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Proteolysis activity of IgM antibodies from rheumatoid arthritis patients' sera: evidence of atypical catalytic site†

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The IgM antibodies from rheumatoid arthritis (RA) patients' sera were screened for peptide hydrolyzing activity. Recovery of structurally intact IgM antibodies (Abs), in a single step, was achieved using a weak anion-exchange methacrylate monolith disk. The IgM Abs from patients' sera hydrolyzed the Pro-Phe-Arg-4-methyl-coumaryl-7-amide (PFR-MCA) substrate appreciably compared to the healthy donors. The apparent K_m values of IgM Abs from patients' sera were between 0.4 and 0.7 mM. Furthermore, IgM Abs displayed 5 to 10-folds greater proteolysis activity than IgG Abs, recovered from the same pathological serum. The proteolysis activity, as a function, was found to be independent of IgM-RF titer value. Affinity labeling approach targeted at the catalytic site histidine was studied, using a specific irreversible inhibitor, N-α-tosyl-L-lysine chloromethyl ketone (TLCK). Despite modification of catalytic His, observation of serine protease like activity suggest presence of an atypical catalytic framework in a few pathological IgM Abs. Copyright © 2010 John Wiley & Sons, Ltd.

Keywords: autoantibodies; IgM purification; monolith chromatography; nucleic acid hydrolysis; peptidase activity; rheumatoid factor

INTRODUCTION

Antibodies (Abs) isolated from autoimmune diseases both human and experimental animals are endowed with catalytic activities. Compelling evidences remarkably support both, the catalytic activity as an intrinsic property of autoimmune Abs and the presence of catalytic site, which are close to enzymes (Tramontano *et al*., 2000). The presence of enzymic property in Abs, their biological role(s) and their occurrence notably in autoimmune diseases are yet fascinating questions. Autoimmune Abs (autoantibodies) hydrolyzing diverse molecules such as peptides, proteins, and nucleic acids are documented (Nevinsky *et al*., 2000; Gabibov *et al*., 2006; Paul *et al*., 2006). Extensive studies with evidences have disclosed different catalytic functions of IgG class Abs recovered from autoimmune patients' sera. Recently, IgM Abs from autoimmune sera are in focus, because they are the first class of Ab produced by plasma cells, besides, the information of protein will be close in sequence to their respective genes (Planque *et al*., 2004).

In rheumatoid arthritis (RA), a systemic autoimmune disease, rheumatoid factors (RFs) present in patients' sera, pertain to IgM Abs. However, IgG class RF Abs are also identified in serum (Randen *et al*., 1992; Soltys *et al*., 1997; Edwards and Cambridge, 1998). The antigenic target for RF is the Cγ2-Cγ3 domain interface in the constant (F_c) region on autologous IgG Abs, this interaction results to an immune complex (Sasso *et al*., 1988; Corper *et al*., 1997; Westwood *et al*., 2006). Despite, an unclear clinical significance of RF, the frequency of RF occurrence is correlated to RA disease severity. Thus, IgM-RF, an autoantibody (AAb), is used as a definite disease biomarker for RA diagnoses (Steiner and Smolen, 2002; Marcelletti and Nakamura, 2003). Qualitative and quantitative recovery of AAbs, IgM class, for further investigations remains an important challenge.

Purification of IgM Ab has proven to be difficult due to the low concentration (0.5–1.5 mg ml^{-1} of serum) and the lower stability of this molecule. Further, the general use of multi-purification step is detrimental to IgM properties (Tornøe *et al*., 1997). A substantially pure IgM, free from IgG, is required for biochemical studies and other applications. Recently, Brne *et al.* (2007) reported purification of IgM Abs, in a single step, using ion-exchange monolithic systems. The new chromatographic media, monolith or Convective Interaction Media (CIM™) is a polymer of methacrylate, cast into a continuous block interlaced with channels/pores. Owing to the

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Abbreviations: *AAb(s), Autoantibody (ies); Ab(s), Antibody (ies); CIM, Convective Interaction media; CV, Column Volume; EDA, Ethylenediamine; PFR-MCA, Pro-Phe-Arg-4-Methyl-Coumaryl-7-Amide; RA, Rheumatoid Arthritis; RF(s), Rheumatoid Factor(s); TLCK, N-α-Tosyl-L-Lysine chloromethyl ketone.*

macroporous structure (no dead end pores), monolithic systems exhibit convection based mass transfer, high flow rates and a low backpressure. In monoliths, the dynamic binding capacity is unaffected by high flow rate, which enables rapid and efficient separation of large molecules (Podgornik and Strancar, 2005). In addition, the rapid purification process does not affect three-dimensional conformation of the molecule.

Here, we report on the proteolysis activity exhibited by IgM Abs recovered from RA patients' sera. Recovery of IgM Abs in a single step using a weak anion-exchange CIM-ethylenediamine (EDA) system is detailed. The proteolysis activity of IgM was compared with IgG recovered from the same RA patient's sera to understand their functional perspective. A preliminary attempt to correlate the IgM-RF titer values with their proteolysis activity was studied. We also report the presence of an atypical catalytic site topology in IgM Abs, investigated by the affinity labeling of the catalytic site histidine using N-α-Tosyl-L-Lysine Chloromethyl Ketone (TLCK).

MATERIALS AND METHODS

Materials

CIM-EDA column was obtained from BIA Separations, Slovenia. Bovine pancreas trypsin, phosphate buffers, Bradford reagent and silver nitrate were purchased from SIGMA (St.Louis, MO, USA). Pro-Phe-Arg-4-Methyl-Coumaryl-7-Amide substrate was brought from Peptide Inc., Osaka, Japan. All other chemicals used were of analytical grade.

Serum collection

RA patients' sera with different RF titer values ($n = 20$) were obtained from Laboratoire d'immunologie biologique, Groupe hospitalier Cochin-Saint, Paris, France, with their informed consent. Healthy donor sera ($n = 5$) were also obtained from the same centre. All patients fulfilled the American College of Rheumatology (ACR) criteria for definite RA.

Antibody purification

IgM Ab from the human sera was purified using CIM-EDA disk (BIA Separations, Slovenia). The specification of the disk is, dimension 12 mm × 3 mm ID and 0.34 ml column volume (CV). All chromatographic procedures were carried out at room temperature with an AKTA FPLC system (GE Health Care, Sweden), at 4 ml min^{-1} flow rate. Total serum (50 μl, ~3 mg) diluted twice in 0.02 M phosphate buffer, pH 7.2 (buffer A) was injected on to the disk that was pre-equilibrated with same buffer. After injection, the disk was washed with 6 CV of buffer A, until the base-line was stable. The bound proteins were eluted, in a linear gradient mode (0–100%), with 16 CV of 0.02 M phosphate buffer, pH 7.2 + 1 M NaCl (buffer B). The disk was regenerated with 1M NaOH (50 CV) followed by water wash, and re-used successively. The unicorn software (in-built) captured all the data.

Analytical methods

Protein concentration in each fraction was determined by Bradford assay. Amicon Ultra-4, 50 K device (Millipore Bioscience, USA) was used for desalting and concentration of proteins present in the eluted peak fractions.

The homogeneity of the recovered protein was analyzed by SDS-PAGE (Biogene, USA), under non-reducing and reducing conditions, followed by silver staining.

Proteolysis assay

The recovered IgM (0.12 μM) was dispensed in 25 μl reaction buffer, composed of 0.05 M Tris-HCl, pH 7.7, 0.1 M glycine and 0.025% Tween 20. Increasing concentrations (10–1000 μM) of PFR-MCA substrate (Peptides Inc., Japan) made in 25 μl reaction buffer were distributed in a 96 well plate. Antibody was mixed with substrate and was incubated at 37°C in a humidified incubator (Sarath *et al*., 2001). Substrate alone (0.2 mM) in the reaction buffer was taken as a blank. A positive control, constituting 0.2 mM substrate (25 μl) and 200 μM (25 μl) bovine pancreas trypsin was included. The fluorescence leaving group, methyl coumaryl amide (MCA), of the substrate was measured at $\lambda_{ex} = 360$ nm, $\lambda_{em} = 470$ nm with a spectrofluorometer plate reader (Flurostar, BMG Labtech, Germany). Substrate hydrolysis was measured at different time intervals. Experiments were carried out in duplicates.

Investigation of the catalytic site histidine by affinity labeling

Affinity labeling reaction targeted at the catalytic site histidine, in IgM, was performed with 1000-fold excess TLCK, (SIGMA, USA). The reaction buffer constituted of 0.05 M Tris-HCl, pH 7.4, 0.1 M glycine and 0.025% Tween 20. About 5 nM, IgM (25 μl reaction buffer) was mixed with 100 μM, TLCK (25 μl) dispensed in 0.05 M Tris-HCl, pH 7.4, 0.01 M calcium chloride, in a 96-well plate. To the above reaction mixture 0.2 mM, PFR-MCA substrate (25 μl reaction buffer) was added and incubated at 37°C in humidified conditions. RA patients IgM, healthy donor IgM and a positive control, bovine trypsin were assayed. For each reaction, control experiments were carried out in the absence of inhibitor. The fluorescence leaving group (MCA) of the substrate was measured at different time intervals using a spectrofluorometer plate reader (λ_{ex} 360 nm, λ_{em} 470 nm).

RESULTS

Antibody purification

To identify the proteolysis activity of RA diseased IgM Abs, the IgM was recovered from the whole sera in a single step using CIM-EDA chromatographic system. Figure 1 shows a typical chromatogram of serum fractionation, where peak 1 is the non-retained proteins of serum, while peaks 2, 3, and 4 are the retained and the eluted proteins. Analysis by SDS-PAGE (Figure 2A) revealed the presence of IgM (900 kDa) only in peak 4 fractions. As expected, the reducing SDS-PAGE showed only two bands corresponding to the heavy chain (70 kDa) and light chain (25 kDa) of IgM, visualized by silver staining (Figure 2B). As peak 4 fractions had homogeneous IgM Ab, these fractions were used for further studies.

Next, the quantitative analysis of protein shows that the IgM recovery from pathological sera was significantly greater than the controls. The mean (± SEM) IgM recovery from RA sera ($n = 15$) and healthy donors ($n = 4$) were 119.5 ± 6.5 μg and 79.2 ± 1.2 μg, respectively ($p < 0.012$, *t*-test). Recovery of great quantity of IgM from the pathological sera can be attributed to the disease condition and in particular, the presence of RF molecules pertaining to IgM class.

Proteolysis activity

The recovered IgM Abs subjected to proteolysis assay cleaved the classical model peptide substrate, Pro-Phe-Arg-4-methyl-coumaryl-7-amide (PFR-MCA), readily detectable. The amide

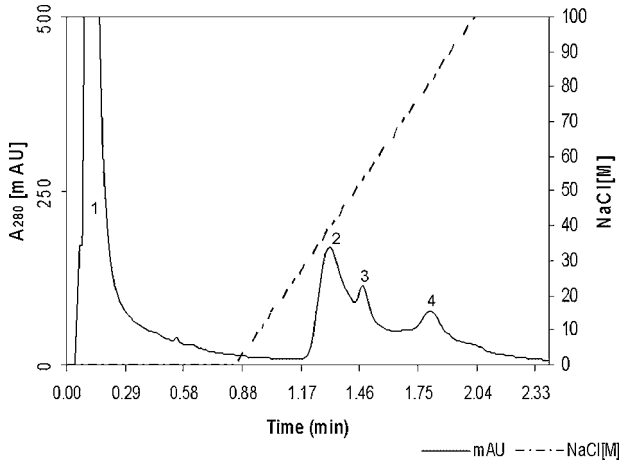

Figure 1. A typical chromatogram of a RA patient serum fractionated on CIM–EDA disk. Conditions: 50 μl (~3 mg) of serum diluted twice in 0.02 M phosphate buffer pH 7.2 was injected at a flow rate of 4ml/min. Peak 1 is non-retained proteins while peaks 2, 3, and 4 are proteins eluted in a linear gradient (0–100%) using 0.02 M phosphate buffer pH 7.2 + 1 M NaCl. —— Absorbance (mAU), ---- NaCl [M].

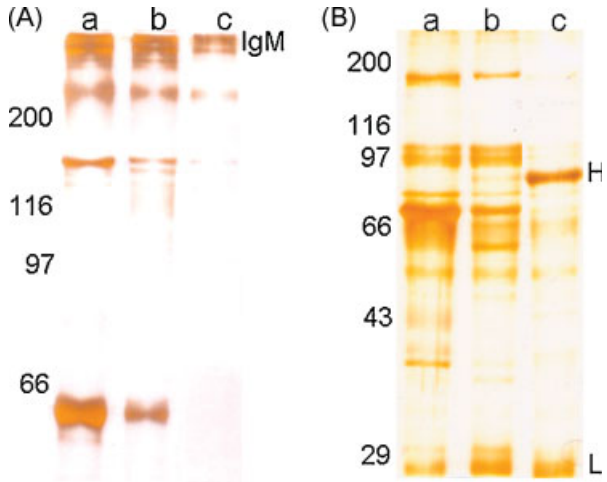

Figure 2. SDS-PAGE of the eluted peak fractions from the chromatogram under (A) non-reducing and (B) reducing conditions (silver staining). Lanes: a-Peak 2; b-Peak 3; c-Peak 4. H; Heavy chain (70 kDa) and L; Light chain (25 kDa).

bond between the basic amino acid arginine and MCA of the peptide substrate has been confirmed as the cleavage site by earlier studies (Sarath *et al*., 2001; Planque *et al*., 2004). Thereby, the release of MCA group results in increase of fluorescence. Substrate hydrolysis by all the samples and by positive control trypsin detected after 2 h of incubation were substantially above blank (Figure 3). In every case, the activity was linear with the incubation time, at a fixed Ab concentration. All Ab catalysis followed the Michaelis–Menten kinetics. Table 1 summarizes the apparent kinetic parameters of a few RA samples studied. The Lineweaver Burk (LB) plot of a pathological and a healthy donor IgM is shown in Figure 4. Interestingly, the apparent K_m values of pathological sera (0.4–0.7 mM) were significantly greater compared to healthy donors (0.45–0.55 mM). The activity of a RA sample with (low) titer 61 U ml^{-1} was 1787.5 μM h^{-1} and K_{cat} was 2.48×10^2 min^{-1}, while that of a high titer sample, >300 U ml^{-1}, was 1666.6 μM h^{-1} with a K_{cat} of 2.31×10^2 min^{-1}. These results indicate that irrespective of titer values, virtually all the samples studied efficiently hydrolyzed the substrate. A preliminary attempt to correlate the titer value and IgM proteolysis activity resulted in no correlation ($r = 0.003$). The correlation analysis indicates that the RF titer and catalytic function are two separate entities. In addition, the results also suggest that proteolysis activity, which is independent of titer value, in turn depends on individual patient.

Next, IgM and IgG Ab proteolysis activity was compared to analyze their molecular function diversity. Previously, we reported proteolysis activity of IgG Abs isolated from RA patients' sera (Kamalanathan and Vijayalakshmi, 2009). The results given in Table 2 and Figure 5, explain the proteolysis activity difference between IgM and IgG Abs recovered from the same RA sera. Further, the results clearly convinced that IgM Ab (μM Ab h^{-1}) had a magnitude of 5 to 10-folds higher activity than IgG Ab (μM Ab h^{-1}). Interestingly, the apparent K_m values of these two different Abs (IgM & IgG) were in close range, which implies virtually same affinity to the PFR-MCA substrate. However, the discrepancy between the valence of IgM (decavalent) and IgG (bivalent) were taken into account while fixing the concentration for the assay. As expected, the IgM Abs from the arthritis sera proves to have a strong protease activity.

Investigation of the catalytic site histidine by affinity labeling

We examined the catalytic site composition of RA IgM Abs by affinity labeling approach. Here, the interest was mainly to investigate the presence of catalytic site His, since IgM Abs displayed serine proteases like activity. Thus, the catalytic site His was chemically modified (alkylation of imidizole side chain) using a specific and a well-known irreversible inhibitor, TLCK (Boden *et al*., 1998). All the IgM preparations studied here showed serine protease like activity by cleaving the PFR-MCA substrate. Observations after 8 h of incubation of the reaction mixture, IgM Abs with TLCK (100 μM) in presence of substrate, revealed striking arguable results (Table 3). Out of the seven IgM preparations studied, no inhibition effect was observed in three samples, while

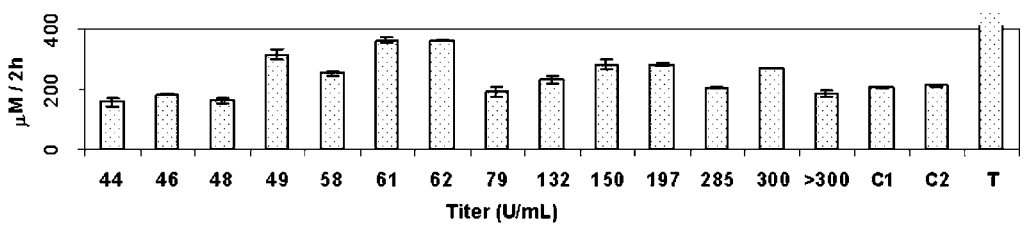

Figure 3. The PFR-MCA substrate hydrolysis by IgM Abs from RA patient samples with their respective titer value (designated on the abscissa). C1 and C2; healthy donors and T; positive control, trypsin. Reaction time, 2 h. Data are mean ± s.d.

Table 1. The proteolysis activity of IgM Abs recovered from different RA samples

Sample ID*	$V_{max} \times 10^{-4}$ M h^{-1}†	$K_m \times 10^{-4}$ M†	K_{cat} (min^{-1})	K_{cat}/K_m (M^{-1} min^{-1})
53	1.08 ± 0.08	3.89 ± 0.69	1.25×10^2	322×10^3
61	2.42 ± 0.16	3.90 ± 0.44	2.48×10^2	636×10^3
70	2.03 ± 0.13	4.75 ± 0.66	2.35×10^2	495×10^3
51	1.51 ± 0.23	4.91 ± 0.61	2.31×10^2	471×10^3

* The apparent kinetic data of pathological IgM Abs was determined using the substrate PFR-MCA, after 2 h of incubation.
† Kinetic values ± SEM was calculated using Michaelis–Menten equation from the values of duplicate experiments.

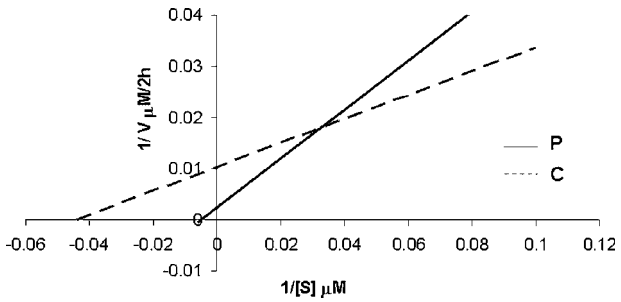

Figure 4. A typical Lineweaver-Burk plot for the peptide hydrolysis by a RA sample and a healthy donor. PFR-MCA substrate (10–1000 µM) was incubated with a fixed concentration of IgM Abs, 0.12 µM. Reaction time, 2 h. P; RA sample, C; healthy donor.

two samples showed 30 and 57% loss in their activity. These results predict, in case of pathological IgM Abs, either His may be absent or disoriented in the active site. Interestingly, one sample showed 92% loss in the activity, suggesting the presence of His in the active site. Further, continuous incubation up to 24 h showed no drastic changes in the results (data not shown). The positive control trypsin was inhibited (97%) by TLCK, while no inhibition or activity was observed in healthy donors.

DISCUSSION

Purification of intact Abs free from contaminates is an essential pre-requisite to understand its structure-function relation. The conventional IgM Ab purification methods are tedious, time-consuming and affect both, yield and purity (Tornøe *et al*., 1997). Here, we demonstrate the use of monolithic chromatography, as a simple, fast and efficient tool, for purifying IgM Abs. In comparison to conventional approaches, the CIM-EDA method proves to be a better choice for the recovery of intact IgM molecules, due to the mild, single step purification conditions and good yield (~85%) (Brne *et al*., 2007). In addition, this system permits to process 2-fold diluted sera at a flow rate of 4 ml min^{-1}, thereby reduces the process time (2–3 min). Thus, CIM-EDA approach remains as a good choice and a potent tool for efficient separation of IgM Abs, in a single step, devoid of other serum proteins.

Electrophoretically homogenous IgM Abs recovered from the pathological sera distinctly hydrolyzed the model peptide substrate. The apparent K_m values in micro molar range indicate a low affinity interaction with the substrate. The specificity constant parameter (K_{cat}/K_m) validates the catalytic efficiency of IgM Abs (Table 1), however, the low values suggest the likelihood of wide substrate specificity. The IgM preparation from different RA samples cleaved the substrate at different rates (Figure 3). This result denotes difference in their binding and catalysis of the same substrate. The non-negligible difference in the activity (V_{max}) and in the K_m values (0.4–0.7 mM), within the samples can be attributed to (i) subtle structural differences in IgM Abs binding site, and (ii) the process of complex formation of RFs, i.e., the interaction of RF with self-Abs (a swapping mechanism) (Westwood *et al*., 2006) resulting in occlusion of the active site. The K_m value in micro molar range and differences in the activity (V_{max}) is in accord to the earlier reports, wherein the studies were performed with various synthetic peptide substrates (Planque *et al*., 2004) and the natural protein (Paul *et al*., 2004). However, in those studies, the IgM was from a different disease and collectively, they suggest that the active site is localized on the V domain. On similar ground, the present results suggest that the active site is perhaps localized to V domain of the IgM Abs. The above argument is strengthened by van Esch *et al.* (2002) report that the VH domain of IgM-RF dominates in specificity and polyreactivity. The kinetic values and SDS gel analysis results

Table 2. Comparison of kinetic parameters of IgM and IgG Abs recovered from same RA sera

	Kinetic constant†			
Patients Abs	V_{max} (µM Ab/h)	K_m (mM)	K_{cat} (min^{-1})	K_{cat}/K_m (M min^{-1})
IgM	904.1–2020.8	0.40–0.70	125–280	$312.5–400 \times 10^3$
IgG	135.2–228.6	0.49–0.89	2.5–4.3	$5.1–4.8 \times 10^3$

† The kinetic constants were calculated from the experimental values using Michaelis–Menten equation. Concentration of IgM, 0.12 µM. Concentration of IgG, 0.6 µM. PFR-MCA substrate was hydrolyzed by the Abs. Assay conditions as described in the methods section.

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Table 3. Effect of TLCK on IgM Abs isolated from rheumatoid arthritis sera

Samples	V_{max} nM IgM h^{-1} *I* (−)	V_{max} nM IgM h^{-1} *I* (+)	% Loss
72	3.50	3.50	0
38	2.25	2.10	0
16	1.75	1.70	0
43	1.95	1.12	30
29	1.5	0.65	57
78	2.1	0.5	77
61	2.69	0.25	92

IgM Abs (5nM) were incubated without [*I* (−)] and with [*I* (+)] inhibitor, TLCK (100 μM) and PFR-MCA peptide substrate (0.2 mM). Reaction time, 8 h. Michaelis–Menten equation was used to determine the kinetic constants. Assay conditions as described in the methods section.

both, led to conclusion that the pathological IgM Abs encompass a serine protease like activity.

Observation of higher activity of IgM Abs (5 to 10-folds) compared to IgG Abs, can be related to valence (10) of IgM Abs (Table 2). Presence of higher activity suggests contribution of more than five valences of IgM Ab toward the substrate catalysis. Further, the explanation proposed by Planque *et al.* (2004), for the difference in activity due to the loss in the valences during IgM to IgG switching mechanism, substantially support the present observations. Another possible explanation for higher activity can be attributed to the diversification in the IgM conformation flexibility (anti-idiotype concept), as a function of RA autoimmune conditions.

Investigation of the catalytic site histidine by affinity labeling

Many authors have reported the presence of typical serine proteases like catalytic triad (Ser-His-Asp) in the catalytic Abs (Kolesnikov *et al.*, 2000; Tramontano *et al.*, 2000). Apart from this, recently, Ramsland *et al.* (2006) has shown the presence of an atypical catalytic site in the Yvo IgM Abs, isolated form Waldenstrom's macroglobulinaemia. Accordingly, the putative site had Ser-Arg-Glu amino acids. Despite this variation, the mechanism exhibited and the spatial arrangement closely resembled the Ser-His-Asp triad.

Given the preceding phenomenon, here, the first demonstration, to our knowledge, about the presence of an atypical catalytic framework in autoimmune IgM Abs is reported. The catalytic activity of pathological IgM Abs was not drastically affected despite modification of the catalytic His using TLCK (Table 3). Consequently, the results challenge the presence of serine proteases like activity of pathological IgM Abs, in absence of the catalytic His. Following explanations can be provided for these observations (i) the general base, His in the catalytic site, should be absent in pathological IgM Abs, and/or (ii) the presence of catalytic variants, wherein the His residue maybe disoriented. The former explanation is further strengthened by a likely situation noted in a few natural serine proteases (Paetzel and Dalbey, 1997; Dodson and Wlodawer, 1998; Karlstrom *et al.*, 2000) and in site-directed mutagenesis studies of trypsin (Corey and Crail, 1992). Here, both, no inhibition effect results and the previous findings, depict to hypothesize that similar conditions prevail in the three IgM Abs samples. Altogether, the presence of catalytic variants in pathological IgM is difficult to exclude, due to the partial inhibition result exhibited by two samples. Further, this view is appreciably supported by the differences in the hydrolysis activity results among the samples (Figure 3). Thus, presence of catalytic property and their structural variants may naturally evolve in Abs, in accord to the anti-idiotype concept (Kolesnikov *et al.*, 2000).

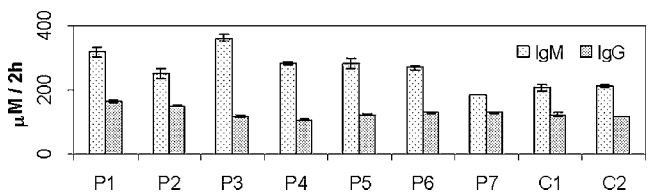

Figure 5. Comparison of PRF-MCA substrate hydrolysis between IgM (0.12 μM) and IgG (0.6 μM) Abs recovered from the same RA serum. IgM was purified using CIM-EDA disk and IgG was purified using L-His-PEVA hollow fiber membrane chromatography. Assay conditions as described in methods section. Data are mean ± s.d.

Conclusion

In conclusion, the results demonstrate with evidence that RA patients' IgM Abs possess proteolysis activity. In general, IgM Abs display greater activity compared to IgG Abs recovered from same pathological sera. The purification method and conditions employed here are simple, efficient and ensure recovery of structurally intact functional IgM Abs, in a single step. The inhibition studies predict presence of an atypical catalytic framework and evoke the question of evolution in immunoglobulins. Similarly, future study of catalytic IgM recognition and degradation of the cartilage collagen is of interest, to investigate their pathological role(s).

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