factor of this enzyme from 5 to 6 and the

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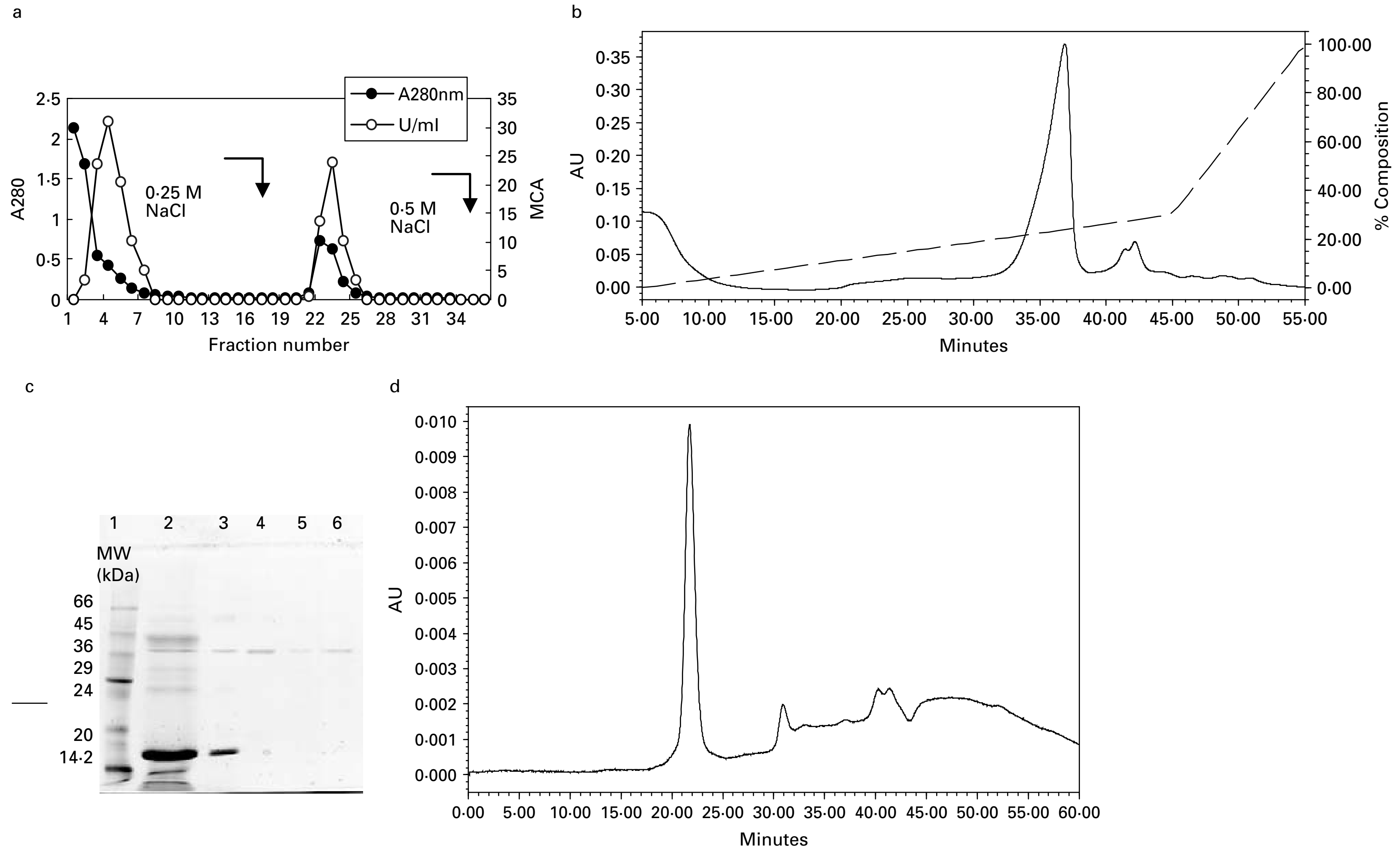

Fig. 1. Purification of kid chymosin. (a) Elution profile from DEAE cellulose ion exchange column. (b) Elution profile from MonoQ HR 5/5 column. ---% 1 M-NaCl (c) SDS-PAGE: MW markers (lane 1), crude extract (lane 2), after the DEAE column (lane 3), after the Sephacryl S200 column (lane 4), fractions with retention time 32–35 min (lane 5) and 36–38 min (lane 6) from MonoQ HR5/5 column (d) Elution profile from SUPERDEX 75HR column.

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Table 1. Purification steps and recoveries of kid chymosin and pepsin

Purification step	Protein (mg)	Total activity (U)	Specific activity (U mg^{-1})	Yield (%)	Purification factor
Crude extract	1238	7700	6	100	1
DEAE cellulose					
Chymosin	266	1660	6	22	1
Pepsin	133	4326	33	56	5
Sephacryl S200					
Chymosin	6·4	484	76	6	13
Pepsin	55	1920	35	25	6
MonoQ HR					
Chymosin	2	340	170	4	28
Pepsin	23	1773	77	23	13

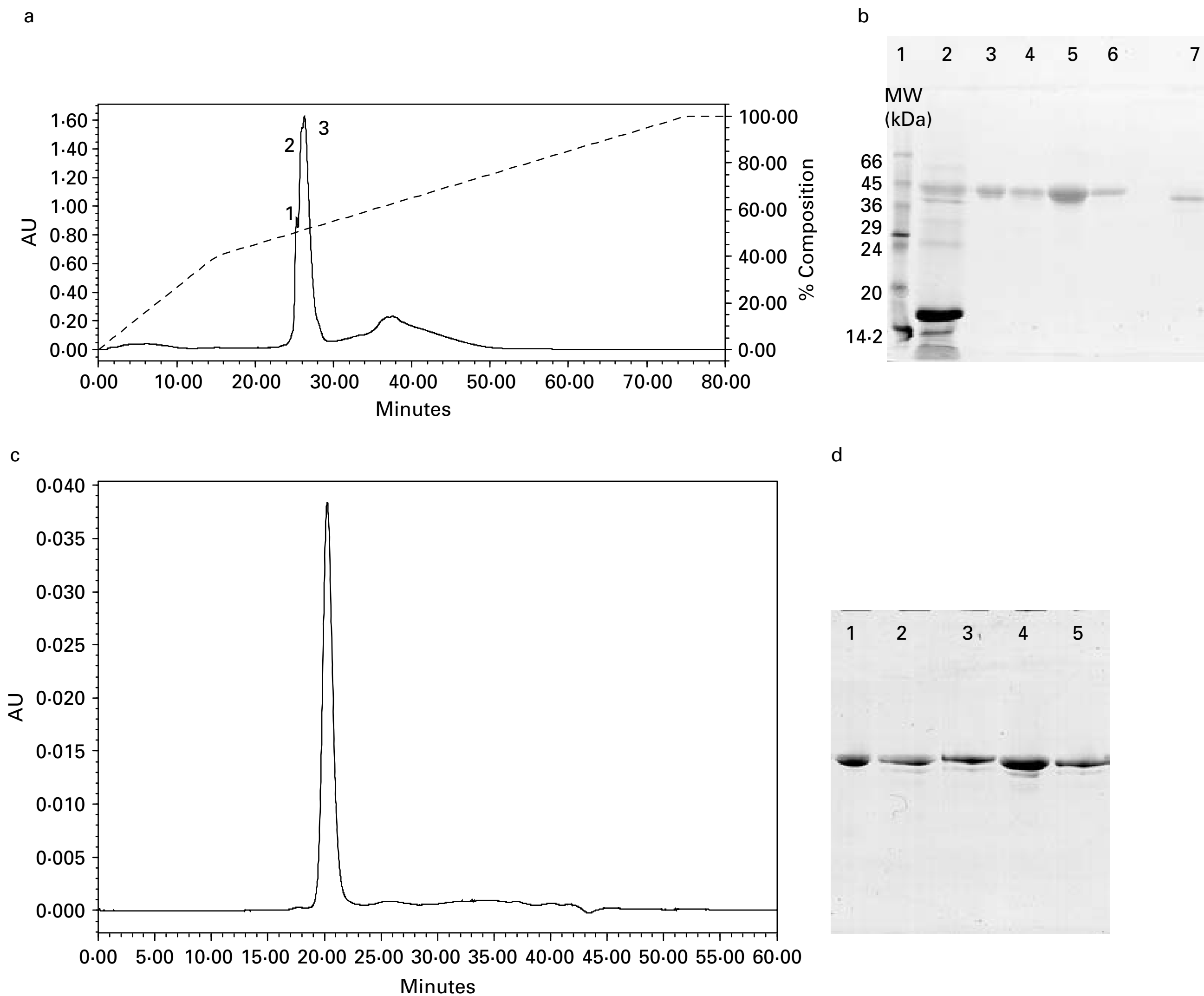

Fig. 2. Purification of kid pepsin. (a) Elution profile from MonoQ HR 5/5 column. ---% 1 M-NaCl (b) SDS-PAGE: MW markers (lane 1), crude extract (lane 2), after the DEAE column (lane 3), after the Sephacryl S200 column (lane 4), peaks 3, 2 and 1 from MonoQ HR5/5 column (lanes 5, 6, & 7 respectively) (c) Elution profile from SUPERDEX 75HR column (d) Urea-PAGE: after the DEAE column (lane 1), after the Sephacryl S200 column (lane 2), peaks 1, 3 & 2 from MonoQ HR 5/5 column (lanes 3, 4 & 5 respectively).

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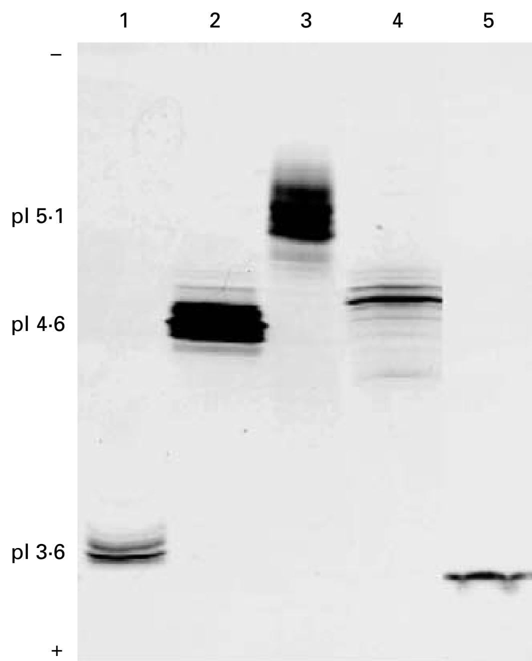

Fig. 3. Isoelectric focusing of purified kid chymosin and kid pepsin: pI marker 3·6 (lane 1), pI marker 4·6 (lane 2), pI marker 5·1 (lane 3), kid chymosin (lane 4), kid pepsin (lane 5).

corresponding specific activity from 33 to 35 units/mg (Table 1). On SDS-PAGE, the pepsin fraction from DEAE cellulose and gel filtration chromatography showed one main band and a second one very close to the first (Fig. 2b, lanes 3 & 4). Elution of kid pepsin from MonoQ column occurred at 0·5 M-NaCl (Fig. 2a) and revealed two to three very closely spaced peaks (sub-peaks 1, 2 and 3) showing milk clotting activity. Peak 4 had no enzyme activity. This profile was similar to that obtained from another purification protocol in which whole abomasum of a kid of the same breed had been used (unpublished data) and also to that reported for lamb pepsin also eluted from MonoQ-FPLC system (Baudys et al. 1988). Each sub-peak was initially collected separately and analysed by SDS-PAGE (Fig. 2b, lanes 5, 6 & 7). The electrophoretic pattern of the MonoQ peak 3 showed two very close zones (Fig. 2b, lane 5), while peaks 2 and 1 (lanes 6 & 7 respectively) showed one zone with similar mobility to those of peak 3 (lane 5). Furthermore, the three sub-peaks were individually applied on the SUPERDEX 75 gel filtration column and had the same retention time (20·3 min, Fig. 2c) confirming that the zones in lanes 5, 6 and 7 (Fig. 2b) were of the same substance. In addition, the urea-PAGE of the pepsin fractions from each purification step showed two zones with different electrophoretic mobility (Fig. 2d). These results suggested that the purified kid pepsin was rather heterogeneous and this heterogeneity might be attributed to its phosphorous content (see below). The purification of kid pepsin achieved by MonoQ column was 13-fold with specific activity 77 units/mg and a yield 23 % (Table 1).

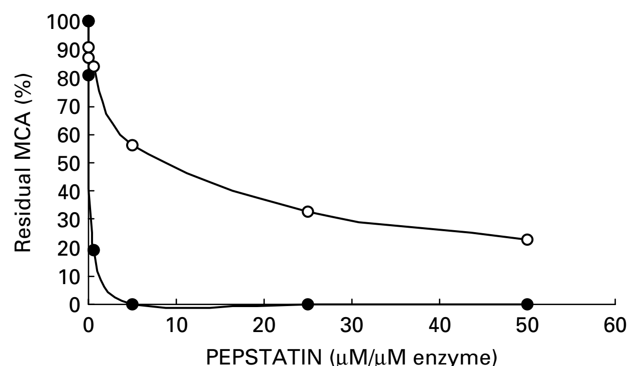

Fig. 4. Influence of Pepstatin A on the milk clotting activity of purified kid chymosin -○- and pepsin -●-.

Values are the means of two trials

Characterization

The molecular weights (M*r*) of the purified kid chymosin and kid pepsin, determined by gel filtration chromatography on SUPERDEX 75 column, were 36 kDa and 40 kDa respectively. Purified kid chymosin was eluted as a single symmetric peak at retention time 21·65 min (Fig. 1d), whereas purified kid pepsin was at 20·3 min (Fig. 2c). The same results were obtained when these enzymes were purified from whole abomasum (unpublished data). Calf chymosin and bovine pepsin were eluted at 21·9 min and 20·3 min respectively. The M*r* of the purified kid chymosin is similar to 36·3–36·5 kDa of calf chymosin (Foltmann et al. 1977; Kleinert et al. 1988), to 36 kDa of lamb chymosin (Baudys et al. 1988) to 35·6 kDa of buffalo chymosin (Mohanty et al. 2003) or even to 37·5 kDa of recombinant lamb chymosin (Rogelj et al. 2001). On the contrary, it differs significantly from the M*r* 44 kDa reported for kid chymosin by Amourache & Vijayalakshmi (1984). The M*r* of sheep pepsin has been estimated 34 kDa (Fox et al. 1977) and 37 kDa (Baudys et al. 1988). To our knowledge so far, there are no data regarding M*r* of goat pepsin.

The isoelectric point of purified kid chymosin was between pH 4·6 and 5·1 (Fig. 3) showing multiple bands (3–6 bands) that might be isoenzymes or degradation products. This result is in contrast to pI value 6·0 reported by Amourache & Vijayalakshmi (1984) for kid chymosin and in agreement with that of lamb chymosin that shows 6 zones-bands at pH about 4·8 (Baudys et al. 1988). It is also similar to pI 4·6 (Foltmann, 1970), 4·7 ± 0·05 (Righetti et al. 1977), 4·5–5·0 (Martin et al. 1982) and 4·5–4·7 (Kleinert et al. 1988) of calf chymosin or to pI of recombinant calf chymosin which is 4·75 for one main zone and 4·55 and 5·0 for two others minor zones (Bines et al. 1989). pI of purified kid pepsin was found at pH value <3·0 (Fig. 3). pI values between 2·8–3·09 with multiple zones have also been reported for bovine pepsin (Righetti et al. 1977; Martin et al. 1982).

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Table 2. Relative proteolytic activity (12% TCA-SN, expressed as equivalent of µg BSA*) of purified kid chymosin and pepsin and commercial calf chymosin on bovine, ovine and caprine casein at pH 5·6 and 32° C for 24 h

Enzyme	Bovine casein	Ovine casein	Caprine casein
Kid chymosin	16·40	29·24	23·89
Kid pepsin	14·50	28·33	23·43
Calf chymosin	14·93	29·15	23·31

* Mean values of two trials

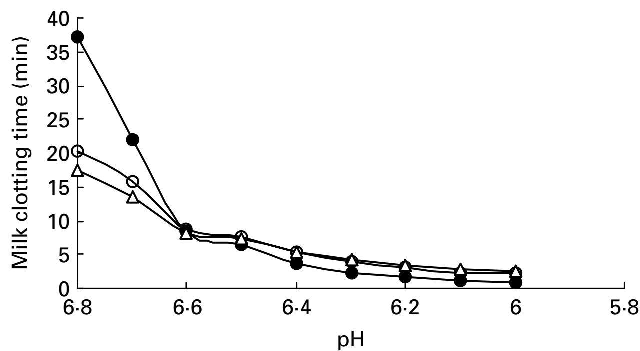

Fig. 5. Influence of substrate pH on the milk clotting activity of purified kid chymosin -○-, kid pepsin -●- and commercial calf chymosin -△-.

Values are the means of two trials

Table 3. Effect of substrate pH on the temperature-inactivation of the purified kid chymosin and pepsin and commercial calf chymosin

	Temperatures* above which the enzymes became inactive, °C		
pH	Kid chymosin	Kid pepsin	Calf chymosin
6·6	52	46	56
6·4	56	52	58
6·2	60	56	62

* Mean values of two trials

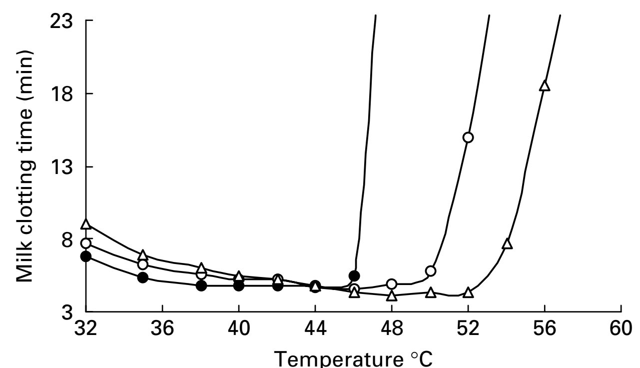

Fig. 6. Influence of substrate temperature on milk clotting activity of purified kid chymosin -○-, kid pepsin -●- and commercial calf chymosin -△- at pH 6·6.

Values are the means of two trials

Analysis of purified kid pepsin for organic phosphorus showed that this enzyme contained about 0·37 mole of phosphorus per mole. Bovine pepsin contains 0–3 phosphate groups per molecule (Meitner & Kassell, 1971; Martin, 1984; Martin & Corre, 1984) and sheep pepsin 1 atom phosphorus per molecule (Fox et al. 1977). Phosphorylation of bovine pepsin is responsible for heterogeneity (Meitner & Kassell, 1971; Martin & Corre, 1984) and the 4 to 6 peaks-isoenzymes of bovine pepsin eluted by HPLC with pH gradient or NaCl gradient have been attributed to the different degree of phosphorylation (Panari et al. 1990; Rampilli et al. 1992; Corradini et al. 1993).

Regarding enzyme inhibition, kid pepsin was completely inhibited by 5 µM-pepstatin A. On the contrary, kid chymosin had a residual activity about 25% of the initial activity at 50 µM-pepstatin A (Fig. 4). Suzuki et al. (1999) have found that goat pepsin is completely inhibited by 10 µM-pepstatin A, while the effective concentrations of pepstatin A for inhibiting bovine pepsin and calf chymosin are 220 nM and 45 pM respectively (Zollner, 1993). From the cheesemaking point of view, pepstatin A could be used in cheese production for inhibiting kid rennet residual activity and controlling cheese proteolysis. The use of pepstatin A in Cheddar cheesemaking with recombinant chymosin has also been suggested by Shakeel-Ur-Rehman et al. (1998).

The relative proteolytic activity at pH 5·6 of the two purified enzymes, as measured by the formation of 12% TCA-SN, did not differ substantially between them and was also similar to that of calf chymosin on all kinds of casein used (Table 2). Similar results have been reported for other enzymes. For example, O'Leary & Fox (1973) measuring the 2% TCA-SN produced from the action of ovine, bovine and porcine pepsins on bovine sodium caseinate at pH 5·5 for 24 h report that porcine pepsin is the most proteolytic, while bovine and ovine pepsins are almost equally proteolytic and only slightly more than chymosin. Also, Alais et al. (1962) have shown that the proteolytic activity, expressed as 12% TCA-SN, of lamb rennet is very similar to that of calf rennet.

The effect of pH and temperature on MCA of the purified enzymes and of commercial calf chymosin is shown in Figs. 5 & 6. The kid pepsin was affected more strongly than the other enzymes at pH 6·7 (Fig. 5). However, it was still active at pH 6·8 and this result was in agreement with those reported by Fox (1969) and O'Leary & Fox (1973) for bovine and ovine pepsin respectively. Kid chymosin and calf chymosin showed similar responses to substrate pH in the range 6·0 to 6·8, increasing the milk clotting time

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sharply from pH 6·6 to 6·8. Recombinant lamb chymosin acts similarly (Rogelj et al. 2001).

The substrate temperature influenced the MCA of the purified enzymes as well as of that of calf chymosin at pH 6·6, as shown in Fig. 6. The three enzymes behaved similarly up to 46 °C. Above this temperature kid pepsin was inactive, whereas kid and calf chymosin were active at least up to 52 °C. However, the purified kid chymosin was more temperature sensitive than calf chymosin, the latter being active up to 56 °C. The thermal inactivation of kid pepsin at temperatures above 46 °C could be an advantage for manufacturing hard cheeses. At pH 6·6, the purified kid pepsin seems to be more temperature sensitive than bovine pepsin and porcine pepsin, which are inactive at temperatures above 47 °C and 57 °C respectively (Fox, 1969).

The temperature at which the three enzymes became inactive was dependent on the substrate pH and the relationship was inverse (Table 3). The inactivation temperature of kid pepsin was more pH-dependent than that of the two chymosins. Kid pepsin was inactive at temperature above 52 °C and 56 °C at pH 6·4 and 6·2-respectively, while kid chymosin was still active up to 60 °C at pH 6·2. However, the purified enzymes did not seem to be so heat resistant as their homologous of bovine origin. Calf chymosin was still active up to 62 °C at pH 6·2. Bovine pepsin is inactive above 57·6 °C at pH 6·45 (Fox, 1969).

In conclusion, in terms of the chromatographic behaviour, molecular characteristics and technological properties, chymosin and pepsin isolated from a Greek indigenous kid goat breed were very close to the corresponding enzymes. This fact is probably due to the close phylogenetic relationship between kid and lamb or calf. For this reason, kid chymosin and pepsin could be an alternative source of milk-clotting enzyme for manufacturing different types of cheeses.

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